Future-proofing Our Food: Increasing Tolerance to Abiotic Stress in Hexaploid Bread Wheat Using Transcriptomics

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Abstract

Over four billion people around the world rely on hexaploid bread wheat (*Triticum aestivum* L.) as a major constituent of their diet. However, a warming climate, with increasingly common fluctuations in temperature and rainfall, threatens wheat yields, and, subsequently, the lives and livelihoods of billions of people who depend on the crop for food. To future-proof wheat against a hostile and variable climate, where periods of heat and drought stress occur more intensely and unpredictably in some regions, a better understanding of how the response to these stresses, and inherent stress tolerance are regulated is required. This thesis introduces the YoGI wheat landrace panel, a diverse selection of 342 accessions taken from several landrace collections, and utilizes them to better understand the regulation of the transcriptional and physiological responses to early heat and drought stress exposure, as well as the transcriptional regulation of inherent thermotolerance. This thesis primarily employs a network approach, weighted gene co-expression network analysis (WGCNA), to identify candidate master-regulators of these processes, whilst comparative transcriptomic analyses provide insights in to how the wheat transcriptome is affected by these stresses. This thesis also examines whether exposure to, and then removal of, early heat stress leads to any physiological changes and yield effects later in development, identifying a novel delayed flowering phenotype after this stress treatment, and potential transcriptional determinants of this delay. In all, this thesis represents an exploratory examination of the hexaploid wheat transcriptome; identifying genes which may determine inherent stress tolerance, or which may act to coordinate the transcriptional and physiological responses to heat and drought stresses – genes which could, one day, aid the production of climate-resilient wheat varieties, better able to grow in an increasingly hostile climate.

Author's Declaration

I declare that this thesis is a presentation of original work. This work has not previously been presented for a degree or other qualification at this University or elsewhere. All sources are acknowledged as references. The entirety of the work described in **Chapter 2** has been published in The Plant Journal (doi.org/10.1111/tpj.16248), and was completed in collaboration with other authors, particularly Zhesi He and Andrea Harper, who mapped and compiled the transcriptome data, and produced the TDTPs. These authors wrote parts of the manuscript pertaining to these processes and analyses. However, the rest of the experimental work (i.e co-expression network analysis and hub gene validation) was conducted entirely by this author, whilst the rest of the manuscript was also written entirely by this author. The entirety of the work described in **Chapter 3** has been published in Frontiers in Plant Science (doi.org/10.3389/fpls.2023.1252885), and was conducted in collaboration with other authors, particularly Sara Franco Ortega, who processed and mapped RNA sequencing data and conducted RT-qPCR, and wrote the section of the manuscript pertaining to these processes. The remainder of the work described in this chapter was completed entirely by this author. The entirety of the work described in **Chapter 6** has been published in Frontiers in Plant Science (doi.org/10.3389/fpls.2023.1212559), and was completed in collaboration with other authors, particularly Isaac Reynolds (with whom this author shares first authorship), who mapped RNA sequencing data and conducted comparative transcriptomic analyses, and who also wrote the parts of the manuscript pertaining to these processes and analyses. Plant growth experiments, as well as RNA extraction and preparation were conducted in collaboration with this author. The rest of the experimental work (co-expression network construction and subsequent analysis) was conducted entirely by this author, whilst the rest of the manuscript was also written entirely by this author. Only slight changes, to improve readability or to ensure that formatting is appropriate for the thesis, have been made to these manuscripts.

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1. Introduction

1.1. Formation and Importance of Hexaploid Bread Wheat

Triticum aestivum L., more commonly known as bread wheat, is an allohexaploid species and member of the *Poaceae* (grass) family of flowering plants whose genome is comprised of 21 homeologous chromosomes organized into three subgenomes, each derived from different progenitor species, referred to as “A”, “B” and “D”, respectively (2*n* = 2*x* = 42; AABBDD; 2*n* is the number of chromosomes in each somatic cell and 2*x* is the basic chromosome number). Formation of hexaploid wheat came as the result of two polyploidization events; the first occurring several hundred thousand years ago, between two diploid species which diverged from a common ancestor ~7 million years ago – *Triticum urartu* (the A genome progenitor, AA) and an unknown species related to *Aegilops speltoides* (the B genome progenitor, BB) – a hybridization which formed the allotetraploid species, *Triticum turgidum* (AABB); subsequently, the second polyploidization between this allotetraploid and the diploid species *Aegilops tauschii* (DD) resulted in the formation of hexaploid wheat, *T. aestivum* (Petersen et al., 2006; International Wheat Genome Sequencing Consortium (IWGSC), 2014, 2018).

One could argue that this polyploidization event formed the most important cultivated plant in human history – a species which has shaped the course of agriculture, food processing and cooking from the outset (Venske et al., 2019). Initially, hexaploid wheat grains were ground and used to make unleavened flatbreads to feed families and local communities, whilst also being used as animal feed. Today these uses persist, however the importance of hexaploid wheat as a food source has significantly expanded beyond just being grown to feed local communities, as the crop now accounts for 20% of the world’s caloric and protein intake, with some regions relying much more heavily on it – for example, 40-50% of the calories consumed per day in Egypt and Turkey are provided by hexaploid wheat (Shewry, 2009; Hawkesford et al., 2013; Pfeifer et al., 2014; Shewry and Hey, 2015; Food and Agriculture Organization of the United Nations et al., 2018; Venske et al., 2019). As well as providing calories and protein, the nutritious grains are also rich in dietary fibres, B vitamins, minerals, and lipids (de Sousa et al., 2021). The vast nutritional benefits of hexaploid wheat mean that it is grown all around the world, from 67° North to 45° South, with global consumption in 2021/2022 reaching almost 800 million metric tonnes (United States Department of Agriculture - Foreign Agricultural Service, 2023; Levy and Feldman, 2022). Such levels of dietary reliance and consumption means the lives, and livelihoods, of billions around the world depend on the success of this single crop.

1.2. The Dynamic Hexaploid Bread Wheat Genome Aided Environmental Adaptability and Global Expansion

Fundamentally, inter-specific hybridization events result in the production of organisms which contain the genomes of different species that have adapted to grow in different environments, subsequently meaning the resulting allopolyploid is able to grow under a wider range of environmental conditions than both of its parent species. This is the case for hexaploid wheat, which shows improved tolerance to salinity, low pH, aluminium and cold stress, relative to its tetraploid parent (Dubcovsky and Dvorak, 2007).

The allohexaploid nature of bread wheat also means its genome is both incredibly large (between 15.4Gb and 15.8Gb; five times the size of the human genome) and complex, comprising 107,891 high-confidence genes across its three subgenomes, as well as almost 4 million transposable elements which account for ~85% of the entire genome (International Wheat Genome Sequencing Consortium (IWGSC), 2018). However, not only is the wheat genome large and complex, it is also dynamic – a factor which also likely aided hexaploid

wheat's adaptation to different temperate environments, and facilitated its spread from the Middle East to all parts of the world, reaching western Europe ~7000 years ago, Britain and Scandinavia ~5000 years ago, China ~3000 years ago, and more recently Mexico (~500 years ago) and Australia (~250 years ago) (Dubcovsky and Dvorak, 2007; Shewry, 2009).

Repetitive sequences and transposable elements, features of a dynamic genome, can insert into genes, causing mutation and gene deletion – subsequently creating genetic diversity which may alter phenotype. The abundance of repetitive sequences and transposable elements in the hexaploid wheat genome increases the likelihood of null mutation generation compared to its parents and progenitor species (Harberd, Flavell and Thompson, 1987; Kidwell and Lisch, 1997; Chantret et al., 2005); indeed, recent work found that the number of miniature inverted-repeat transposable element (MITE) insertions in hexaploid wheat varieties was significantly greater than observed in its tetraploid and diploid progenitors (Ubi et al., 2022). The epigenetic regulation of transposable element activity suggests such insertions may occur as a direct response to changing environmental stimuli, and thus the genetic alterations generated by transposable element movement, present in different early hexaploid wheat varieties, were likely selected for as they conferred improved fitness in new environments (Kidwell and Lisch, 1997; Fedoroff, 2012; Levy and Feldman, 2022) – a suggestion supported by the observation that MITE insertion into the 3'-UTR of the heat-responsive gene *TaHSP16.9-3A* causes increased upregulation of gene expression after exposure to heat stress treatment, and thus likely aids growth under elevated temperatures (Li et al., 2014).

Insertion of these repetitive elements not only likely affected early hexaploid wheat's ability to tolerate different environmental stresses, but also allowed it to grow and, crucially, reproduce in these new environments. Early hexaploid wheat required a period of prolonged cold, called "vernalization", in order to flower – however, the insertion of repetitive elements into the regulatory regions of *VRN1* and *VRN3*, paired with four large independent deletions within the first intron of *VRN1*, led to the removal of this vernalization requirement, and subsequently meant hexaploid wheat was able to spread to regions where this vernalization period did not occur (Yan et al., 2004; Fu et al., 2005; Loukoianov et al., 2005; Yan et al., 2006; Dubcovsky and Dvorak, 2007). The effects of such mutations are still felt today, with winter (requires vernalization to flower) and spring (does not require vernalization to flower) habit wheat varieties being grown in different regions around the world, depending on each region's environmental conditions and crop growth cycles.

Mutations, such as those caused by repetitive sequence insertion, can often be deleterious in diploid species, however null mutations in one of the homeologous gene copies in the hexaploid wheat genome can lead to more subtle phenotypic changes, due to functional redundancy between the homeologues on each sub-genome allowing for such loss of function to be compensated for. For example, a null mutation in the *GPC-B1* gene in tetraploid wheat's B genome led to a small difference in the number of days to seed maturity, whilst reduced expression via RNAi against the ortholgous gene in diploid rice caused almost complete seed sterility. Similarly, in hexaploid wheat, dosage-dependent mutation effects on phenotype have been observed for traits such as grain protein content, grain size and red pericarp colour, whereby the phenotypic effect becomes more severe as the number of mutated homeologues increases (Dubcovsky and Dvorak, 2007; Himi et al., 2011; Avni et al., 2014; Wang et al., 2018b; Borrill, Harrington and Uauy, 2019). The scope for adaptive variation via this kind of subtle phenotypic change is incredible when one considers the amount of different combinations of null mutations that could occur across the 107,891 genes in the hexaploid wheat genome, and has undoubtedly bolstered the ability of hexaploid wheat to grow in regions with vastly different environmental conditions.

Similarly, functional redundancy between homeologues permits even greater degrees of genetic variation between hexaploid wheat varieties that, again, would lead to drastic phenotypic changes in diploid species. For example, complete chromosomal loss or gain (known as aneuploidy) can occur relatively frequently, however the compensatory phenotypic buffering effect provided by homeologous genes means that removal of an entire chromosome only results in minor phenotypic changes, and is therefore likely to be inherited by the next generation (Zhang et al., 2013a; Renny-Byfield and Wendel, 2014). Such large-scale genetic changes can improve environmental adaptability, however, with chromosomal translocation events – occurring after chromosomes break and re-attach to other broken chromosomes – improving tolerance to heat and drought stress in hexaploid wheat (Monneveux et al., 2003; Pradhan and Prasad, 2015; Zhai et al., 2021)

Although possessing multiple copies of each gene permits dosage-dependent mutation effects, the production of multiple functionally redundant proteins is costly. Therefore, to retain multiple gene copies, many of the homeologues in the hexaploid wheat genome have likely undergone subfunctionalization or neofunctionalization, whereby protein functionality is split across homeologues (subfunctionalization), or whereby one homeologue retains its original protein functionality, whilst the remaining homeologues accumulate mutations which are selected for as they confer novel, beneficial protein function (neofunctionalization). Neofunctionalization has likely played a major role in the retention of genes within key families, and the subsequent development of novel traits (Wang, Wang and Paterson, 2012; Schilling et al., 2020; Wu et al., 2022). For example, homeologue retention for *MIKC-type* genes was significantly higher than the genome-wide homeologue retention rate suggesting homeologous genes within this family are both functionally important and not redundant – indeed, the authors found that several *MIKC-type* genes showed novel expression patterns, particularly in response to environmental stimuli such as biotic stress, which led them to suggest that homeologues within this gene family have been disproportionally retained due to high levels of neofunctionalization, and that this process may have played an important part in the adaptation of wheat to a range of environments (Schilling et al., 2020). Further functionally redundant gene copies can also initially be formed via small-scale gene duplication events; much like homeologous gene copies, retention of these duplicated genes is costly for the plant, and so they also provide an opportunity for mutation accumulation and subsequent retention via neofunctionalization (Glover et al., 2015; Du, Ma and Mao, 2023). This sequence of events has led to the functional diversification of many duplicated genes, and has also likely contributed to the adaptability of hexaploid wheat. For example, an accumulation of mutations in the regulatory regions of the duplicate genes *TaMSL4-A1* and *TaMSL7-A* has meant that although they share an expression response to drought stress, *TaMSL4-A1* is downregulated under heat and salinity stress, whereas *TaMSL7-A* is upregulated – suggesting these duplicated genes play different roles in the response to various abiotic stresses (Kaur et al., 2022).

The generation of new genetic diversity, via the mechanisms discussed here, occurs frequently in the dynamic wheat genome, and likely has been occurring since the hexaploid's formation thousands of years ago. This diversity can lead to subtle phenotypic changes in the plant, thanks to the compensatory action of homeologous genes, but also likely played a crucial role in the expansion of wheat around the world, as plants adapted to grow well under a wider range of conditions, and became more tolerant to various environmental stresses.

1.3. Wheat Diversity: From Landraces to the Green Revolution and Beyond

As wheat naturally spread to new environments from the Middle East, and then began to be cultivated in these regions, and beyond, the spontaneously-produced genetic diversity occurring rapidly in the dynamic hexaploid wheat genome, that conferred improved growth

under new environmental conditions, began to be selected for – initially by natural selection, but then subsequently by early wheat farmers via artificial selection, as wheat began to be cultivated further afield. These selection events, occurring independently around the world, resulted in the production of a great number of different wheat landraces – varieties containing unique genetic variation that allowed successful growth in the specific combination of environmental challenges present in the area of their cultivation (Charmet, 2011; Peng, Sun and Nevo, 2011; Lopes et al., 2015). However, although yield traits such as seed size, seed shattering, plant height and spike number were also being selected for by these farmers (Fuller, 2007; Peng, Sun and Nevo, 2011; Lopes et al., 2015), the total yields from the landraces was only sufficient to sustain local communities.

Therefore, as the global population began to grow rapidly in the 19th and 20th centuries, the demand for key crops, such as wheat, simultaneously grew – necessitating a move away from locally-adapted landrace varieties, towards varieties which produced significantly higher yields. The Green Revolution during the mid-20th century resulted in 1% yield improvements per year, and a 208% increase in yield per hectare in developing countries between 1960 and 2000 (Evenson and Gollin, 2003; Food and Agriculture Organization of the United Nations, 2004; Pingali, 2012), via the production of semi-dwarf wheat varieties containing mutant forms of *Rht* genes which impeded the plant's ability to respond to gibberellic acid and subsequently stunted growth, reducing yield losses to lodging, and increasing the amount of resources allocated to grain development (Allan, Vogel and Craddock, 1959; Gale and Gregory, 1977; Evenson and Gollin, 2003; Hedden, 2003).

Due to the yield improvements these high-yielding varieties (HYVs) presented, they were widely adopted around the world, being grown in the majority of all cropland (63%) by 1998, and reaching an adoption rate as high as 82% in China by the same year (Byerlee and Moya, 1993; Evenson and Gollin, 2003; Renkow and Byerlee, 2010; Pingali, 2012). This level of adoption meant an increased level of uniformity in the varieties being grown around the world, and, subsequently, reduced genetic diversity as vast numbers of locally adapted landraces were largely replaced by a handful of HYVs, all produced by crosses between a select number of wheat varieties. This created an issue known as genetic vulnerability, whereby the widespread adoption of HYVs left fields full of genetically identical HYV plants, all suited to identical environmental conditions, and subsequently all susceptible to the same perturbations. Before this move towards HYVs, fields of wheat plants more closely resembled natural populations, as different landraces, and spontaneously generated mutants, were sown together as if they were identical. This practice, however, protected yields from adverse effects, as if some genotypes failed to survive certain conditions, other genotypes within the population were likely to tolerate these conditions, thus ameliorating the effect of any yield losses – something that would not be possible in a monoculture of genetically identical HYVs (Keneni et al., 2012).

In the pursuit of improved yields during the production of these HYVs, linkage drag resulted in unintended phenotypic consequences that persist amongst modern wheat varieties, and subsequently contribute to the genetic vulnerability of these varieties due to genetic uniformity. This is especially true for less obvious, or non-visible, phenotypes such as root growth – for example, three of the main Green Revolution founder lines produce smaller root systems than several landrace varieties; whilst *Rht* genes, fundamental during the production of HYVs in the Green Revolution, control root proliferation as well as plant height, and selection for the heading date trait during the production of European bread wheat eliminated a combination of haplotype variants which confer increased root biomass, via linkage drag (Waines and Ehdaie, 2007; Bai, Liang and Hawkesford, 2013; Voss-Fels et al., 2017). Similarly, this was also the case for traits not screened during the production of these HYVs, as subsequent work has

found that the mutated *Rht* genes contribute to a reduced rate of seedling emergence and poor establishment in warm, dry environments where seeds need to be sown deeply to access the limited soil moisture (Rebetzke et al., 2014) – traits which would have been selected for, naturally or artificially, in locally-adapted landraces.

Unfortunately, because many of the HYVs produced during the Green Revolution, or their descendants, are still widely used in breeding programmes and grown in monocultures around the world today, current wheat crops are still faced with this level of genetic vulnerability, and the adverse effects of selecting for yield traits only. Therefore, continued widespread use of a small number of HYVs may be seen as a "risk-reward" strategy, as although the potential yields of these crops are higher than locally-adapted landraces, their phenotypic uniformity, stemming from low levels of genetic diversity, mean wheat crops around the world are vulnerable to damage in the face of unexpected challenges to their growth.

1.4. A Rapidly Growing Population Demands Rapid Increases in Crop Yields

After its formation, and during the early stages of its cultivation, demand for hexaploid wheat was relatively localized to wheat-growing areas, as the crop was grown by local farmers to feed their families and communities. The expansion of crop cultivation, termed the "Neolithic Revolution", precipitated the first major leap in the growth of the world's population – growing from only 4 million people to 190 million by the start of the 1st century CE (Bowles and Choi, 2019; Ritchie and Roser, 2023). Despite this leap, the global population only grew, on average, by 0.04% per year between the years 10,000 BCE and 1700, resulting in a global population of 600 million by the start of the 18th century (Ritchie and Roser, 2023). This period of population growth, although relatively steady, demanded an increased supply of food, and thus crop production – however, when the agricultural practices of the day were insufficient to meet these demands, this steady rate of population growth was impeded in the form of famines, such as the famine which swept across the English countryside between 1315 and 1322 (Kershaw, 1973).

The steady rate of population growth seen between 10,000 BCE and 1700, however, ceased from the beginning of the 18th century, with the global population reaching almost 1 billion people by 1800, 1.65 billion by 1900, and 3 billion by 1960 (Ritchie and Roser, 2023) (**Figure 1a**). This dramatically increased the pressure on global food supplies, necessitating the adoption of HYVs (discussed previously), widespread use of chemical fertilizers and pesticides, and increased mechanization of agriculture brought about by the Green Revolution of the mid-20th century. These technologies and practices meant cereal crop yields in some countries increased at a faster rate than their population grew; for example, in India, between 1961 and 1990, cereal production increased by 121.94% whilst population increased by 90.74% (**Figure 1b**), whereas in China cereal production and population increased by 275.64% and 76.07%, respectively, during the same time period (Ritchie, 2017) (**Figure 1c**). Therefore, the Green Revolution has subsequently been credited with the prevention of famines, and the permission of the fastest doubling of the world's population ever observed, which occurred in just 37 years between 1950 (2.5 billion people) and 1987 (5 billion people) (Ritchie and Roser, 2023).

The global population has continued to grow rapidly in more recent times, reaching 7 billion by 2011 and 8 billion in 2023. This rapid population growth is also expected to be sustained over the coming years, with the global population expected to reach 9 billion people by 2036 and 10 billion by 2058 (Ritchie and Roser, 2023). Although current agricultural practices are largely adequate to support the needs of the current population, sustaining the expected growth of the global population will be a significant challenge as it is predicted that the global production of key crops will need to double by the year 2050 (Ray et al., 2013). To achieve

this, increases of between 2-3% in the supply of these crops will be required each year, however, the yields of major cereals, such as rice, wheat and maize, have been fairly stagnant in recent times (Hawkesford et al., 2013). Wheat is showing the steadiest rate of yield increase (0.9%), which will see only a 38% increase in global production by the year 2050 – far below the level required to meet the predicted demand (Ray et al., 2013). Therefore, increasing the yield potential of wheat varieties cultivated around the world will significantly alleviate pressure on the global food supply. Achieving such improvements in the yield potentials of these varieties, however, can be achieved via different approaches, depending on where the crop is being grown. In regions with well-established and modernized agricultural practices, such as the United Kingdom or the United States of America, increases in wheat yield potential can likely only be achieved via genetic improvements in the varieties being grown, whereas in other regions, such as China, improved soil and crop management practices, paired with genetic improvement of wheat varieties, is likely the most optimal and practical way to achieve the necessary yield increases (Hawkesford et al., 2013). A tailored regional approach should therefore be taken in an attempt to improve crop yield potential, ensuring future demands for key crops, such as wheat, are met.

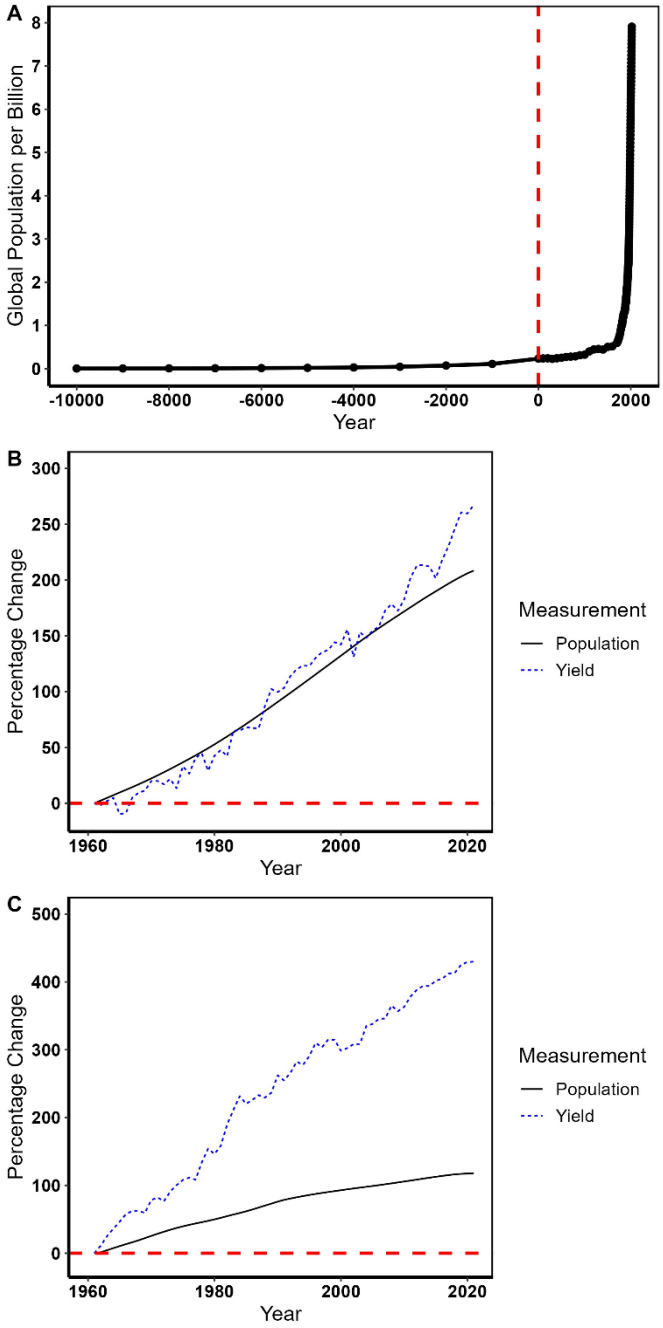

Figure 1.1: Global population and cereal yields have risen dramatically in recent times. A) The global population showed very steady growth between the years 10,000 BCE and 1700, before increasing rapidly from then on. This has resulted in the global population reaching 7.9 billion people in 2021. The dashed red line marks the transition from BCE to CE. Data taken from Ritchie and Roser, 2023. Cereal yields (tonnes per hectare) increased more rapidly than populations grew in India **(B)** and China **(C)** since the advent of the Green Revolution in the 1960s – suggesting that the production of HYVs during the Green Revolution played a key part in supporting this population growth. Dashed red line represents a percentage change of zero. Data taken from H. Ritchie, 2017.

1.5. Climate Change will Significantly Change Crop Growth Conditions

Although improved yield potential is a key way of meeting future demands for food, protecting this yield potential from various stresses and perturbations will also be crucial in a world

experiencing increasingly drastic changes to its climate. In France, for example, the gains made to hexaploid wheat yield potential via genetic improvements, since the 1980s, have been almost totally nullified by the damaging effects of climate change (Oury et al., 2012; Hawkesford et al., 2013), whilst the latest Intergovernmental Panel on Climate Change (IPCC) report on trends in global climate change explicitly states that although agricultural productivity has increased since the mid-20th century, climate change over the past 50 years has significantly slowed this growth (Calvin et al., 2023).

The term “climate change” refers to long-term shifts in global temperature and weather patterns – shifts which can either be natural, as a result of factors such as large volcanic eruptions, or a direct consequence of anthropogenic activities. In recent times, especially since the advent of the Industrial Revolution in the mid-18th century, anomalous long-term temperature and weather patterns, relative to historical records, have been observed. For example, the mean global surface temperature between 2011 and 2020 was 1.1°C higher than was observed between 1850 and 1900, meaning the planet is now warmer than at any time in the last 100,000 years, whilst the increase in global surface temperature since 1970 has occurred faster than in any other 50-year period in the last 2000 years (Calvin et al., 2023).

The IPCC state that the observed warming is caused by excessive anthropogenic emission of greenhouse gases, such as carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), whose concentrations have risen drastically since 1750 (47%, 156% and 23%, respectively) – reaching CH_4 and N_2O levels unprecedented in 800,000 years, whilst the current concentration of atmospheric CO_2 is predicted to be higher than at any point in the last 2 million years (Calvin et al., 2023). The emission of these greenhouse gases comes largely from major processes supporting human life, such as the energy sector, industry, agriculture, and transport, which account for 34%, 24%, 22% and 15% of all net greenhouse gas emissions, respectively (Calvin et al., 2023). The accumulation of these gasses in the Earth’s atmosphere effectively absorbs the heat radiated to the Earth’s surface from the Sun and prevents it escaping into space – this is known as the greenhouse effect. Although the greenhouse effect has supported the evolution of life on Earth, increasing and stabilizing temperatures, excessive accumulation of these greenhouse gases can subsequently accentuate the greenhouse effect, leading to increased warming such as that observed in the last few decades.

This increased rate of global warming, as a result of anthropogenic greenhouse gas emissions, has meant that periods of extreme heat (such as heatwaves) have become more frequent and intense across 44 of the 46 global land regions (IPCC AR6 WGI reference regions) since 1950, whilst periods of extreme cold have become less frequent (Calvin et al., 2023). Similarly, the changing climate has meant that precipitation patterns have changed significantly since 1950, with heavy precipitation occurring more frequently in 19 of the 46 regions, whilst periods of agricultural and ecological drought have occurred more frequently in 12 regions, and less frequently in one (Northern Australasia) (Calvin et al., 2023). These periods of extreme temperature and precipitation are also likely to become more common as the climate continues to change; for example, the IPCC predicts that high latitude regions will experience greater levels of precipitation, whilst mid-latitude, subtropical arid, and semi-arid regions will likely receive less precipitation. This means that regions such as the Mediterranean, states in the southwest of the USA, countries in the south of Africa, and parts of northeast and southwest South America will likely experience decreased soil moisture content and longer dry periods, and, subsequently, will be at an increased risk of agricultural drought in the coming years (Intergovernmental Panel on Climate Change (IPCC), 2014). Increased global temperatures are also predicted to continue, with temperatures between the years 2081 and 2100 likely to be 1.5 to 2°C higher than the mean global temperature between 1850 and 1900. As well as this, the IPCC claims that it is virtually certain that there will be

more hot, and fewer cold, temperature extremes in most parts of the world, as the climate continues to warm, with increases in the frequency, duration and magnitude of these hot temperature extremes (also known as heatwaves) occurring as a direct consequence of increased mean temperatures (Intergovernmental Panel on Climate Change (IPCC), 2014).

As well as this, there is evidence that the changing climate is causing shifts in the length and onset of the conventionally-defined seasons in the northern hemisphere, with the length of summer extending at a rate of 4.2 days per decade and starting 1.6 days per decade earlier between 1952 and 2011, whilst winter shortened by 2.1 days per decade and started 0.5 days per decade later (Wang et al., 2021a). The authors also predict that, given current trends in greenhouse gas emissions and increasing global temperatures, these seasonal shifts will continue throughout the 21st century, potentially resulting in the onset of spring on the 18th of January (compared to the 19th and 17th of February in 1952 and 2011, respectively) and a summer lasting 166 days, in 2100 (Wang et al., 2021a). Such seasonal shifts, as described by J. Wang et al. (2021), are essentially caused by temperature increases in these seasons beyond thresholds defined by historical temperature data – these temperature increases, or periods of extreme elevated temperature, are sometimes referred to as “unseasonal temperatures”. In recent years many countries in the northern hemisphere have experienced unseasonal temperatures during the spring months, providing further evidence that these seasonal shifts are currently taking place; for example, May 2022 saw record temperatures reached across Southern, Central and Western Europe, whilst temperatures approaching record levels (in excess of 40°C in some cases) were observed in some states of the USA (NOAA National Centers for Environmental Information, 2022a, 2022b). Globally, there is also evidence for this shift towards shorter, or unseasonably warm, springs with the period from March to May (Northern Hemisphere’s meteorological spring) in 2022 being the fifth warmest on record (NOAA National Centers for Environmental Information, 2022a), whilst the ten warmest January to May periods have all occurred since 2010 (NOAA National Centers for Environmental Information, 2022a).

Not only are these spring months becoming warmer, but water shortage and periods of drought stress are also likely to become more common in some parts of the Northern hemisphere during this time of the year, as the climate continues to change. Evidence of such a shift materialized in recent times, as April 2022, for example, was an incredibly dry month for many of the world’s largest wheat-producing countries, with almost 50% of the United States experiencing moderate to exceptional drought (NOAA National Centers for Environmental Information, 2022d), whilst large parts of Europe (including major wheat-producing nations such as the United Kingdom) experienced a drier month than normal (NOAA National Centers for Environmental Information, 2022c).

Therefore, not only will excessive anthropogenic greenhouse gas emission lead to increased mean global temperatures, but also increased severity and duration of periods of extreme heat stress (heatwaves) in some regions, and effective changes in the duration and onset of the four seasons – likely posing major challenges to crop growth around the world. As well as this, the changing climate will pose further challenges in some regions, with the amount of rainfall and soil moisture contents both expected to decrease across large swathes of the globe, placing the crops grown in these regions under increased risk of severe drought stress. It is these environmental stresses, heat and drought, which will be the focus of the present work, and thus will be discussed at length hereafter.

1.6. Climate Change is Likely Already Causing Crop Damage and Yield Loss around the World

Although many of the predictions made about the future of the planet's climate paint an incredibly worrying picture for future crops, the effects of climate change have likely been causing demonstrable damage to crop yields around the world for some years, with the Food and Agricultural Organization of the United Nations (FAO) deeming that all cultivated areas, globally, are currently affected by changes in the climate, whilst other work has found that climatic changes have reduced crop yields by up to 70% (Boyer, 1982; van Velthuizen, 2007). More recently, Beillouin and colleagues compiled 82,000 sets of regional yield data from 17 European countries between 1901 and 2018, and utilized machine learning models to measure the effect of weather anomalies in these regions on the yields of nine crop species (Beillouin et al., 2020). They found that extreme temperatures and levels of precipitation are both associated with negative yield observations, but that this effect varied across Europe in this period. As well as this, they found that the yield losses and simultaneous crop failures observed in Northern and Eastern Europe in 2018 were associated with a combination of extremely low rainfall and high temperatures during the spring and summer months, whilst the improved yields seen in Southern Europe in the same year were associated with higher than average levels of rainfall in the region (Beillouin et al., 2020). Similarly, modelling has been used to simulate two sets of climate data in West Africa – one set simulating actual climate data, and another simulating climate data without consideration of anthropogenic influences on the climate – whilst modelling was also used to simulate yield trends across West Africa for two crops, millet and sorghum (Sultan, Defrance and Iizumi, 2019). The authors found that, between 2000 and 2009, consideration of anthropogenic effects resulted in ~1°C warming, as well as more frequent heat and rainfall extremes, and subsequently explained yield losses of up to 20% and 15% in millet and sorghum, respectively (Sultan, Defrance and Iizumi, 2019).

Open-source climate and yield data were also utilized by Ray et al. (2019) who aimed to assess the impact of climate change on the yields of ten key crops, including maize, rice and wheat. They found that the relationship between weather changes and crop yields was statistically significant (as tested by linear regression analysis, $p < 0.05$) in between 54% (sorghum) and 88% (rice) of the harvested areas for each of the ten crops, and that global climate change accounts for yield losses of up to 13.4% (in oil palm), whilst the impacts of climate change on crop yields tended to vary depending on geographical location – with yields in regions such as Europe, Southern Africa and Australia being the most significantly detrimentally affected (Ray et al., 2019). Worryingly, the authors also found that the changing climate has already had an impact on crop yields in countries where hunger is prevalent, as the amount of available consumable calories from crop yields in 27 of the 53 countries with a hunger index of either "serious", "alarming", or "seriously alarming" decreased, with countries such as Zimbabwe (-7.2%), Malawi (-6.5%), Mali (-3.9%) and Ghana (-3.8%) being affected especially severely (Ray et al., 2019). Therefore, not only does climate change pose a significant threat to future crops, but it also appears that changes in temperature and precipitation levels around the world have already had a significant detrimental effect on the yields of key crops, and thus has already jeopardized global food security.

1.7. Elevated Temperatures during Reproductive Development Cause Yield Losses via Disruption to Processes Crucial for Grain Formation and Development

The elevated global temperatures, as well as increasingly common and severe periods of extreme temperature, caused by climate change, pose a significant threat to wheat crops thanks to the effect that heat stress has on the growth and physiology of the plant. As a cool season crop, wheat has an optimal growth temperature of around 20°C and shows a 3-6% reduction in yield for every degree above this optimum (Chowdhury and Wardlaw, 1978; Kobza and Edwards, 1987; Wardlaw et al., 1989; Nagai and Makino, 2009; Ray et al., 2013;

Zhao et al., 2017; Tian et al., 2018). Often, these yield reductions come as a result of exposure to elevated temperatures during reproductive development – the stage of the wheat lifecycle most vulnerable to damage by heat stress, which has an optimum temperature of between 15°C and 20°C (Shewry, 2009).

Elevated temperatures during reproductive development have repeatedly been shown to cause yield losses in wheat, both under glasshouse and field conditions, for example; exposure to 35°C heat stress ten days after anthesis caused up to 51% yield loss in a range of commercially-popular winter wheat varieties (Bergkamp et al., 2018), whilst heat stress of up to 33°C at anthesis led to reduced grain number per unit area and grain size (Liu et al., 2016). Similarly, grain weight and the duration of the grain filling period were both significantly reduced by heat stress treatment (an average daytime temperature of 31°C) after anthesis (Dias and Lidon, 2009); grain yield (43%) and individual grain weight (44%) both showed significant reductions after exposure to 34°C/26°C (day/night) heat stress ten days after anthesis (Pradhan and Prasad, 2015), and five days of heat stress treatment (35°C/26°C, day/night) 14 days after flowering was sufficient to reduce grain weight by up to 36%, and shorten the duration of the grain filling period by up to 12 days (Vignjevic et al., 2015). More recently, across 108 elite wheat cultivars, the application of heat stress (36°C/30°C, day/night) from heading until maturity resulted in a 53.05% reduction in grain yield (Qaseem, Qureshi and Shaheen, 2019), whilst heat stress (32°C/22°C, day/night) exposure during anthesis and grain filling reduced grain yield per plant by 29% and 44%, respectively, in the cultivar Seri82 (Djanaguiraman et al., 2020). This trend is also clear in the field, as grain yield was reduced by an average of 57.3%, across three years (2015, 2016, 2017), in 32 winter wheat varieties exposed to maximum air temperatures of 45.7°C, 45.4°C, and 47.2°C for 14 days after anthesis (Schittenhelm et al., 2020), whilst delayed sowing, by 53 and 58 days, respectively, in the field for two successive seasons resulted in exposure to elevated temperatures during reproductive development, and subsequently, 40.17% and 41.19% reductions in grain yield per m^2 (Shenoda et al., 2021).

Heat stress during reproductive development is so destructive to yields, as not only does it significantly disrupt the development of reproductive organs and gametophytes, it also detrimentally affects pollination and fertilization – all of which are essential in reproduction and the formation of grains. Floral organ and gametophyte development are susceptible to damage by high temperatures, with reduced floret fertility, as well as desiccated pollen grains, stigmas, and ovaries all being symptoms of exposure to 36°C/26°C (day/night) heat stress at booting (Prasad and Djanaguiraman, 2014), whilst exposure to heat stress (30°C for 3 days) during meiosis in pollen mother cells led to pollen abortion, abnormal anther locules and sterility (Saini, Sedgley and Aspinall, 1984), as well as abnormal embryo sac development and complete absence of the gametophyte (Saini, Sedgley and Aspinall, 1983). These findings came after Saini and Aspinall had shown that this stress treatment caused reduced grain yield by reducing the number of grains formed (Saini and Aspinall, 1982), and so, when viewed together, the work done by these researchers unequivocally linked the reduced yields seen after heat stress exposure during reproductive development, at least in part, to abnormal gametophyte development. Further connections between heat-induced damage to gametophytes and reduced grain yield have been made more recently, as delayed sowing, leading to terminal heat stress exposure, resulted in meiotic abnormalities, such as precocious chromosome migration, laggard chromosomes, micronuclei, and an absence of metaphase plate in pollen mother cells, subsequently reducing the number of grains per spike (Omidi et al., 2014), whilst exposure to elevated temperatures at meiosis and anthesis caused both reduced pollen viability, and reduced grain yield per spike (Bokshi et al., 2021).

Contrary to some of these findings, however, recent work by Choudhary and colleagues found that elevated temperatures during reproductive development did not have a significant effect on pollen viability, with the authors instead arguing that the reductions in grain number they observed after heat stress exposure came as a result of heat-induced reductions in ovule viability, and/or disrupted fertilization and subsequent formation of viable embryos (Choudhary et al., 2022). Support for this observation and hypothesis is provided by the recent finding that combined heat and drought stress caused damage to stigma functionality (Fábián et al., 2019), further suggesting that male sterility and damage to pollen grains and pollen development are not the only factors which may lead to reduced grain formation, and yields, under heat stress exposure during reproductive development.

Due to the incredibly damaging effect of heat stress of wheat gametophytes, and on both sets of reproductive organs, pollination and fertilization are also detrimentally affected by elevated temperatures. The desiccation of the stigma which occurs under heat stress results in reduced adherence of pollen grains, and subsequently reduced pollination (Prasad and Djanaguiraman, 2014), whilst heat stress also causes desiccation of the style and ovary as well as undirectional cues from female cells, leading to disorientated pollen tube growth, and subsequently reduced rates of fertilization (Prasad and Djanaguiraman, 2014).

Not only can elevated temperatures during reproductive development damage gametophytes, but such periods of heat stress can also cause abortion of any embryos which are able to form. Hays and colleagues found that exposure to 38°C heat stress during early kernel development led to kernel abortion and subsequently reduced kernel weight, but also resulted in elevated ethylene accumulation, suggesting the phytohormone plays a key role in determining embryo abortion under stress. Support for this hypothesis was then provided when application of 1-methylcyclopropane (an ethylene receptor inhibitor) prior to heat stress resulted in the heat-induced kernel abortion and reductions in kernel weight being blocked (Hays et al., 2007).

As well as having a devastating impact on the processes essential for sexual reproduction, and subsequent grain formation, periods of heat stress during reproductive development can also cause damage to other processes which also determine grain yield – chiefly, photosynthesis. Photosynthesis is one of the main determinants of yield, with the synthesis of sugars being essential to support biomass and grain production (Simkin, López-Calcagno and Raines, 2019). However, heat stress disrupts photosynthesis by reducing the activities of key enzymes as well as damaging proteins and structures fundamental to the reaction – resulting in reduced yield (Djanaguiraman et al., 2018, 2020).

Biosynthesis of chlorophyll, the pigment which plays a fundamental role in photosynthesis thanks to its light absorption, is disrupted under heat stress, via head-induced damage caused to key enzymes involved in its production, such as 5-aminolevulinate dehydratase, mg-protoporphyrin IX methyltransferase, and protochlorophyllide oxidoreductase, whose activity and efficiency are all significantly reduced during periods of high temperature (Tewari and Tripathy, 1998; Mohanty, Grimm and Tripathy, 2006). Not only is chlorophyll biosynthesis reduced, but its degradation is increased under heat stress exposure, thanks to improved efficiency of key degradation enzymes, such as chlorophyllase (Tewari and Tripathy, 1998; Feng et al., 2014). Combined, this results in chlorophyll loss under heat stress, and subsequently in reduced light absorption, and reduced rates of photosynthesis. Similarly, the key enzyme in photosynthesis, Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), is also affected by elevated temperatures as a result of differential activity of its activator chaperone, Rubisco activase (RCA), under heat stress. High temperatures cause thermal denaturation of RCA, and subsequently it is unable to reactivate Rubisco, leading to reduced

enzymatic activity and reduced photosynthetic potential under heat stress (Crafts-Brandner and Salvucci, 2000; Salvucci et al., 2001; Feng et al., 2014; Perdomo et al., 2017; Kumar et al., 2019b). Heat stress can also cause damage to other photosynthetic machinery, such as the two photosystems, I and II (PSI and PSII). PSII is known to be particularly heat-labile (Yamamoto, 2016; Wang et al., 2018a; Hu, Ding and Zhu, 2020), with heat stress causing damage to thylakoid membranes by changing their fluidity, subsequently resulting in inactivation of PSII (Yamamoto, 2016), whilst also resulting in PSII light-harvesting complexes falling off of thylakoid membranes, subsequently reducing the efficiency of electron transfer, and reduced photosynthesis (Janka et al., 2013; Mathur, Agrawal and Jajoo, 2014; Hu, Ding and Zhu, 2020).

Therefore, periods of heat stress during reproductive development are likely to cause yield losses, such as those described above, chiefly via damaging effects on gametophyte production, sexual organ development, pollination, fertilization, and photosynthesis – processes which are all crucial for the formation of grains, and high yields.

1.8. Heat Stress Causes Altered Morphology, and Cellular and Physiological Damage

Although the occurrence of heat stress during reproductive development can have serious implications for yield, periods of elevated temperatures throughout the lifecycle of hexaploid wheat can cause equally devastating effects on the growth and development of plants. For instance, seed germination was significantly negatively affected by heat stress exposure, across three different varieties, with the number of days to germination being longer by up to 50% (Hossain and da Silva, 2012), whilst the same authors also found that late sowing in the field, resulting in heat stress exposure, also caused significantly advanced developmental speed, with the three varieties all booting, heading, flowering and maturing significantly faster than their counterparts under control conditions. Similarly, other work has also found that wheat plants proceed through their life cycle faster after/during exposure to elevated temperatures, with traits such as number of days to heading, number of days to flowering, and number of days to maturity all being reported to be shorter after exposure to high temperatures (Rahman et al., 2009; Nahar, Ahamed and Fujita, 2010; Hakim et al., 2012; Hossain et al., 2012, 2013). Periods of high temperature can also affect leaf morphology, with leaf rolling occurring to protect the activities of PSI and PSII, maintaining relatively high levels of photosynthesis whilst also ensuring more efficient stomata-mediated rates of transpiration (Sarieva, Kenzhebaeva and Lichtenthaler, 2010). Similarly, leaf area can be affected, with exposure to heat stress reducing leaf extension (Lal et al., 2022), whilst 30°C/25°C (day/night) caused reduced green leaf area (Rahman et al., 2009). Exposure to elevated temperatures also leads to wax deposition on the leaf surface, in an attempt to prevent water loss and limit the absorption of excess solar radiation (Farhad et al., 2023). Other tissue is also affected by exposure to heat stress, with plants able to accumulate less biomass, compared to cooler conditions, via the production of fewer tillers, as well as shorter stems and roots (Rahman et al., 2009; Gupta et al., 2013).

One of the main ways that high temperatures cause cellular and physiological damage to wheat plants is via denaturation of protein structure, and the subsequent disruption of protein function. Heat stress disturbs normal cellular protein-folding machinery, and thus leads to the accumulation of misfolded or unfolded proteins (Lohani, Singh and Bhalla, 2022) – an accumulation of which can lead to the formation of toxic protein aggregates due to the exposure of hydrophobic residues on polypeptide chains, and can result in cell death (Vabulas et al., 2010; Nakajima and Suzuki, 2013; Ueno et al., 2019). Besides causing cell death, disruption of protein folding can significantly diminish protein functionality, as described above for RCA (Crafts-Brandner and Salvucci, 2000; Salvucci et al., 2001; Feng et al., 2014;

Perdomo et al., 2017; Kumar et al., 2019b), and subsequently lead to biochemical and physiological damage.

Heat stress disrupts the protein structures, functionalities and activities of many key enzymes involved in photosynthesis (described above), and thus the reduced photosynthetic potential that occurs after exposure to heat stress during reproductive development, via the mechanisms described above, can also occur during earlier stages of development, with the results being largely the same – reduced sugar production via photosynthesis meaning fewer resources are available to fuel growth, development and biomass production. The heat-induced damage done to photosynthetic machinery, and to chlorophyll in particular, often results in premature leaf senescence whereby leaves become discoloured (due to the degradation, and reduced synthesis of the chlorophyll pigment), shrivelled, and, thus, less functional, as, besides the reduced chlorophyll content impeding photosynthetic potential, less total leaf area is also available for gas exchange, meaning CO_2 assimilation decreases, further reducing the photosynthetic potential of the plant (Sarkar et al., 2021). The activity of Rubisco itself is also affected by high temperatures, thanks to the reduced solubility of CO_2 in the atmosphere under these conditions, relative to O_2. This, subsequently, means that the enzyme more readily conducts oxygenation under heat stress – increasing the rate of photorespiration at the expense of photosynthesis, diminishing the pool of available ribulose-1,5-bisphosphate and thus the production of photoassimilates to fuel growth and development (Yadav et al., 2022; Farhad et al., 2023).

This damage to photosynthetic apparatus and the formation of protein aggregates can both also occur as a result of oxidative damage which comes after the accumulation of reactive oxygen species (ROS), such as super-oxides, hydroxyl radicals, and hydrogen peroxide during periods of heat stress (Savicka and Škute, 2010; Mathur, Agrawal and Jajoo, 2014; Lévy et al., 2019). Heat stress was found to cause elevated levels of H_2O_2, with the authors suggesting that this increase resulted in reduced levels of photosynthetic pigments, and increased levels of membrane damage, compared to control condition plants (Mohi-Ud-Din et al., 2021). The damaging effects of ROS on cellular components translates to phenotypic effects, with heat stress causing reduced plant height, leaf area and biomass production in wheat plants, however application of compounds which aid the scavenging of ROS ameliorated this physiological damage – suggesting ROS accumulation is a key contributor towards the damage caused by elevated temperatures (Zhang et al., 2023).

The accumulation of ROS under heat stress also leads to damage of cellular and organellar plasma membranes, which can, in turn, affect cellular function (Savicka and Škute, 2010) – for example, thylakoid membranes are damaged by ROS during periods of heat stress, leading to impeded electron transport, and reduced rates of photosynthesis (Wang et al., 2018a). Phospholipid molecules within such membranes are the sites of ROS attack during a period of heat stress, with the unsaturated double bond between two carbon atoms, and the ester linkage between glycerol and the fatty acid molecule being particularly susceptible to ROS-mediated peroxidation (Sharma et al., 2012). This sort of damage can result in 54% reductions in wheat membrane stability under heat stress (Savicka and Škute, 2010), and subsequently, electrolyte leakage, impairment of organellular function, and ultimately cell death (El-Basyoni et al., 2017; Farhad et al., 2023) – meaning, therefore, that varieties which show high levels of membrane thermostability are also able to produce more biomass and higher grain yields under heat stress (Shanahan et al., 1990; Blum, Klueva and Nguyen, 2001; El-Basyoni et al., 2017; Islam et al., 2017).

1.9. Amelioratory Molecular Responses to Heat Stress

In an attempt to limit the amount of damage caused by heat stress, plants employ several tactics which are activated in direct response to elevated temperatures. For instance, heat-shock proteins (HSPs) are rapidly expressed in response to high temperatures (amongst other abiotic stresses, including drought); a phenomenon first discovered in soybean exposed to 40°C for four hours (Key, Lin and Chen, 1981), but since identified many times over in wheat, with rapid expression responses to heat stress (as little as one hour) being observed in more recent times (Qin et al., 2008; Kumar et al., 2018; Vishwakarma et al., 2018; Erdayani et al., 2020; Lee et al., 2022). These proteins act as molecular chaperones to regulate protein folding, and subsequently prevent the formation of toxic aggregates of misfolded or denatured proteins, and can even take part in the re-folding of proteins during periods of heat stress (Wang et al., 2004; Al-Whaibi, 2011). 753 HSPs have been identified in hexaploid wheat, and are split across several protein sub-families, each with their own specialized function; sHSPs, HSP40s, HSP60s, HSP70s, HSP90s and HSP100s (Kumar et al., 2020). Increased expression and accumulation of these proteins, therefore, have been shown to improve the ability of plants, including species such as rice, maize and *Arabidopsis thaliana*, to tolerate periods of heat stress (Queitsch et al., 2000; Hong and Vierling, 2001; Tonsor et al., 2008; Gao et al., 2012; Sun et al., 2012b; Zhai et al., 2016; Guo et al., 2020a; Rahman et al., 2022; Do et al., 2023), whilst the beneficial effect of HSPs on hexaploid wheat growth under elevated temperatures have also been widely observed (Chauhan et al., 2012; Erdayani et al., 2020; Vishwakarma et al., 2018; Kumar et al., 2020).

Similarly, to combat the cellular and organellar damage caused by ROS production and accumulation under heat stress, the expression of genes encoding antioxidant enzymes, and the activity of these enzymes, are upregulated under heat stress exposure in hexaploid wheat (Gupta et al., 2013; Kumar et al., 2018; Lee et al., 2022). These genes, such as superoxide dismutase (SOD), catalse (CAT), glutathione peroxidase (GPX), guaiacol peroxidase (POX), peroxiredoxins (Prxs), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR), are able to detoxify the cellular environment by either stabilizing or deactivating free radicals and other ROS before they attack cellular components (Caverzan, Casassola and Brammer, 2016). Subsequently, the induction of such genes, and the activity of the encoded enzymes have been shown to protect hexaploid wheat against the effects of heat stress-induced oxidative damage, with increased SOD activity being associated with improved membrane stability under heat stress in the variety HDR77 (Kumar R R et al., 2013), whilst FER-5B is able to improve tolerance to heat stress via its effect on the activities of CAT and GR (Zang et al., 2017).

Phytohormones play key roles in allowing plants to sense and perceive changes in their external environment, and are subsequently involved in almost every aspect of growth and development – however, their involvement in signal perception and transduction means they are key mediators of the response to various abiotic stresses, such as heat. Accumulation of phytohormones are able to cause phenotypic changes in hexaploid wheat and alleviate the damaging effects of heat stress exposure via action on gene expression and signalling pathways. For instance, ABA is a key stress signalling phytohormone, involved in the perception of many abiotic stresses, and has been shown to be a key player in the heat stress response, via its activation of the expression of antioxidant and HSP genes (Tao et al., 2022). Heat shock transcription factors (discussed in more detail later) are also affected by ABA accumulation under heat stress, with HsfC2a acting as part of an ABA-induced pathway to activate the expression of various thermo-protectant genes, such as HSPs (Hu et al., 2018), whilst overexpression of *HsfA6f* resulted in increased thermotolerance thanks, in part, to elevated ABA levels and increased sensitivity to the phytohormone (Bi et al., 2020). The

amelioratory effect of ABA has also been observed via exogenous application, with this reducing the damage done to photosynthesis and growth in wheat plants exposed to 6 hours of 40°C heat stress every day for 15 days, whilst inhibition of ABA led to increased heat-induced damage (Iqbal et al., 2022). Similarly, exogenous application of the ubiquitous phytohormone auxin was found to limit the damage done to wheat yields by moderate heat stress, with grain number and yield both showing improvements compared to the stressed negative control (Abeysingha et al., 2021). Other stress phytohormones have been found to accumulate differentially in wheat after heat stress exposure, with gibberellic acid and salicylic acid showing decreased levels in root and shoot tissue, whereas cytokinins accumulated in wheat seedlings after exposure to 40°C heat stress (Kosakivska et al., 2022) – suggesting differential roles for these phytohormones as part of the response to heat stress.

As well as phytohormones, the accumulation of ROS and Ca^{2+} that occurs after membrane damage during periods of heat stress also play key roles in the perception of heat stress, and influence targets downstream which help ameliorate the damage done by elevated temperatures. For example, ROS and Ca^{2+} accumulation triggers the activity of HsfA1 transcription factors – the master-regulators of the heat stress response – which activate the expression of heat-inducible transcription factors (such as other heat shock transcription factors, and dehydration-responsive element binding (DREB) transcription factors), subsequently leading to the expression of thermo-protectant genes, such as those encoding HSPs (Ohama et al., 2017; Abhinandan et al., 2018). Other work, however, has suggested that this activation cascade occurs independently of HsfA1 transcription factor activity, and in direct response to Ca^{2+} and ROS accumulation under heat stress, which causes increased levels of IP3, and the activation of DREB2A – subsequently leading to the expression of HSPs and other thermo-protectant genes (Zheng et al., 2012; Ohama et al., 2017; Abhinandan et al., 2018).

1.10. Drought Stress Causes Significant Yield Loss when Applied at Different Developmental Stages

Not only is climate change expected to increase the prevalence and intensity of heat stress in some regions over the coming years, but drought stress (a prolonged period of abnormally low rainfall, leading to water shortage) is also expected to occur more frequently in some parts of the world, as the climate continues to change. This prospect threatens global wheat yields, with the reproductive part of the crop lifecycle, in particular, also being vulnerable to significant damage by water shortage (Yu et al., 2018a). For example, the application of drought stress from heading to maturity led to reduced yield, with losses of 44.66% being seen across 108 varieties (Qaseem, Qureshi and Shaheen, 2019), whilst other work found that drought stress exposure during grain filling resulted in yield losses of up to 78% (Guóth et al., 2009), whereas water shortage 12 days after heading caused a 57% reduction in grain yield (Balla et al., 2011). Similarly, drought stress application at anthesis and maturity caused yield losses between 58% and 92% across 30 varieties (Dhanda and Sethi, 2002), with similar losses being seen in other work where drought stress was applied at anthesis – resulting in a mean yield loss of 27% across 12 varieties (Jatoi et al., 2011), and 43% and 51% yield reduction for two different varieties (Gupta, Gupta and Kumar, 2001). Water shortage after anthesis, during grain formation or filling, can also result in similar yield losses – up to 38% (Majid, Asghar and Murtaza, 2007; Eskandari and Kazemi, 2010).

As with heat stress, exposure to drought stress during reproductive development results in yield loss largely via the effect of the stress on gametophyte development and sexual reproduction. For example, drought stress during anthesis and early grain formation causes abnormal development of the floral organs, subsequently reducing the amount of successful pollination and fertilization, as well as inducing the abscission of flowers and the abortion of

grains early in their formation (Saini, 1997). However, exposure to water stress slightly earlier in reproductive development, during the young microspore stage of pollen development, results in the production of abnormal pollen, and even pollen abortion (Saini, 1997; Ji et al., 2010). Pollen sterility is likely a consequence of abnormal degeneration of the tapetum in anthers, leading the microspores to lose orientation and subsequently possess a dilute cytoplasm, as well as causing them to have an impaired ability to accumulate starch – a key feature of fertile grass pollen – due to drought-induced reductions in invertase activity, meaning the developing pollen is unable to metabolize sucrose into hexose sugars (Dorion, Lalonde and Saini, 1996; Lalonde, Beebe and Saini, 1997; Saini, 1997). Additionally, drought-induced accumulation of abscisic acid (ABA) in the anthers also contributes to pollen sterility, with drought tolerant varieties able to synthesise less of the phytohormone, whilst exogenous application of ABA to the spike leads to increased levels of pollen sterility (Ji et al., 2011). These processes cause yield reductions as increased pollen abortion and sterility leads to diminished grain formation, however the occurrence of drought after anthesis reduces yield due to its shortening of the grain filling period, whereby grains grow in size and weight due to the biosynthesis and accumulation starch (Wardlaw and Willenbrink, 2000; Altenbach et al., 2003; Plaut et al., 2004).

However, the application of drought stress during other developmental stages can also directly result in yield loss, with recent work identifying stem elongation and booting as particularly susceptible to damage by moderate drought stress, resulting in large yield effects (Riedesel et al., 2023). A recent meta-analysis found that drought significantly reduced grain yield by 57.32%, however this effect varied depending on whether plants were exposed to prolonged (water shortage throughout development) or terminal (water shortage during reproductive development) drought, with these regimes causing 83.6% and 26.4% reductions in yield, respectively (Wan et al., 2022). Another meta-analysis found that wheat yields showed significant reductions after exposure to drought stress at different stages throughout their development, with losses of 27.4%, 21.4%, 16.8% and 16.3% occurring after plants experienced water shortage during tillering, jointing, heading, blooming and grain filling, respectively (Zhang et al., 2018). Similar work found a concurrent trend, that the later drought stress was applied, the less severe the yield effects were, with drought at stem elongation, booting and grain filling resulting in yield losses of 81%, 53% and 40%, respectively (Shamsi and Kobraee, 2011). Work in the field also found that, over the course of three years, yield traits such as spike length, hundred grain weight and grain yield were all significantly reduced by drought stress (Abou-Elwafa and Shehzad, 2021), whilst prolonged withholding of water in the field led to yield losses ranging from 7.95% to 39.35%, across 15 varieties (Zhang et al., 2022c).

As described previously for heat stress, yield losses during these stages of development are likely to be consequences of the drought-induced reductions in photosynthetic capacity and damage to photosynthetic machinery. However, drought stress is able to reduce photosynthetic capacity via a mechanism not previously discussed for heat – the reduction of CO_2 available for photosynthesis, via drought-induced closure of stomata. Stomatal closure will be discussed in more detail later, however their closure under drought stress significantly reduces the rate of photosynthesis, as less CO_2 is able to diffuse into mesophyll cells and be captured by Rubisco, resulting in a reduced rate of photosynthesis and subsequently less accumulation of photoassimilates (Flexas et al., 2004). Not only this, but drought stress can reduce the activity of Rubisco via the binding of inhibitors, and also cause reduced levels of the protein over the course of the drought period (Parry et al., 2002), whilst further reductions in photosynthetic capacity can be caused by a shortage of ribulose-1,5-bisphosphate, thanks to diminished regeneration of the molecule under severe drought stress (Bota, Medrano and Flexas, 2004). ROS are also produced under drought stress via the Mehler reaction, and again

attack photosynthetic apparatus, such as thylakoid membranes, as described above for heat stress, further impeding photosynthesis (Asada, 2006; Cruz de Carvalho, 2008; Farooq, Hussain and Siddique, 2014).

1.11. Drought Stress Causes Altered Morphology, and Cellular and Physiological Damage

Not only can drought stress cause yield losses when applied throughout development, but it can also lead to altered morphology, as well as causing extensive cellular and physiological damage. Water limitations can significantly inhibit seed germination, whilst also causing reduced seed reserve utilization, subsequently decreasing seedling growth and vigour (Soltani, Gholipoor and Zeinali, 2006; Duan et al., 2017; Guo et al., 2017; Sharma et al., 2022), whilst also causing reduced shoot length (Guo et al., 2017; Ahmed et al., 2022; Sharma et al., 2022) and reduced biomass production (Ahmed et al., 2020, 2022) in wheat seedlings. Similar morphological and developmental effects are also seen when drought is applied later in development, with traits such as plant height, tiller number and leaf area all being reduced (Gupta, Gupta and Kumar, 2001; Sharma et al., 2022). Development of the plant is also sped up by drought stress exposure, like it is during or after exposure to heat stress, with the number of days to heading and maturity both being reduced by water limitation (Dhanda and Sethi, 2002; Sharma et al., 2022). This acceleration of development is known as the drought escape, whereby plants reduce the amount of time spent in vegetative development in order to flower faster, with the hope of either producing seed before the drought becomes more severe and causes death, or to minimize exposure to severe drought stress during the susceptible reproductive developmental stages, subsequently protecting yield from the stress (Riboni et al., 2013; Shavrukov et al., 2017; Bader et al., 2023).

Many of these morphological and developmental changes, as with yield, will be due, in large part, to the reduced photosynthetic potential seen under drought stress, as a result of damaged photosynthetic machinery, reduced Rubisco activity and abundance, and reduced CO_2 capture, as described previously, meaning fewer photoassimilates are available to fuel growth and development. For instance, drought stress has been observed to decrease the rate of photosynthesis, and net photosynthesis in wheat, likely via reduced intercellular carbon concentration in mesophyll cells, increased chlorophyll degradation, reduced PSII electron transport, and the inhibition of Rubisco via up to 60% of active sites being blocked by inhibitor binding (Parry et al., 2002; Shah and Paulsen, 2004; Zivcak et al., 2013; Sharifi and Mohammadkhani, 2016; Zhao et al., 2020)

However, wheat growth, morphology and physiology will also be significantly affected by changing water relations, with many of these changes being symptoms of stomatal responses to water availability, and of turgor changes in stomatal guard cells. Stomata are microscopic pores on the epidermal leaf surface which control gas exchange with the atmosphere, and subsequently allow atmospheric CO_2 to access leaf mesophyll cells and be captured by Rubisco for use in photosynthesis (Hetherington and Woodward, 2003; Kollist, Nuhkat and Roelfsema, 2014; Bertolino, Caine and Gray, 2019). This process, termed stomatal conductance, also allows the loss of water from the leaf surface via transpiration – meaning these pores are a key part of the physiological response to drought stress. Under drought stress, the lack of water within plant tissue, as well as the accumulation of other factors such as ABA and ROS, leads to a loss of turgor pressure in guard cells, and the closure of stomatal pores, reducing stomatal conductance and limiting the extent of water loss via transpiration (Hetherington and Woodward, 2003; Pirasteh-Anosheh et al., 2016; Agurla et al., 2018; Bertolino, Caine and Gray, 2019). This lack of transpiration on leaf surfaces reduces both the water potential and negative pressure gradients between leaf and root tissue, subsequently leading to reduced water uptake (Aston and Lawlor, 1979). The lack of available water,

stemming from reduced stomata-mediated evapotranspirational water uptake, impairs mitosis and cell elongation, resulting in reduced growth and leaf expansion (Fahad et al., 2017), as well as elevated leaf surface temperatures, leaf wilting and curling (Ali et al., 2022), further reducing the photosynthetic potential of the plant.

Not only does the reduced soil water availability seen under drought stress lead to changing water relations within the plant, but growth and development can be further impeded by the effect of this diminished soil moisture on soil nutrient availability and uptake. For instance, under drought conditions, the radius of water-filled pores in the soil decreases, causing tortuosity to increase and phosphorus to become less mobile – subsequently reducing phosphorus uptake, and foliar phosphorus content (Sardans and Peñuelas, 2004; Faye et al., 2006; Noman et al., 2018). Drought stress can also reduce the active transport of and membrane permeability for potassium, calcium and magnesium ions, again leading to reduced uptake from the soil (Sallam et al., 2019), and reduced accumulation in above-ground tissue (Sardans, Peñuelas and Ogaya, 2008; Noman et al., 2018). Micronutrient availability is also affected by soil moisture content, with micronutrients such as manganese, iron and molybdenum being more soluble under well-watered conditions – subsequently, drought has been observed to cause reduced accumulation of such elements in plants (Hu and Schmidhalter, 2005; Sallam et al., 2019).

Periods of drought stress also cause an accumulation of ROS, with ROS production and the severity of drought stress having a linear relationship in durum wheat (El Keroumi, Dihazi and Naamani, 2019). As discussed previously, generation and accumulation of ROS can cause serious cellular harm via disruption to photosynthetic machinery, and reductions in enzyme activity – but particularly via phospholipid membrane peroxidation, which reduces the membrane's ability to control ion flux into and out of the cell, and subsequently reduces organellar or cellular function, potentially resulting in cell death. Under drought stress, membrane stability is not only threatened by ROS production, but also by the activity of lipoxygenase, which acts to peroxidise polyunsaturated fatty acids, and whose activity is heightened during periods of drought (Sánchez-Rodríguez et al., 2010; Alam et al., 2013).

1.12. Amelioratory Molecular Responses to Drought Stress

As discussed previously, drought-induced stomatal closure is a key part of the response to water limitation as the plant attempts to prevent water loss via a reduced rate of transpiration from the leaf surface. Although this closure is regulated by cellular moisture content causing stomatal guard cells to lose turgor pressure, other factors also play key roles in this process. ABA accumulates under drought stress, and, amongst other functions, has long been held as the key regulator of stomatal closure, with high correlations being observed between leaf ABA concentration and stomatal conductance in wheat (Loveys and Kriedemann, 1974; Quarrie, 1980; Henson, Jensen and Turner, 1989; Munns and Sharp, 1993; Saradadevi et al., 2014, 2015). However, ABA alone is unable to completely determine stomatal conductance; for example, when excised wheat leaves were exogenously fed solutions without ABA, partial stomatal closure was observed (Dodd and Davies, 1994) – suggesting roles for other compounds in this process. ROS are also able to influence stomatal conductance, acting in unison with ABA, as ROS accumulation in the apoplast and chloroplasts under drought stress is triggered by a complex ABA-mediated signalling cascade which ultimately results in transmission of the ABA signal via OST1 and, subsequently, stomatal closure (Song, Miao and Song, 2014; Sierla et al., 2016; Postiglione and Muday, 2020). Similarly, other phytohormones have been identified as regulators of stomatal conductance, with jasmonic acid prompting stomatal closure, largely to prevent pathogen invasion during periods of biotic stress (Förster et al., 2019; Zamora et al., 2021), whilst cross talk between jasmonic acid and ABA has been observed with respect to stomatal conductance, as application of an ABA

inhibitor led to reduced methyl-jasmonate-mediated stomatal closure (Hossain et al., 2011). JA has been found to accumulate in wheat under drought stress too, however, thanks to the upregulation of genes involved in the phytohormone's biosynthesis (Wang et al., 2021c) – suggesting such a response may illicit stomatal closure under drought stress.

Although ABA plays a fundamental role in stomatal closure under drought, its accumulation also affects the expression of genes such as *ERF3*, which acts to reduce endogenous H_2O_2 levels, thus limiting the amount of cellular damage done under drought stress (Rong et al., 2014). Perception of ABA also leads to downstream amelioration of drought stress damage, as greater expression of the ABA receptor *PYL1-1B* in wheat led to increased ABA sensitivity, as well as improved growth and yield under drought stress, thanks to enhanced photosynthetic capacity and water-use efficiency (Mao et al., 2022a). The beneficial impacts of phytohormone accumulation has also been shown via exogenous application; for instance, JA and salicylic acid treatment improved the ability of wheat seedlings to grow under drought stress conditions, thanks to increased activities of antioxidant enzymes, and a subsequent reduction in the extent of ROS-mediated cellular damage (Kang et al., 2013; Shan, Zhou and Liu, 2015). Some phytohormone signalling pathways are actually depleted under drought stress – this is particularly true of auxins, which largely act to regulate processes essential for normal development, and so auxin receptors and transporters are downregulated to ensure more resources are allocated to processes involved in the stress response, such as those controlled by the aforementioned phytohormones (Krugman et al., 2011; Abhinandan et al., 2018).

Much like the expression of HSPs is a key feature of the plant's molecular response to heat stress, the expression of genes encoding late embryogenesis abundant (LEA) proteins is fundamental to the molecular drought response. LEAs have no enzymatic activity and instead act as chaperones to prevent the formation of toxic protein aggregates during periods of drought stress, whilst also acting to protect the function of other proteins, as well as stabilizing cellular and organellar membranes (Tolleter, Hincha and Macherel, 2010; Hanin et al., 2011; Cuevas-Velazquez, Rendón-Luna and Covarrubias, 2014; Liu et al., 2019). A total of 179 LEAs have been identified in wheat (Liu et al., 2019), however one group in particular, the dehydrins, are particularly involved in ameliorating the effects of drought stress. At least 54 putative dehydrins have been identified in wheat, with the expression of many of these known to respond to drought stress, ultimately leading to increased protein levels as soil moisture decreases during drought stress (Lopez et al., 2003; Zhang et al., 2013b; Wang et al., 2014; Hassan et al., 2015; Hao et al., 2022). High levels of such accumulation and expression under drought stress are associated with improved growth in hexaploid wheat (Hassan et al., 2015), with dehydrin expression under drought stress leading to improved shoot growth (Lopez et al., 2003), whilst the transcription factor bHLH49 was able to confer improved relative water content, chlorophyll content and membrane stability under drought stress thanks to its positive regulation of *WZY2* dehydrin expression (Liu et al., 2020a). Not only are dehydrins able to illicit improved growth under drought stress via their chaperone activity, but recent evidence suggests that they may also act to control the expression of other genes which act as part of the drought response, whilst also playing an indirect role in drought-induced epigenetic modifications (Tiwari and Chakrabarty, 2021).

As with heat stress, increased expression and activity of antioxidant enzymes are a large part of the molecular response to drought stress, as the plant attempts to prevent oxidative damage to proteins and membranes. For example, drought stress was found to increase the expression and activity of CAT by up to 50% (Luna et al., 2005; Devi, Kaur and Gupta, 2012), whilst similar increases in activity were seen for APX, GR and POD in the endosperms and shoots of wheat seedlings under drought stress (Devi, Kaur and Gupta, 2012). The same authors also found that the varieties able to grow well under drought stress were those which

showed upregulation in the activity of any three of these antioxidant enzymes (Devi, Kaur and Gupta, 2012), whilst other work has found that increased activity of APX and CAT resulted in improved growth and reduced lipid peroxidation under drought stress (Sairam, Deshmukh and Saxena, 1998), and similarly, improved ROS homeostasis, thanks to increased antioxidant activity, led to longer survival under drought stress, as well as a less severe reduction in shoot biomass, compared to control plants (Wang et al., 2008).

Under drought stress, plants often also accumulate osmolytes, such as proline and sugars, to lower the osmotic potential of cells and increase water uptake – subsequently leading to maintenance of cellular turgor, as well as improved carbon intake and assimilation, and, ultimately, improved growth (Subbarao et al., 2000; Ullah et al., 2022). Not only this, but proline accumulation under drought stress can also lead to improved membrane stability, and reduced levels of oxidative damage (Mahajan and Tuteja, 2005; Farooq et al., 2018). Such drought-induced accumulation has been observed in wheat (van Heerden and de Villiers, 1996; Ullah et al., 2022; Kamruzzaman et al., 2023), with increased accumulation of proline being seen in wheat varieties able to grow well under drought stress (van Heerden and de Villiers, 1996; Ullah et al., 2022).

1.13. Regulation of Molecular Responses to Heat and Drought Stress

The responses to heat and drought stress, described previously, allow plants to survive for longer under these sub-optimal growing conditions – therefore, understanding how these responses are regulated is important if the key genes, proteins and processes underpinning these responses are to be identified. These amelioratory responses are largely mediated by changing levels of protein accumulation within cells under heat and drought stress, with this change in the cellular proteome subsequently leading to significant molecular and physiological effects. The first step in this proteomic change is differential gene expression, with varying levels of transcript abundance subsequently leading to differences in the amounts, and types, of proteins produced. Much work has been done on the differential expression of genes under heat and drought stress in wheat, identifying that the stresses cause huge transcriptomic shifts at different stages of development (Qin et al., 2008; Aprile et al., 2009; Liu et al., 2015; Ma et al., 2017; Chaichi et al., 2019; Iquebal et al., 2019; Jin et al., 2020; Chu et al., 2021). This transcriptomic shift under abiotic stress is often largely the result of transcription factor activation or repression, as these proteins recognize and bind to conserved sequence motifs in the regulatory regions of these genes, subsequently recruiting or blocking the binding of the transcriptomic machinery. Different transcription factor families bind to different sequence motifs, and subsequently allow tailored regulation of gene expression in response to different stresses.

Due to the accumulation of ABA under both stresses, but particularly under drought stress, transcription factors which bind to the ABA response element (ABRE) motifs, a group of proteins called ABA response element binding proteins, are key regulators of gene expression under drought stress (Shen and Ho, 1995; Yoshida, Mogami and Yamaguchi-Shinozaki, 2014). These transcription factors often coordinate the transcriptomic responses of key genes which act to ameliorate the effects of drought stress in wheat, such as LEAs and dehydrins – for instance, *AREB3* was able to improve growth under drought stress when transgenically expressed in Arabidopsis, thanks to regulation over the expression of a suite of LEAs including *AtRD29A* and *AtRD29B* (Wang et al., 2016), whilst wheat dehydrins have repeatedly been shown to respond to ABA treatment, likely due to the fact that the ABRE was found in the promoter sequence of every wheat dehydrin gene (Borovskii et al., 2002; Wang et al., 2014; Zhu et al., 2014; Yang et al., 2015; Yu, Wang and Zhang, 2018; Hao et al., 2022). Regulation of transcription under drought also occurs independently of ABA accumulation, particularly through the action of dehydration-responsive element binding proteins (DREBs), which bind

to conserved dehydration response elements in the promoter region of genes. 210 DREBs have been identified in wheat (Niu et al., 2020), with these authors identifying that transgenic overexpression of *DREB3* in Arabidopsis led to improved growth under drought stress, thanks to increased chlorophyll and proline content, increased activity of enzyme detoxifiers POD, SOD and CAT, and, subsequently, reduced electrolyte leakage as a result of improved membrane stability (Niu et al., 2020). This overexpression resulted in the significant upregulation of key genes involved in the response to drought stress, such as LEAs and dehydrins – suggesting that the expression of these gene families are also regulated by DREB binding under drought stress in wheat. Another group of transcription factors heavily involved in regulating the wheat drought response are the NAC transcription factors; proteins identified by a conserved N-terminal DNA-binding domain (Ooka et al., 2003). As with the other transcription factor families described here, NAC transcription factors have been shown to improve growth when overexpressed in Arabidopsis (Mao et al., 2012; Huang et al., 2015; Mei et al., 2021; Ma et al., 2022). This improved growth likely comes as a result of NAC transcription factors' ability to regulate the expression of key genes such as LEAs and dehydrins, as well as genes controlling stomatal conductance and membrane stability. For example, overexpression of *NAC5D-2*, *SNAC4-3A* and *NAC2* resulted in increased expression of dehydrins and LEAs (Mao et al., 2012; Mei et al., 2021; Ma et al., 2022), whilst *NAC5D-2* and *SNAC4-3A* overexpression resulted in reduced stomatal aperture and thus less water loss under drought stress – a response likely caused by changes in gene expression mediated by the transcription factors, with *NAC5D-2* overexpression leading to repression of *AtABI1* and *AtABI2*, which themselves act as negative regulators of stomatal closure (Ma et al., 2022). Again, this suggests that these transcription factors regulate similar genes in wheat – a hypothesis supported by the observation that overexpression of *NAC29* in wheat led to improved membrane stability and growth under drought stress, as well as higher levels of SOD and CAT activity (Chen et al., 2018).

Transcriptional regulation is also a key determinant of the physiological and molecular response to heat stress in wheat, with one family, in particular, playing an incredibly large role. Heat shock transcription factors (Hsfs) bind to heat shock element (HSE) motifs in the promoter regions of heat-responsive genes, and subsequently either activate or repress their expression. Although Hsfs are involved in regulating gene expression under ambient temperatures, their main roles lay in the sensing and signalling of heat stress, as well as the regulation of HSP gene expression under elevated temperatures (Akerfelt et al., 2007; Scharf et al., 2012; Kumar et al., 2018). For example, *HSFA6e* was found to target *HSP17*, *HSP70* and *HSP90* and induce their expression under heat stress in wheat (Kumar et al., 2018), whilst overexpression of *HsfA2-1* in Arabidopsis led to more significant upregulation of *AtHSP18.2*, *AtHSP32*, and *AtHSP70b* (Liu et al., 2020b), and similarly, the overexpression of *HsfA2e-5D* led to increased expression of various HSPs as well as some genes encoding antioxidant enzymes in Arabidopsis (Bi et al., 2022). This regulation of HSP gene expression, as well as the regulation of other heat-responsive genes, means these transcription factors are a key part of the protection against protein misfolding and aggregation under heat stress, with their importance being shown in the previously described work, as increased upregulation of *HSFA6e* was correlated with improved growth under heat stress as well as increased antioxidant enzyme capacity and decreased lipid peroxidation (Kumar et al., 2018), whilst *HsfA2-1* overexpression in Arabidopsis led to increased chlorophyll content, membrane thermostability, and survival rate under heat stress (Liu et al., 2020b), as did overexpression of *HsfA2e-5D* (Bi et al., 2022).

Not only can the activity of transcription factors lead to regulation of the response to heat and drought stress, but protein structure and modification are also key features of such regulation. For instance, although *HSP* gene expression is controlled by Hsf binding, before the

transcription factors can bind to HSE in the *HSP* promoter regions, they must first undergo some structural changes. Under normal temperatures, Hsf proteins are present in an inactive form as they are bound in a complex by HSP90 and HSP70 proteins – however, under heat stress this complex breaks down, releasing Hsf transcription factors from the chaperone complex, subsequently allowing them to oligomerize, bind HSEs and regulate heat-responsive gene expression (Lee et al., 2000; Hahn et al., 2011; Gomez-Pastor, Burchfiel and Thiele, 2018; Kumar et al., 2018). Similarly, post-translational modifications to proteins can play large roles in the response to these stresses, with phosphorylation of proteins being a key part of many signalling cascades which ultimately lead to the regulation of these molecular responses. For example, to permit ABA-induced regulation of gene expression under drought stress (or any stress where ABA accumulates), ABA first binds to PYL receptors which leads to the inhibition of the phosphotase activity of PP2Cs via the formation of ABA-PYL-PP2C complexes. Reduced PP2Cs phosphatase activity subsequently leads to the accumulation of phosphorylated class III SNF-1-related protein kinases 2 (SnRK2s), which then directly phosphorylate members of the AREB transcription factor family, activating them to bind to ABRE in promoter regions of ABA-responsive genes, and subsequently allowing the regulation of their expression (Cutler et al., 2010; Duarte et al., 2019).

Modifications to proteins can also affect gene expression in plants under heat and drought stress if they are made to histones – the proteins which bind DNA to form chromatin. Post-translation modifications to these histones will cause different effects on gene expression, depending on the residue modified, and the modification made, as the DNA either becomes more or less exposed, subsequently affecting transcription factor and transcriptional machinery binding. For instance, methylation of H3K4 and acetylation of H3K9ac residues under severe drought stress facilitated increased expression of LEAs and dehydrins (Kim et al., 2008, 2012a), whilst addition of the H3K4me3 mark by AtATX1 under drought stress is required for the upregulation of ABA-induced LEAs *AtRD29A* and *AtRD29B* (Ding, Avramova and Fromm, 2011). Similarly, DNA methylation can also affect transcription factor and transcriptional machinery binding, thus affecting gene expression. In wheat, the level of DNA methylation has been found to increase as drought progressed, however the level of DNA methylation was also different between varieties, leading the authors to suggest that some varieties are able to more rapidly respond to drought stress, thus affecting their ability to survive under such conditions (Duan et al., 2020). Other work, however, found that removal of DNA methylation marks under drought stress may also determine the ability of different varieties to grow well under drought stress (Kaur, Grewal and Sharma, 2018), whilst recent work found that demethylation of the ATG-proximal regions in the promoters of *P5CS* and *BADH* promoted the accumulation of the osmolytes proline and betaine in wheat under drought stress (Li et al., 2023).

1.14. Inherent Tolerance also determines a Plant's Ability to Survive under Heat and Drought Stress

Although a plant's ability to quickly respond to changing environmental conditions will determine its ability to survive under heat and drought stress, natural genetic variation can also predispose plants to tolerate these stresses, or be susceptible to their damaging effects, via control over key traits – with tolerant varieties often being defined as those which show little difference in growth, or in a key trait, when grown under the stress, compared to when grown under optimal conditions. For instance, the level of cuticular wax accumulation on wheat leaves was found to be associated with yield under drought stress, with those varieties producing wax on their leaf surface showing increased yield and photosynthetic rate when exposed to drought stress (Guo et al., 2016a). Similarly, allelic variation in the *WD40-4B.1* gene accounts for differing levels of drought tolerance, as those varieties possessing a nonsense mutant allele in the gene showed a greater extent of leaf wilting under drought

stress, likely due to increased H_2O_2 accumulation and oxidative damage, because the mutant allele encodes a truncated protein which is unable to interact with catalases and promote their activity via oligmerization (Tian et al., 2023). Genetic variation can often also lead directly to increased gene expression under control conditions, which can have subsequent effects on inherent drought tolerance; for example, a mutation in the promoter region of the wheat *PYL1-1B* gene has resulted in the presence of a MYB transcription factor recognition site which is bound by MYB70, leading to increased expression of *PYL1-1B* under control conditions and, subsequently, higher yields under drought stress (Mao et al., 2022a).

Similarly, genetic variation under control conditions has been found to be associated with inherent thermotolerance in wheat; for example, a screen of 2111 spring wheat varieties identified several single nucleotide polymorphism (SNP) markers significantly associated with variation in cell membrane stability under heat stress – meaning those varieties which showed high levels of cell membrane stability under heat stress (i.e. thermotolerant varieties) could be identified via the presence of these SNPs (El-Basyoni et al., 2017). 30 SNPs within a quantitative trait locus (QTL) on chromosome 4a that affects thermotolerance (called *HST1*) have been identified as quantitative trait nucleotides, with the presence or absence of these sequence variants being significantly associated with the level of reduction in shoot and root weight seen under heat stress exposure (Khan et al., 2022). Deletion polymorphisms have also been found in this QTL amongst two breeding lines; one of which was thermotolerant (E6015-4T), whilst the other was susceptible to damage by heat stress (E6015-3S), as E6015-4T showed less severe leaf wilting, as well as increased chlorophyll levels, membrane stability and photosynthetic rate after heat stress exposure. The susceptible variety showed heightened levels of gene deletion at the distal end of the QTL, removing 19 genes and thus impairing the thermotolerance action of the *HST1* QTL – explaining the leaf wilting, chlorophyll damage, membrane damage and decreased photosynthetic rate seen in this variety under heat stress (Zhai et al., 2021).

1.15. Methods used to understand the Regulation of the Responses and Tolerance to Heat and Drought Stress

Since the advent of faster and cheaper sequencing technologies in the 21st century, researchers have been able to use a wide variety of powerful approaches to identify genes which may be acting to regulate both tolerance to and the responses to heat and drought stress in hexaploid wheat. For example, genome-wide association studies (GWAS) have been used to identify regions of sequence variation (SNPs) associated with traits which may confer improved tolerance to these stresses, subsequently identifying candidate genes which may confer these beneficial traits (Mathew et al., 2019; Li et al., 2019; Maulana et al., 2020; Abou-Elwafa and Shehzad, 2021; Khan et al., 2022). Despite this approach proving to be successful, other researchers have opted to use different approaches to gain a clearer relationship between marker and trait, as those SNPs identified by GWAS are often not causal, and are instead merely in high linkage disequilibrium with a genomic region, in which the gene controlling the beneficial trait lies. Gene expression markers, such as those provided by differential expression analysis of RNA sequencing data, perhaps provide a clearer link, with quantitative changes in gene expression being seen between varieties which show different traits, or between plants exposed to different conditions (Qin et al., 2008; Liu et al., 2015; Ma et al., 2017; Lv et al., 2018; Iquebal et al., 2019; Jin et al., 2020; Chu et al., 2021). However, these studies, much like GWAS, often provide a wealth of potentially causative candidate genes, all of which contribute a small amount to the trait – therefore meaning screening these genes to identify large-effect regulators can be incredibly time-consuming. The use of network analyses, however, circumvents this, as relationships between factors in a system are predicted, and a more concise list of potential regulators of gene expression, and subsequently of a trait, are identified (Pavlopoulos et al., 2011). Weighted gene co-expression network

analysis (WGCNA) (Langfelder and Horvath, 2008, 2012), is an example of this approach which establishes a correlation gene network by grouping genes with similar expression patterns, across all samples, into groups where genes are deemed to be connected if they are significantly co-expressed across samples. These groups, called modules, therefore often contain genes involved in the same, or similar, biochemical or physiological processes, meaning the most central and well-connected gene within modules may be good candidates for master-regulators of this process – these genes are called hub genes. Screening this concise list of hub genes is therefore much less time-consuming, whilst the co-expression network also provides researchers with information which can be measured during the screening process – such as, whether disruption of hub gene expression results in changes in expression of the genes it is connected to within the module, and whether such changes have an effect on the trait under study. However, despite the power and attractive qualities of such a method, network approaches have been relatively underutilized in the identification of genes which may regulate tolerance, or the response, to heat and drought stress (Girousse et al., 2018; Lv et al., 2020; Mishra et al., 2021; Du et al., 2022; Tian et al., 2022).

1.16. Current Breeding Efforts are Aiming to Increase Heat and Drought Tolerance by Introducing Genetic Diversity into Elite Wheat Varieties

Due to the aforementioned lack of genetic diversity within elite wheat varieties cultivated around the world, relative to wheat landraces, for example, they often do not exhibit traits, such as those described above, which would predispose them to better tolerate periods of heat or drought stress. The current efforts of wheat breeders around the world, therefore, are focussed on introducing new genetic diversity into these varieties, with the hope of improving heat and drought tolerance. This genetic diversity can come from natural sources, or can be induced via mutation, manipulation of gene expression levels, expression of transgenes, or gene editing.

Manipulating the expression of existing genes in wheat can be can done via *Agrobacterium tumefaciens*-mediated transformation of the plant with an expression vector containing the gene under the control of a constitutive promoter, such as the ubiquitin or cauliflower mosaic virus promoters. Such an approach was used to overexpress the wheat *PYL1-1B* and *FER-5B* genes, with overexpression of the former resulting in increased ABA sensitivity, photosynthetic capacity and water use efficiency, and subsequently improved drought tolerance and grain yields under drought stress (Mao et al., 2022a), whilst overexpression of the latter led to improved thermotolerance, thanks to increased membrane stability and PSII efficiency under heat stress (Zang et al., 2017). *Agrobacterium tumefaciens*-mediated transformation can also be used to introduce genes from other species, called transgenes, into wheat with the hope of improving stress tolerance. For example, overexpression of the *OsSNAC1* gene from rice led to improved water and chlorophyll contents in leaves under drought stress, as well as increased survival under drought (Saad et al., 2013), whilst transgenic overexpression of the *Escherichia coli* gene *mtlD* in wheat led to improved tolerance to water limitation thanks to increased mannitol accumulation, as transgenic plants showed smaller reductions in fresh weight, dry weight, plant height and flag leaf length compared to the negative control plants (Abebe et al., 2003). Such findings, however, have not really aided wheat breeders in their production of stress tolerant wheat varieties, as legislation in many parts of the world, especially in the European Union, prevent the growth of crops containing genetic variation induced by such methods.

However, the advent of gene-editing technologies, such as CRISPR-Cas9, has increased the likelihood that crop varieties produced via genetic variation induced in the laboratory could one day be grown in the field, as governments around the world are more amenable to the growth of such crops – for example, the United Kingdom recently voted the Genetic

Technology (Precision Breeding) Act into law, stating that crop varieties produced using gene editing technologies, such as CRISPR-Cas9, could be cultivated within the country. Although such technology has successfully been used to specifically mutate drought-responsive genes *ERF3* and *DREB2* in wheat (Kim, Alptekin and Budak, 2018), and to confirm the role of *NAC071-A* in determining drought tolerance via inducing mutations and subsequently reducing the gene's expression (Mao et al., 2022b), there is little evidence of the technology unequivocally being used to improve heat and drought stress tolerance in wheat.

Genetic variation can also be induced via exposure to chemical mutagens which cause changes in the genome sequence, and can subsequently change gene expression and protein functionality. The approach has been used to generate varieties with improved agronomic yield traits for a range of species, including rice, barley, and oilseed rape (Parry et al., 2009), however has also led to the production of more stress tolerant wheat varieties. For instance, exposure to the mutagen ethyl methanesulfonate (EMS) caused point mutations across the genome, and led to the production of the variety BIG8-1, which can endure 21 days without water, and maintain its relative water content, chlorophyll content and stomatal conductance during seven days of drought stress (le Roux et al., 2021). Similarly, exposure to the alkylating mutagen sodium azide again resulted in the production of random point mutations throughout the genome, and, subsequently, the variety RYNO3936. The variety showed increased expression of Rubisco, LEAs and dehydrins, and was also able to maintain increased chlorophyll content, photosynthetic rate and stomatal conductance under seven days of drought stress (le Roux et al., 2020).

Restrictions on the genetic diversity of hexaploid wheat have been in place since the formation of the species, as the genetic diversity possessed by those early hexaploids depended entirely on that possessed by its tetraploid and diploid parent plants. Therefore, in an attempt to remove some of these constraints, and capture more genetic diversity amongst hexaploid wheat's tetraploid and diploid parents, the polyploidization event between *T. turgidum* and *A. tauschii* has been artificially repeated by researchers – resulting in the formation of synthetic hexaploids. Because researchers are conducting the hybridization themselves, they are able to select *T. turgidum* and *A. tauschii* varieties which show certain desirable traits, or are adapted to grow well under certain environmental conditions; hopefully resulting in the formation of a hexaploid wheat variety that inherits these traits, and the genetic diversity which encodes them. For example, crossing the *T. turgidum* variety Langdon with 17 different *A. tauschii* accessions with a range of drought tolerance levels led to the production of 17 synthetic hexaploid varieties, which also showed differing levels of drought tolerance, with those hexaploids descended from drought tolerant *A. tauschii* accessions being significantly more drought tolerant than those descended from *A. tauschii* accessions susceptible to damage by drought stress (Kurahashi, Terashima and Takumi, 2009). Similarly, crossing *T. turgidum* with 67 *A. tauschii* varieties with a range of ABA-responsiveness levels, resulted in the formation of hexaploids that showed varying levels of ABA-inducible gene expression, and drought tolerance – likely stemming from the inherited genetic diversity on their D genome (Iehisa and Takumi, 2012).

The progenitor species used to make these synthetic hexaploid, as well as the wild relatives of these progenitors, can also be used, in their own right, to introduce genetic diversity into hexaploid HYVs, and enhance fitness under different environmental perturbations. For example, a recent screen of 113 diverse wild wheat relatives identified several species which showed extreme susceptibility or extreme tolerance to Fusarium head blight, with the authors identifying that introgression wheat lines, where a terminal portion of the 3B chromosome had been replaced by a similarly sized portion of the 3G chromosome from *T. timopheevii*, showed significantly enhanced resistance to the disease (Steed et al., 2022).

Diversity does exist within hexaploid wheat varieties, however, with hexaploid landraces showing extensive genetic and phenotypic diversity thanks to their adaptation to different environments via natural and small-scale artificial selection by local farmers, as described previously (Zeven, 1998; Reynolds, Dreccer and Trethowan, 2007; Corrado and Rao, 2017; Schmidt et al., 2019; Cseh et al., 2021; Tehseen et al., 2022). Seeds of such varieties are often housed in large collections, gathered by breeders and/or researchers to preserve diversity, and to be used as a tool in the generation of new crop varieties. Such germplasm can, therefore, easily be sourced by fellow researchers around the world for their own work. The A. E. Watkins collection of wheat landrace varieties, for example, shows remarkable genetic and phenotypic diversity when compared to a collection of modern elite varieties (Wingen et al., 2014), as traits such as flowering time and plant height showed larger ranges, whilst d_{PIC}, Nei's gene diversity, and Shannon-Weaver Diversity Index scores (measures of genetic diversity) were all lower in the modern varieties. The extensive genetic diversity across the landrace collection was shown, with an average allele number per locus of 22.4, ranging from 3 to 61 alleles, being seen across 45 loci (Wingen et al., 2014). Similarly, work assessing the genetic diversity of a Central European landrace collection containing varieties tolerant to drought and heat stress found that the landrace collection showed greater nucleotide diversity than the modern varieties, and that these varieties contained a higher average number of polymorphisms (Cseh et al., 2021). As well as this, a set of novel alleles were identified in the landrace collection, many of which were co-localized with genes which may determine heat or drought stress tolerance, such as those encoding DREB transcription factors (Cseh et al., 2021).

As well as showing increased levels of phenotypic and genetic diversity, compared to modern varieties, adaptive traits likely conferring improved drought tolerance have also been found in wheat landraces, further suggesting such varieties may be more adaptable to the changing climate than the widely used HYVs, which are increasingly showing yield losses under more frequent and intense periods of heat and drought stress (as discussed previously in 1.7. and 1.10.). For example, one Mexican landrace has been found to show increased water extraction at deep soil levels, which, if paired with the water use efficiencies of elite varieties via crossing, could confer up to 36g m^{-2} more biomass production under water-limited conditions (Reynolds, Dreccer and Trethowan, 2007). Such traits are likely results of genetic variation in these landrace varieties, with other work identifying that favourable alleles, conferring improved combined heat and drought tolerance, were widespread amongst Asian and African landraces, with the authors suggesting that these varieties be used in breeding programmes to improve the stress tolerance of modern elite varieties, via the introduction of this novel genetic diversity (Schmidt et al., 2019). For example, the introduction of a single novel locus from chromosome 6a, which confers higher grain wheat, harvest index, thousand kernel weight, and grain number under combined heat and drought stress, into modern elite lines may result in the production of a variety whose yields are resistant to the effects of climate change, and therefore would represent a large step towards achieving the necessary yield increases to support the projected rate of population growth, despite the changing climate.

Both of these works emphasize the inherent value of the genetic and phenotypic diversity held by these landraces to wheat breeders, and much further downstream, to those who depend on wheat for food. Namely, the adaptive traits they exhibit, conferred by novel genetic diversity, can be captured and transferred to HYVs via conventional breeding, to improve the climate-resilience of these lines, and mitigate the damaging effect of the changing climate on future yields, thus maintaining food security.

The value of these climate-adaptable landrace varieties to breeders is further heightened because, relative to the other methods of introducing novel genetic diversity into HYVs

discussed previously, the genetic diversity possessed by wheat landraces exists naturally, so capturing and utilizing this diversity, via conventional breeding with modern varieties, is, at least in theory, less complicated than generating new diversity in hexaploid wheat via the production of synthetic hybrids, or by mutagenesis. Similarly, because the genetic diversity possessed by landraces occurred and accumulated naturally over many years of adaptation, their use to introduce novel genetic diversity into modern elite varieties will not encounter the same legislative restrictions as the use of transgenics, for example, would – meaning varieties produced by this method would have potential for more widespread use.

Issues may arise when conducting such crosses, however, due to the random nature of recombination during sexual reproduction, as crossing landrace varieties, carrying sequence variation conferring an adaptive trait of interest, with a HYV may not only result in the production of F1 offspring carrying the adaptive trait, but may also result in unintended phenotypic consequences, thanks to recombination at distant non-target loci, or as a result of linkage drag at nearby loci (Waines and Ehdaie, 2007; Bai, Liang and Hawkesford, 2013; Rebetzke et al., 2014; Voss-Fels et al., 2017). Such unintended changes, and the traits/phenotypes they confer, can mitigate the effect of the sequence variation at the target locus, and even lead to reduced environmental adaptability and fitness. Backcrossing to the HYV parent will temper the effects of the distal non-target genetic changes – reverting these sequence changes back to their HYV allele – however, this is not the case for genetic changes, and phenotypes, introduced by linkage drag. Such changes are made at loci physically linked to, and therefore inherited with, the target locus, therefore backcrossing the hybrid with its HYV parent will result in the removal of both the target and non-target loci. Such linkage drag can therefore be mistaken for a poorly performing genetic marker during the breeding process, with countless promising markers likely to have been discounted by breeders over the years due to the silent effect linkage drag is having on a trait, or the fitness of the hybrid progeny.

These same challenges face wheat breeders using progenitor species and their wild relatives (which, like hexaploid landraces, also adapted to specific conditions in their environment of origin) to introduce genetic diversity into hexaploid HYVs, and enhance fitness under different environmental perturbations (Dempewolf et al., 2017; Renzi et al., 2022). Although there has been success using this method, such as recent work identifying that introgression wheat lines, where a terminal portion of the 3B chromosome had been replaced by a similarly sized portion of the 3G chromosome from *T. timopheevii*, showed significantly enhanced resistance to Fusarium head blight (Steed et al., 2022), further complications arise due to the extensive genetic differences between these species and hexaploid HYVs (Dempewolf et al., 2017; Renzi et al., 2022). Differences in chromosome number and/or ploidy level often mean that hexaploid HYVs are reproductively distinct from their progenitor species and their wild relatives, with crosses often producing unfit or infertile offspring, unable to pass on any beneficial genetic diversity to the next generation – a serious issue, unseen when crossing landraces with HYVs, which cannot easily be remedied, and a major barrier preventing more widespread utilization of this approach among wheat breeders.

Therefore, although crossing HYVs with landraces does present some challenges for breeders, the approach should not be neglected, due to the extreme, and, as yet, relatively untapped, diversity held by these landrace varieties, which may hold the key to creating climate-resilient wheat varieties.

1.17. Aims and Approach of this Thesis

With all of this in mind, this thesis had the over-arching aim of identifying genetic markers associated with the regulation of either tolerance, or the response to heat or drought stress in hexaploid wheat landraces. To do this, the present work employed various transcriptomic

analyses to identify genes differentially expressed between plants under control and stress conditions, and genes whose variation in expression between landrace accessions was significantly associated with variation in stress tolerance. Gene expression data were also used to construct weighted gene co-expression networks, and subsequently to identify hub genes within stress-associated modules which may act as master-regulators of stress tolerance or the stress response. As discussed previously, hexaploid wheat landraces have not been extensively used in such comparative transcriptomic analyses, whilst the use of gene expression data from hexaploid wheat landraces to identify candidate master-regulators of stress tolerance or the response to stress is even less common, with no examples currently available in the published literature. The present work therefore represents a novel and exploratory approach to identify these candidate master-regulators, providing a better understanding of how the transcriptional response to these stresses may be regulated in hexaploid wheat. Ultimately, it is hoped that the markers identified in this work could, one day, be used in breeding programmes to aid the production of drought or thermotolerant wheat varieties which are better able to grow in the increasingly hostile climate.

To meet these aims, this thesis first introduces the YoGI wheat landrace panel, and its accompanying transcriptome data, before using these data to identify candidate master-regulators of basal thermotolerance in hexaploid wheat seedlings (**Chapter 2**). A selection of landraces from the panel are then used to identify candidate master-regulators of the response to heat stress in wheat seedlings, as transcriptome data under control and heat stress conditions are gathered, and the transcriptomic response is assessed (**Chapter 3**). This work also identifies a large number of genes putatively involved in promoting flowering and the floral transition which are downregulated after heat stress exposure, so, next, this thesis describes the effect of exposure to, and then removal of, early heat stress on flowering time, whilst also using the gene expression data gathered under control and heat stress conditions to identify gene expression markers significantly associated with flowering time after exposure to, and then removal of, early heat stress (**Chapter 4**). The thesis will then focus on the response to drought stress, with novel analyses being applied to open-source gene expression data to draw new insights – ultimately resulting in the identification of candidate master-regulators of the drought response, as well as potential candidate master-regulators of the ABA-mediated responses to other abiotic stresses (**Chapter 5**). Finally, this thesis will then describe how gene expression data from wheat landrace seedlings exposed to control conditions and drought stress were generated, before the transcriptomic response to this stress was assessed and candidate master-regulators of the response were identified (**Chapter 6**).

2. Co-expression Network Analysis of Diverse Wheat Landraces Reveals Marker of Early Thermotolerance and Candidate Master-regulator of Thermotolerance Genes

2.1. Introduction

Triticum aestivum L. (bread wheat) is one of the most important crops worldwide, accounting for 20% of all calories consumed annually (Pfeifer et al., 2014; Food and Agriculture Organization of the United Nations et al., 2018). However, with the global population expected to increase to 9 billion by 2050, at least a 50% increase in crop yields must be achieved within the next three decades (Godfray et al., 2010; Tilman et al., 2011; Ray et al., 2013). A major obstacle in the way of this increase is the changing climate, as rising global temperatures has meant seasonal periods of extreme heat stress are becoming more common and water supplies are becoming heavily depleted (Hansen et al., 2006). This is particularly worrying for those dependent on wheat yields for their nutrition and/or income, as heat stress is especially damaging to wheat growth. As a cool season crop, wheat has an optimal growth temperature of around 20°C and shows a 3-6% reduction in yield for every degree above this optimum (Chowdhury and Wardlaw, 1978; Kobza and Edwards, 1987; Wardlaw et al., 1989; Nagai and Makino, 2009; Ray et al., 2013; Zhao et al., 2017; Tian et al., 2018). With the Intergovernmental Panel on Climate Change (IPCC) predicting that global mean surface temperatures towards the end of the century will be between 0.3 and 4.8°C higher than they were a century prior (Collins et al., 2013), and some models predicting the earth's average global temperature may rise by 2-5°C by the year 2060 (Wigley and Raper, 2001; Murphy et al., 2004; De Costa, 2011), wheat crops are likely to be in considerable danger to damage by heat stress in the coming years. Therefore, the cultivation of thermotolerant wheat varieties is of paramount importance, if crops are to be protected against an increasingly more hostile climate.

High-yielding elite varieties, produced during and after the Green Revolution, are used both as commercial food crops and in breeding programmes around the world but show reduced genetic diversity and an absence of alleles encoding novel traits, such as abiotic stress tolerance (Tanksley and McCouch, 1997; Keneni et al., 2012; Pingali, 2012; Fu, 2015). This loss of genetic diversity comes as a result of breeding programmes, which are significant genetic bottlenecks, reducing genetic diversity in search of favourable traits, such as high yields (Tanksley and McCouch, 1997; Keneni et al., 2012; Fu, 2015). Wheat landraces, however, are locally-grown varieties that have adapted, through a combination of natural selection and small-scale selection by farmers, to grow successfully in their local climate (Zeven, 1998). These landraces originate from a variety of different countries, and subsequently, climates, so exhibit great variation in their degree of abiotic stress tolerance. This phenotypic diversity is borne out of the remarkable genetic diversity shown by landrace varieties, as a result of their wide geographical distribution into distinct populations, and the absence of major genetic bottlenecks, such as intense breeding programmes, in their recent ancestry. The combination of phenotypic and genetic diversity means wheat landraces are a valuable resource for breeders in the production of varieties more resilient to the challenges posed by a changing climate.

Traditionally, conventional breeding approaches have been used in an attempt to improve the abiotic stress tolerance of wheat varieties (Schmidt, 1983; Pfeifer et al., 2005; Manès et al., 2012). The improvements made using such methods, however, are often marginal and slow, largely due to their untargeted nature (Pfeifer et al., 2005; Manès et al., 2012). More recently, powerful statistical genetics approaches such as genome-wide association studies (GWAS) have been employed to identify genetic markers (SNPs) associated with traits of interest, enabling the identification of candidate genes, and the selection of beneficial germplasm at

the seedling stage using marker-assisted breeding (MAB) (Mathew et al., 2019; Li et al., 2019; Maulana et al., 2020; Abou-Elwafa and Shehzad, 2021). Although such approaches have proved successful, the sequence markers identified using GWAS are often not causal – instead highlighting regions of the genome in high linkage disequilibrium with the causal gene. This approach, therefore, can frequently provide more questions than answers in terms of understanding the biological mechanism linking marker and trait.

The link between marker and trait is much clearer for gene expression markers, however, with differences in gene expression invariably affecting traits directly. The identification of such markers has become increasingly common over the past decade, due to improvements in RNA-seq technology allowing comprehensive studies into the effect of abiotic stress on the expression of the entire wheat transcriptome (Ma et al., 2017; Lv et al., 2018; Iquebal et al., 2019; Chu et al., 2021).

Although such studies can identify a wealth of differentially expressed genes, selecting and screening the most promising candidates for abiotic stress tolerance improvement can be laborious and time-consuming. To circumvent this, and quickly identify the most promising candidate genes, network approaches have become increasingly used in crop species. The creation of these networks has allowed the relationships between key factors in biological processes to be elucidated, and master regulators within systems to be identified (Pavlopoulos et al., 2011). Weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008, 2012) is the most commonly-used network approach, which establishes a correlation gene network to group genes with similar expression patterns, across all samples, into modules. These modules often house genes involved in the same, or similar, biochemical or physiological processes, and, subsequently, the identification of the most central genes within a module provides an insight into which genes may be the master regulators of the other genes in the module, and in turn, these processes. Manipulating the activity or expression of these central genes (or, “hub genes”), therefore, is likely to have a dramatic effect on phenotype, as the expression of the genes under the regulator’s control will also be affected, as a result of the manipulation. Despite the power of this method, however, network approaches have been used sparingly to study thermotolerance in wheat (Girousse et al., 2018; Mishra et al., 2021).

In the present work, transcriptomes of a panel of bread wheat landraces (hereon referred to as the YoGI panel), selected from multiple germplasm collections to represent a large selection of wheat-growing regions and environmental conditions, were sequenced. Thermotolerance hub genes housed within stress-associated modules, from a co-expression network created using expression data from the YoGI panel, were then identified and validated as markers for early thermotolerance. In addition, Transcriptome Display Tile Plots (TDTPs) were interrogated to discern whether naturally occurring rearrangements and homoeologous exchanges have occurred in any of the landrace accessions. Homoeologous exchanges, where recombination occurs between homoeologous (chromosomes from different subgenomes) rather than homologous chromosomes, can result in segmental deletions or duplications in one of the subgenomes, which could potentially affect thermotolerance if they affect hub gene locations.

2.2. Materials and Methods

2.2.1. YoGI Landrace panel

The YoGI panel constitutes 342 *T.aestivum* accessions sourced from: wheat collections held at the Germplasm Resource Unit (GRU), John Innes Centre, Norwich, UK; CIMMYT, Mexico; and the Crop Research Institute, Prague, Czechia (**Supplementary Data S2.1**). Landraces were selected to maximise diversity and representation of countries across all global wheat mega-environments (Sonder, 2016), and include both spring and winter habit accessions. A single plant from each accession was grown for RNA extraction with the second or third leaf harvested from seedlings at the midpoint of the day. RNA was extracted using the E.Z.N.A Plant RNA kit (Omega Bio-tek) according to manufacturer's instructions, including the recommended DNase I step. Leaf transcriptome data was generated using the Illumina HiSeq platform by Oxford Genomics Centre, Wellcome Centre for Human Genetics. Reads were trimmed to 127bp length using fastx_trimmer (Gordon, A. , Hannon, G. J., 2010) to meet the requirement of Maq program. The trimmed reads were mapped by Maq (Li, Ruan and Durbin, 2008) to the IWGSC RefSeq transcriptome v1.0 representative CDS models, with default parameters, meaning that reads with no more than two mismatches with summed Q >= 70 were mapped. Using perl scripts described in (Higgins et al., 2012), transcript abundance was quantified and normalized as reads per kb per million aligned reads (RPKM) value for each CDS model for each sample. All plants used in subsequent experiments were grown from selfed seed from the sequenced plant.

2.2.2. Transcriptome Display Tile Plots

RPKM values for each of the homoeologues triplets were rescaled between 1 and 0, where the individual with the lowest RPKM value = 1, the individual with the highest expression value = 0. These values were then converted to RGB hexcodes, where the A genome homoeologue is represented by cyan, B genome by magenta and D genome by yellow as previously (Harper et al., 2016; He et al., 2017). In addition to the landraces, representative AA (*Triticum urartu*), BB (*Aegilops speltoides*) and DD (*A. tauschii*) genome diploid species samples, and produced tetraploid (AABB, BBDD, AADD) and hexaploid (AABBDD) lanes were included *in silico* to aid analysis of colour variations.

2.2.3. Co-expression Network Construction and Module Detection

The WGCNA package (Langfelder and Horvath, 2008, 2012) was employed to construct a co-expression network in R (version 3.6.3.; R Core Team, 2021) using RPKM data from the YoGI landrace panel. Five accessions were removed after sample clustering, whilst 16,733 genes were removed due to too many zero values, leaving 94,057 genes from 337 accessions for network construction. The blockwiseModules() function conducted blockwise network construction according to the function's default parameters, except the following: maximum block size = 5000, soft threshold power = 8 (the first power to exceed a scale-free topology fit index of 0.85), minimum module size = 30, merge cut height = 0.25. After module detection, edge and node files were created using the "exportNetworkToCytoscape()" function with a threshold of 0.1; filtering out weak connections between genes (nodes).

2.2.4. GO Term Enrichment Analysis

To identify gene ontology (GO) terms significantly enriched in each module, the genes present in each module were collated and submitted to the agriGO Singular Enrichment Analysis tool (Du et al., 2010; Tian et al., 2017). A Fisher's exact test was performed for each module, using the GO terms possessed by all the genes used for network construction as background; 0.05 as the *p*-value threshold; Hochberg (FDR) as the multi-test adjustment method (Benjamini and Hochberg, 1995), and 5 as the minimum number of mapping entries threshold. A GO term was considered enriched in a module when its FDR-adjusted *p*-value was < 0.05. GO annotation terms of IWGSC RefSeq transcriptome v1.0 were retrieved from:

https://opendata.earlham.ac.uk/wheat/under_license/toronto/Ramirez-Gonzalez_etal_2018-06025-Transcriptome-Landscape/data/TablesForExploration/FunctionalAnnotation.rds (Ramírez-González et al., 2018).

2.2.5. Network Visualization and Hub Identification

To calculate degree (connection) scores for each gene, network modules were either visualized in Cytoscape (version 3.9.1.; Shannon et al., 2003) and analysed using the Cytoscape network analyser tool (Assenov et al., 2008), or the number of connections to and from each gene were counted in R. Those genes in a module which showed the highest degree scores (most connections) were deemed to be the central hubs (**Table S2.1**). In some cases, however, multiple genes in one module shared the highest degree score, whilst in other modules, the most well-connected gene was poorly characterized, with little known about its function. In these cases, the characterization of genes were used to determine which of the other well-connected genes in each module would most likely have a regulatory role on gene expression or physiological processes, based on our knowledge of the gene's function, or our knowledge of an orthologous gene in other species, such as *Arabidopsis*. In these instances, those genes which had high degree scores and appeared to potentially play a regulatory role were selected as the hub gene for further study. In other modules, multiple promising candidates were amongst the most well-connected genes, with only small differences in degree score between them. In this case, all well-connected promising candidate genes were taken forward for further study.

2.2.6. Hub Gene Validation

After hub gene identification, the expression of these genes were tested as markers for early thermotolerance in spring habit accessions. All hub genes with a mean RPKM >1 across the entire panel were taken forward as potential markers (*n* = six). Winter habit accessions were not used in validation experiments to prevent potential complications regarding heat stress exposure during early development interfering with the need to vernalize winter habit accessions.

15 spring habit landrace accessions were selected from the panel for use in the thermotolerance plant growth assay, based on their expression of the six hub genes (**Table S2.2**). It was hypothesized that because these accessions showed a range in hub gene RPKM values, they would also show varying degrees of early thermotolerance, and that there would be a significant relationship between the two factors. If such a relationship was observed, it would suggest that expression of the hub gene could be used as a marker of early thermotolerance.

Seeds of these accessions were sown in Levington Advance Seed & Modular F2S compost mixed with Aggregate Industries Garside Sands 16/30 sand (80:20 ratio), treated with CaLypso insecticide (Bayer CropScience Ltd., 0.083ml mixed with 100ml water, applied to each litre of compost). The seeds used in the present work were from plants selfed through at least three generations. Plants were grown in a Percival AR-75L growth cabinet at 22°C/16°C (day/night) on an 18 hour day/night cycle. All plants were exposed to these control conditions until the three-leaf stage of development was reached. At this point, half the replicates of each accession were moved to a separate Percival AR-75L growth cabinet and exposed to 35°C/30°C (day/night), with all other conditions being the same as in the control cabinet. Stressed plants were exposed to these conditions for 14 days following the three-leaf stage, before being returned to control conditions for three days to serve as a recovery period. The remaining half of the replicates of each accession remained under control conditions for the duration of the experiment. All plants were harvested 17 days after they had reached the three leaf stage, by cutting the stem at the soil surface. Dry biomass measurements were taken

after harvested tissue was dried at 65°C for two days. Due to the size of the cabinets, a block design was employed whereby two blocks each contained four replicates of each accession in each condition, with the same cabinets being used for both blocks of the experiment.

2.2.7. Data Processing and Analysis

After all data had been collected, outliers were identified and removed from the dataset using the Tukey method, whereby all values more than 1.5 inter-quartile ranges (IQRs) away from the first and third quartile are removed (Tukey, 1977). After data processing removed extreme outliers from the dataset, the remaining dry biomass measurements were used to calculate mean trait scores for each accession in each condition, from which normalized biomass loss scores, between stressed and control conditions, were calculated as follows: 1 - (mean control dry biomass / mean stressed dry biomass). The normalized loss of mean dry biomass was used as a measure of thermotolerance, with tolerant accessions showing less biomass loss between conditions, and vice versa. To determine whether there was a significant relationship between the expression of any of the hub genes, and dry biomass loss, a linear regression analysis was performed. Similarly, to test whether there was a significant relationship between the expression of hub genes within the same module, linear regression analysis was again employed. A relationship was considered significant when $p < 0.05$.

2.3. Results

2.3.1. Co-expression Network Construction and Network Conformation Statistics

RNAseq data was mapped to the IWGSC RefSeq transcriptome v1.0 CDS models; mapping statistics are provided in **Supplementary Data S2.2**. Normalised transcript abundance (RPKM) for each of the landraces and diploid relatives is provided in SRA data library: accession number PRJNA912645. The co-expression network contained 324 modules, housing 94,057 genes. Mean module size was 291 genes, whilst median module size was 56 genes. Three modules shared the smallest number of genes (30), whilst the largest module contained 6,923 genes.

2.3.2. Identifying Stress-Associated Modules

Modules enriched in GO terms related to response to heat, abiotic stress or abiotic stimuli likely contain genes which determine a plant's degree of stress tolerance. 13 of the 324 modules were found to be significantly enriched in such GO terms (**Table 2.1**), with the blue and lavenderblush1 modules being enriched in GO terms directly related to thermotolerance ("response to heat", FDR = 0.00011 and 1.5e-09, respectively). Three of the 13 modules were enriched in the GO term "response to water" – these modules were studied in the present work, as heat and drought stress can often occur simultaneously during periods of high temperature, so tolerance to drought stress may also bring thermotolerance.

2.3.3. Hub Gene Identification

Within the 13 stress-associated modules, several hub genes showed sequence similarity to stress tolerance genes in wheat or other species (**Table 2.2**). These hub genes were particularly promising as they have been either directly implicated in thermotolerance (in the case of the heat shock protein (HSP) genes); involved in regulating normal growth and development; regulating stress hormone signalling, or are transcription factors which will likely have far-reaching effects on the expression of other genes.

Six of the 13 promising hub genes had a mean RPKM >1 across the panel, so these were the hub genes tested as markers of thermotolerance (**Figures 2.1** and **S2.1**). Despite being unable to be tested as markers in the present work, the remaining seven hub genes represent valuable targets for wheat breeders in the development of thermotolerant varieties where

these genes are expressed at a higher level, as their centrality within stress-associated modules in the network suggests that manipulation of their expression may have large effects on global gene expression, and potentially on thermotolerance.

Table 2.1: 13 modules were significantly enriched in stress-related GO terms, according to GO enrichment analysis using the Singular Enrichment Analysis tool on AgriGO (Du et al., 2010; Tian et al., 2017). The names of these modules and the enriched stress-related GO terms are listed. The FDR-adjusted Fisher test *p*-values associated with each GO term are given in brackets.

Module Name	Enriched GO Term
Blue	Response to Heat (0.0001)
Darkgrey	Response to Stress (0.009)
Darkmagenta	Response to Oxidative Stress (0.0002)
Darkorange	Response to Water (1.4e-05)
Lavenderblush1	Response to Heat (1.5e-09)
Lightyellow	Response to Water (2.8e-19)
Mediumpurple	Response to Stress (0.004)
Navajowhite3	Response to Stimulus (0.031)
Purple	Response to Stress (9.4e-06)
Skyblue	Response to Water (5.9e-06)
Skyblue2	Response to Stress (0.02)
Steelblue	Response to Stress (0.0008)
Thistle	Response to Stress (0.022)

Table 2.2: Hub genes identified in stress-associated modules. These hub genes were those deemed to be particularly promising, based on the known function of the hub gene itself, its orthologue in other species, or the general functions of its protein family. The hub genes in bold were studied further as part of validation experiments. BLAST percentage identity scores are given in brackets after each BLAST hit.

Gene	BLAST Hit	Function	Reference
TraesCS5A01G105900.1	***T. aestivum UBP12 (100%)***	**Regulation of jasmonic acid and abscisic acid signalling**	(Jeong et al., 2017; Liu et al., 2022)
TraesCS7A01G050400.1	*TaClpB1-like (99.95%)*	Long-term acquired thermotolerance	(Mishra and Grover, 2016)
TraesCS6A01G158100.1	*TaMADS-box transcription factor 29-like (100%)*	MADS box transcription factor	(Castelán-Muñoz et al., 2019)
TraesCS5A01G369900.1	*TaCS66-like (100%)*	Cold shock protein	(Park et al., 2009)
TraesCS5D01G125500.1	***A. tauschii dnaJ A7A, chloroplastic-like (100%)***	**Chloroplast development**	(Zhu et al., 2015)
TraesCS1A01G314200.1	*TaABI4-like (100%)*	Regulation of stress hormone signalling	(Chandrasekaran et al., 2020)
TraesCS3B01G270800.1	*TaLPP2 (100%)*	Regulation of ABA signalling	(Paradis et al., 2011)
TraesCS3B01G285100.1	*TaLEA14 (97.79%)*	Protection against abiotic stress	(Hong-Bo, Zong-Suo and Ming-An, 2005; Jia et al., 2014)
TraesCS2A01G447400.1	*TaMYB34-like (100%)*	Stress-responsive transcription factor	(Wang, Niu and Zheng, 2021)
TraesCS2B01G205600.1	***TdPELPK1-like (97.33%)***	**Positive regulation of growth**	(Rashid and Deyholos, 2011)
TraesCS4D01G207500.1	***AtHSP70-1 (78%)***	**Negative regulator of heat stress tolerance**	(Tiwari, Khungar and Grover, 2020)
TraesCS7B01G149200.1	***TaHsp90.2-B1 (100%)***	**Long-term acquired thermotolerance**	(Kumar and Rai, 2014)
TraesCS7D01G241100.1	***TaHsp90.2-D1 (100%)***	**Long-term acquired thermotolerance**	(Kumar and Rai, 2014)

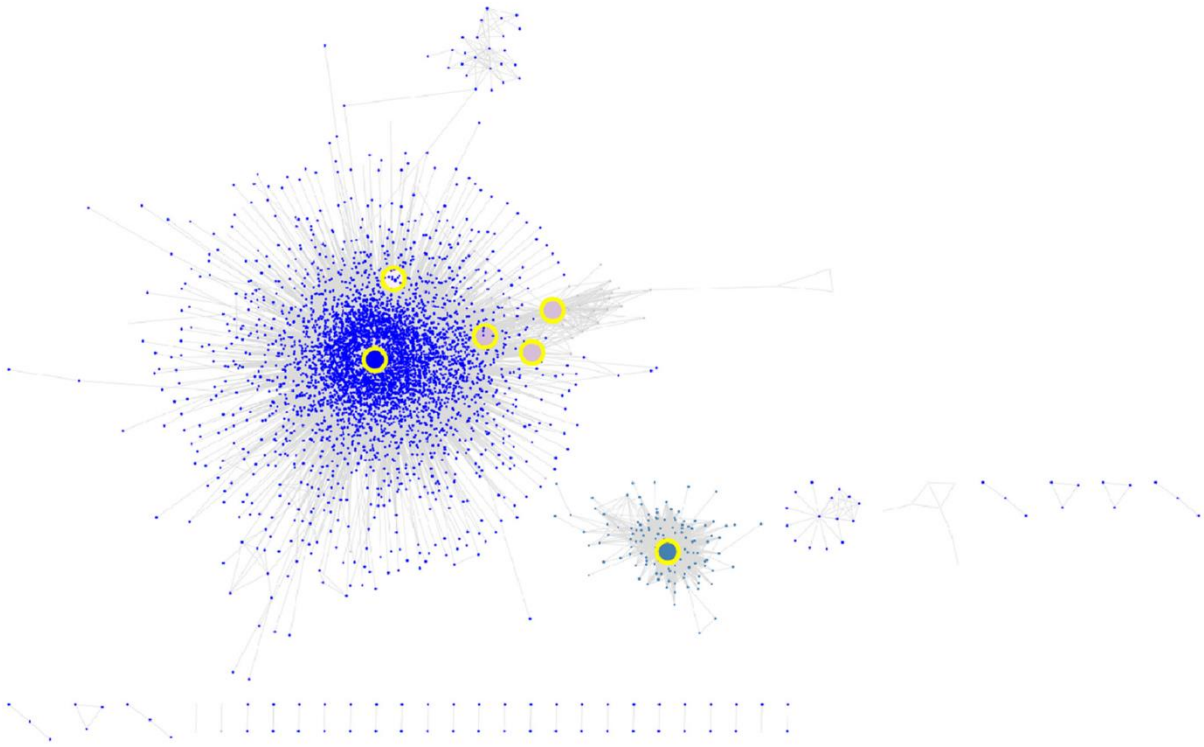

Figure 2.1: Subnetwork of the modules containing the six stress-associated hub genes used to predict thermotolerance. Stress-associated modules housing six genes used to predict thermotolerance were exported together using the "exportNetworkToCytoscape()" function with a threshold of 0.1, before the subnetwork was visualized in Cytoscape. Node colour corresponds with each gene's module membership, whilst the six hub genes are enlarged and highlighted by a yellow border. Each node represents a gene in the subnetwork, whilst each line between nodes represents a connection between the genes.

2.3.4. Transcriptome Display Tile Plots

To assess whether naturally occurring rearrangements or homoeologous exchanges (HEs) in the wheat genome may have the potential to affect thermotolerance of different landraces, candidate hub gene locations with these events were compared using Transcriptome Display Tile Plots (TDTPs), which enable visual comparison of expression for the three wheat genomes to identify regions where HEs or other rearrangements have occurred. As in previous studies (Harper et al., 2016; He et al., 2017) transcript abundance of homoeologous triads (21,073 in total) was visually examined after being assigned to colour space where the A genome is represented by cyan, B genome by magenta and D genome by yellow (**Figure 2.2**).

As expected for a panel of diverse lines, a large number of structural rearrangements were detected in the landraces, varying greatly in size. Of the six hub genes, four were found in rearranged regions in the landrace panel. In YoGI_272, it appears that a rearrangement has occurred in the collinear region encompassing *TraesCS4D01G207500.1*, with only the B and D genome copies of this region being present. *TraesCS7B01G149200.1* and *TraesCS7D01G241100.1* are homoeologues, and this collinear region also appears to be affected by rearrangements in YoGI_030 and YoGI_126 accessions. YoGI_030 appears to be missing the entire 7A chromosome, and a segmental rearrangement appears also to have affected YoGI_126, removing the B genome homoeologue of this gene. Finally, large rearrangements were found in the collinear region surrounding *TraesCS2B01G205600.1* in

six of the landraces. YoGI_004, 013, 229 and 308 appear to possess only the A and D genome in this region; YoGI_302 appears to have only A genome expression in this region; YoGI_338 has only B genome expression in this region. Where these rearrangements have occurred, in some cases the TDTPs also indicate potential increased contribution from one of the remaining genomes.

However, although not outliers, in all cases, total triad transcript abundance (RPKM) for lines with these rearrangements is lower than the average for lines without them, consistent with reduced hub gene dosage in accessions affected by these rearrangements (**Table 2.3**). Despite the potential for RNA-Seq reads to align to all subgenomes when there is high sequence similarity between homoeologues, in general, RPKM values are also lower for the homoeologue that the TDTP indicates is missing. As expression of the hub genes are positively correlated with heat tolerance, average or poorer levels of thermotolerance is predicted in all of these accessions. In addition, as relatively few accessions have rearrangements in these regions, accessions used to validate the predictive capability of the gene network hubs were chosen based on hub RPKM values only.

2.3.5. Hub Gene Validation

15 spring habit landrace accessions were selected from the panel for use in a thermotolerance validation assay, based on the variation they showed in hub gene RPKM (**Table S2.2**). These accessions showed an average normalized loss in mean dry biomass of 0.364 when exposed to heat stress during early development, compared to their counterparts under control conditions. The most tolerant accession showed a normalized loss in mean dry biomass of 0.135 (YoGI_155), whilst the most susceptible accession (YoGI_268) showed a score of 0.513.

To determine whether hub gene RPKM could be used as a marker of early thermotolerance, regression analyses were conducted, comparing the RPKM of each hub gene, shown by each accession, with the normalized loss in mean dry biomass shown by each accession. It was hypothesized that if expression of a hub gene under control conditions could be used as a marker for early thermotolerance, a significant relationship between hub gene RPKM and normalized dry biomass loss would be observed. This was the case for three of the six hub genes (**Figure 2.3**), all of which were members of the same module; *TraesCS4D01G207500.1* (R^2 = 0.36, p = 0.011), *TraesCS7B01G149200.1* (R^2 = 0.33, p = 0.015) and *TraesCS7D01G241100.1* (R^2 = 0.29, p = 0.021). There was no significant relationship observed between hub gene RPKM and normalized dry biomass loss for the remaining three hub genes, however (p > 0.05, **Figure S2.1**). A significant relationship was observed when comparing expression of the most central gene in the module, *TraesCS4D01G207500.1,* with *TraesCS7B01G149200.1* (R^2 = 0.94, p = 1.11e-09) and *TraesCS7D01G241100.1* (R^2 = 0.96, p = 9.18e-11), suggesting that *TraesCS4D01G207500.1* may regulate the expression of the two homoeologous hubs.

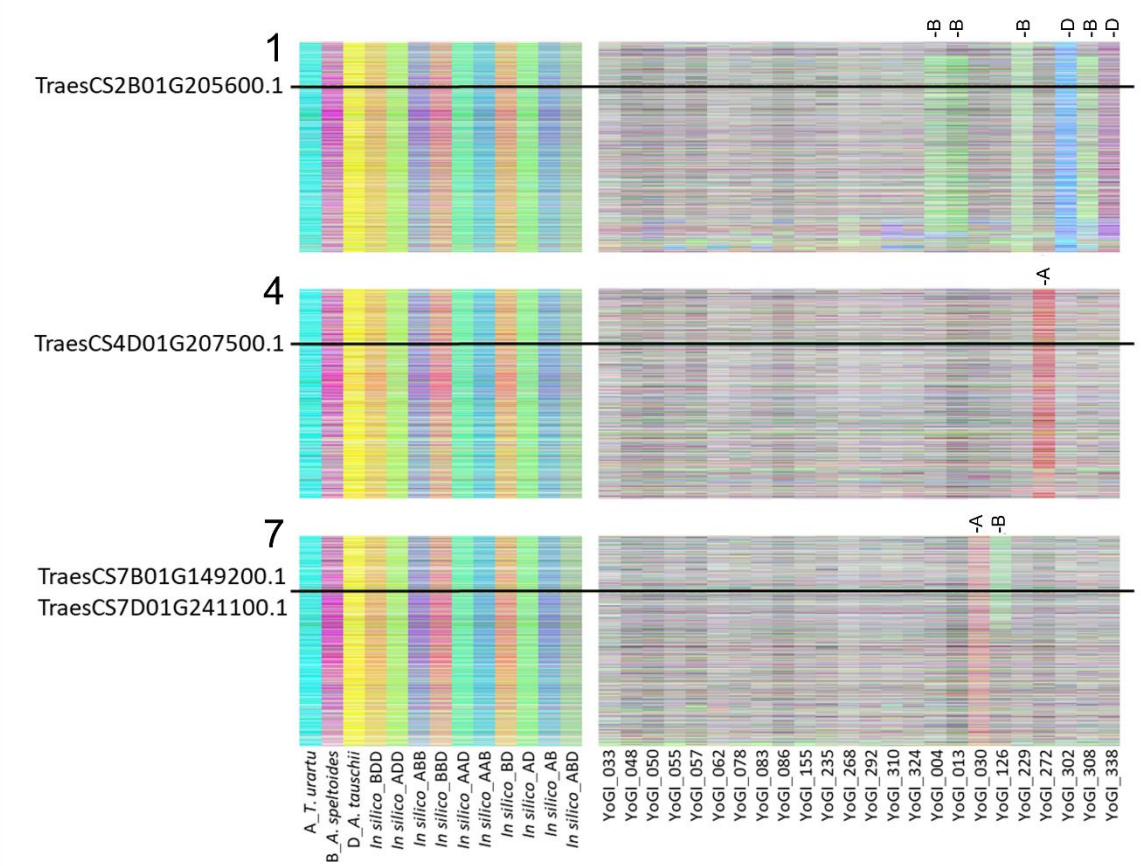

Figure 2.2: Transcriptome Display Tile Plot for the YoGI wheat landrace panel. Tile plots illustrate relative transcript contributions for the A, B and D copies of 15,527 triplets of homoeologous genes on linkage group 2. Represented are 342 bread wheat accessions, diploid ancestors *Triticum urartu* (AA), *Aegilops speltoides* (BB) and *Aegilops tauschii* (DD), tetraploid ancestor *Triticum dicoccoides* (AABB), and in silico tetra- and hexaploid combinations. The A genome is represented by cyan, B genome magenta and D genome yellow. The homoeologous genes are arranged in A genome order.

Table 2.3: RPKM values for hub gene triads with chromosome rearrangements detected. Hub genes detected using WGCNA are indicated in bold, and missing genome contributions predicted by the TDTP are indicated next to accession numbers.

	TraesCS4A01G097900.1	*TraesCS4B01G206700.1*	***TraesCS4D01G207500.1***	Total
Mean RPKM (no rearrangement; n=341)	51.148	74.773	131.740	257.661
RPKM YoGI_272 (-A)	13.879	60.184	110.299	184.361

	TraesCS2A01G178600.1	***TraesCS2B01G205600.1***	*TraesCS2D01G187300.1*	Total
Mean RPKM (no rearrangement; n=340)	29.359	38.707	28.629	96.695
RPKM YoGI_030 (-A)	15.327	34.286	27.003	76.616
RPKM YoGI_126 (-B)	36.317	22.785	33.141	92.244

	TraesCS2A01G178600.1	***TraesCS2B01G205600.1***	*TraesCS2D01G187300.1*	Total
Mean RPKM (no rearrangement; n=336)	0.003	4.053	5.242	9.298
RPKM YoGI_004 (-B)	0.000	0.000	3.438	3.438
RPKM YoGI_013 (-B)	0.000	0.000	3.779	3.779
RPKM YoGI_229 (-B)	0.000	0.327	1.014	1.341
RPKM YoGI_302 (-D)	0.000	2.721	0.000	2.721
RPKM YoGI_308 (-B)	0.000	0.000	3.635	3.635
RPKM YoGI_338 (-D)	0.000	1.525	0.660	2.186

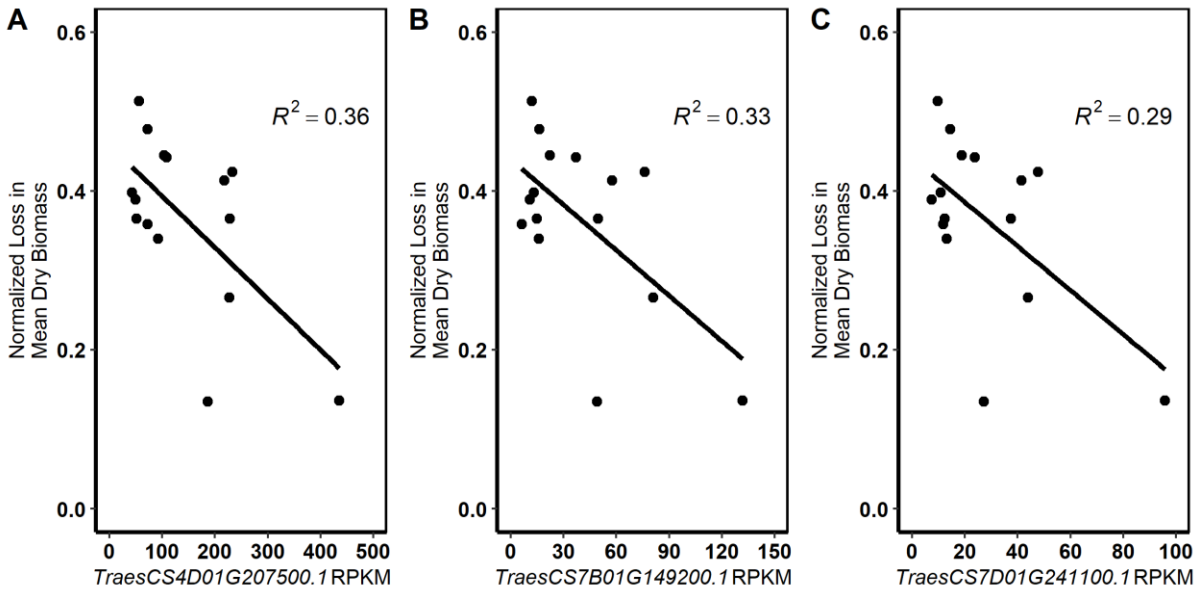

Figure 2.3: Expression of three HSP hub genes were significantly associated with early thermotolerance. The associations between hub gene RPKM and normalized loss in mean dry biomass shown by a small panel of landrace accessions were analysed by linear regression. Significant associations were seen for three hub genes: **A)** *TraesCS4D01G207500.1* (R^2 = 0.36, p = 0.011), **B)** *TraesCS7B01G149200.1* (R^2 = 0.33, p = 0.015) and **C)** *TraesCS7D01G241100.1* (R^2 = 0.29, p = 0.021).

2.4. Discussion

The transition of agriculture towards genetic uniformity may have resulted in higher, more reliable yields under optimal conditions, but as the climate becomes increasingly hostile for cereal crop cultivation, the need to recapture some of the genetic diversity lost during this transition is essential to prepare future varieties for growth under such challenging conditions. Landrace varieties are a valuable source of both phenotypic and genetic diversity, due to their adaptation to climates around the world, and lack of major genetic bottlenecks in their recent ancestry. The value of wheat landraces in the production of stress tolerant varieties has been exploited by some researchers to study the mechanisms underlying boron (Paull, Nable and Rathjen, 1992), heat (Zhang et al., 2022b) and drought tolerance (Naderi et al., 2020; Lin et al., 2019), but global landrace collections remain largely under-utilized in the study of abiotic stress tolerance in wheat. This chapter presents the YoGI landrace panel, and its accompanying transcriptome data, as a resource for researchers and breeders to utilize in the production of stress tolerant wheat varieties, and the study of stress tolerance mechanisms in this crucial crop.

In contrast with the extensively used methods of GWAS, RNA-seq and microarray studies, network approaches, such as WGCNA, provide a more refined list of high impact candidate genes for further study. The role of these candidate genes as hubs within modules suggests they likely regulate the expression of a suite of genes, so manipulating their expression or activity will have far-reaching effects on gene expression. The consequences of these global gene expression changes, as a result of hub gene manipulation, cumulate to produce a large phenotypic effect on the trait under study. Identifying, and manipulating, such hub genes is crucial if complex quantitative traits, such as abiotic stress tolerance, are to be altered in a significant way. These traits are controlled by hundreds, or thousands, of genes in the

genome, so manipulating a poorly-connected effector gene is likely to have little or no impact on the trait, due to the action of the other genes which remain undisturbed. These hub genes, therefore, serve as valuable targets for breeders working on quantitative traits, as large-scale gene expression changes, and subsequently, phenotypic changes, can be achieved via targeted manipulation of a single hub.

Despite their seeming importance to breeders working on quantitative traits, such as thermotolerance, there is relatively little work on the use of network approaches to identify heat stress-related hub genes in wheat (Girousse et al., 2018; Mishra et al., 2021). These works have facets in common that are different to the present work, however. Firstly, the published works use RNA samples taken from plants exposed to stress as well as plants exposed to control conditions, whereas the present work uses only RNA from plants grown under control conditions for network construction. This means that although heat-responsive hub genes will be missed in the present work, those hubs whose expression under control conditions may pre-dispose accessions to be more, or less, thermotolerant will be identified. A further difference is that the published works use far fewer different accessions (maximum of two) than the 337 landrace accessions used for network construction in the present work – a major difference in the amount of genetic diversity exploited.

The present work is also distinct from much of the above published works, as it concerns heat stress exposure and thermotolerance at an early developmental stage. The study of the genetic control of wheat seedling thermotolerance is fairly limited, with only a handful of published works identifying key genes or genomic regions associated with the trait (Abd El-Daim, Bejai and Meijer, 2014; Khatun et al., 2018; Khan et al., 2022). Much of the study on the effect of heat stress on wheat concerns its effect at, or around, anthesis and grain filling, and its subsequent effect on yield, as these are the developmental stages when heat stress commonly occurs in some major wheat producing countries (Stone and Nicolas, 1994, 1995, 1996).

However, shifts in global temperature patterns has meant that months aligned with the early stages of spring habit wheat growth in many countries are becoming a lot warmer. For example, the March to May period (Northern Hemisphere's meteorological spring) of 2022 was the fifth warmest on record (NOAA National Centers for Environmental Information, 2022a). In fact, May 2022 saw record temperatures reached across Southern, Central and Western Europe, whilst temperatures approaching record levels (in excess of 40°C in some cases) were observed in some Southern and North-eastern states of the USA, a major producer of spring wheat (NOAA National Centers for Environmental Information, 2022a, 2022b). This shift in global temperature patterns has been accelerating in recent times with the ten warmest January to May periods all occurring since 2010 (NOAA National Centers for Environmental Information, 2022a), whilst, as predicted by climate variability studies (Easterling et al., 2000; Thornton et al., 2014; Haokip, Shankar and Lalrinngheta, 2020), periods of unseasonal spring heat stress are likely to occur more frequently in the coming years. Such periods have been observed in recent times – for example, May 2020 saw sustained daytime temperatures exceeding 30°C in the USA, Italy and Türkiye (NOAA National Centers for Environmental Information, 2020), whilst 2018 saw temperatures approaching record levels reached in major spring wheat producing states in the American Midwest, such as Minnesota which experienced temperatures of up to 38°C (NOAA National Centers for Environmental Information, 2018). The need, therefore, to prepare future wheat crops to tolerate heat stress during early development is imperative as spring temperatures continue to rise and unseasonal periods of extremely highly temperatures become more common around the world in the coming years.

Ensuring wheat varieties are thermotolerant during early development will not only protect crops against rising spring temperatures and unseasonal heat stress events, recent evidence has emerged that those accessions which are thermotolerant at the seedling stage also showed higher yields when exposed to heat stress after anthesis (Lu et al., 2022). This work showed there was a significant positive correlation between thermotolerance at the two developmental stages, and so suggests breeding thermotolerant seedlings will also protect these plants from heat stress yield damage, if exposed to high temperatures later in development. These findings are particularly promising when viewed in tandem with the present work, as this chapter has presented three hub genes whose expression under control conditions can be used as markers of seedling thermotolerance – but perhaps also, as markers of thermotolerance at yield.

The hub genes tested as predictive markers for early thermotolerance were selected based on their function, or the function of their orthologous genes in other species, as well as their centrality within modules deemed to be particularly stress-associated, thanks to their abundance of genes possessing stress-associated GO terms. Three of the six hub genes were selected due to their roles as HSPs, and subsequent likely direct involvement in thermotolerance, whilst the remaining three hubs were selected due to their likely involvement in processes that may confer thermotolerance or improved growth under heat stress.

The three predictive hub genes were perhaps obvious actors in determining thermotolerance, due to their roles as heat-shock proteins. It was hypothesized that *TraesCS4D01G207500.1* may act as a master regulator, and repressor, of thermotolerance in wheat due to its similarity to *AtHSP70-1/Hsc70-1*. *Arabidopsis* mutant lines showed increased basal thermotolerance, whilst overexpressoion led to increased heat sensitivity, suggesting the gene negatively regulates thermotolerance (Tiwari, Khungar and Grover, 2020). The protein acts on AtHsfA1d/A13/A2, activators of thermotolerance, which subsequently represses the expression of thermotolerance chaperone, *AtHSP101*. The hub gene was validated as a predictive marker of thermotolerance, however unlike its *Arabidopsis* orthologue, the gene appears to have a positive effect on thermotolerance, as accessions with higher levels of expression tended to be more thermotolerant (**Figure 2.3**).

TraesCS7B01G149200.1 and its homeologue *TraesCS7D01G241100.1* have already been described as actors in the biotic stress response, and named *TaHSP90.2-B1* and *TaHSP90.2-D1*, respectively (Wang et al., 2011). However, it was hypothesized these genes may also play a positive role in thermotolerance, as the homeologues show sequence similarity to *AtHSP81.4*, a gene known to be highly expressed in the thermotolerant relative of *Arabidopsis*, *Thellungiella salsuginea* (Taji et al., 2004; Higashi et al., 2013). As well as this, the genes also share extreme sequence similarity to *TaHSP90*, a gene found to be highly expressed under heat stress in a heat and drought tolerant wheat cultivar, C306, and which increased thermotolerance when overexpressed in *E. coli*, suggesting the gene plays a key role in determining thermotolerance in wheat (Vishwakarma et al., 2018). The hub genes have 99.9% and 97.4% similarity, respectively, with the *TaHSP90* sequence (accession number: MF383197) in this study, with *TraesCS7B01G149200.1* being the top BLAST hit against the IWGSC reference transcriptome. This allelic variation, likely a result of population structure differences between C306 and Chinese Spring reference variety, does not affect amino acids within TaHSP90's active site, therefore is unlikely to affect protein function – suggesting these hub genes fulfil the same function as *TaHSP90* and improve thermotolerance in wheat, as observed in the present work (**Figure 2.3**).

The three validated hub genes are all members of the same module, so their shared relationship between expression and early thermotolerance is likely a result of transcriptional

co-regulation. *TraesCS4D01G207500.1* was identified as the most central gene within the module, followed by *TraesCS7B01G149200.1* and *TraesCS7D01G241100.1* (**Table S2.1**), and significant relationship between *TraesCS4D01G207500.1* expression and expression of the homeologous hubs was found, suggesting that *TraesCS4D01G207500.1* may regulate the expression of the previously characterised thermotolerance hub genes.

This chapter proposes that *TraesCS4D01G207500.1* expression can be used as a marker of early thermotolerance due to its inferred function as a regulator of thermotolerance genes. As well as being connected to characterised thermotolerance hub genes, and validated markers, *TraesCS7B01G149200.1* and *TraesCS7D01G241100.1*, *TraesCS4D01G207500.1* may also act to regulate the expression of a suite of other HSPs, being connected to 11 other HSPs, as well as five members of the dnaJ HSP sub-family – groups of proteins both heavily associated with the heat stress response and thermotolerance (Bourgine and Guihur, 2021). The hub was also connected to five heat shock transcription factors (Hsfs), which suggests that, consistent with observations of its orthologue in *Arabidopsis* (Tiwari, Khungar and Grover, 2020), the hub may be able to determine thermotolerance due to regulation of various Hsfs. Here, however, increased expression of the hub likely leads to the upregulation of these genes, and subsequently, increased thermotolerance. *TraesCS5A01G437900.1* is *TaHsfA2-1*, a gene found to play a key functional role determining thermotolerance in wheat seedlings (Liu et al., 2020b). *TraesCS5D01G445100.2* is *TaHsfA2e-5D*, a transcription factor previously reported to confer heat and drought stress tolerance when expressed in yeast and *Arabidopsis* via its activation of HSPs and other stress-related genes (Bi et al., 2022). *TraesCS1A01G375600.2* is another Hsf transcription factor (*TaHsfA6e*) that acts to regulate HSP expression as part of the thermotolerance network (Kumar et al., 2018). *TraesCS1B01G396000.3* and *TraesCS1D01G382900.1* are poorly-characterized homeologues, *Triticum aestivum heat stress transcription factor A-2c-like*. As well as HSPs, Hsfs and dnaJ proteins, the hub gene is also connected to all three homeologous genes which showed sequence similarity to *Triticum dicoccoides serine/arginine-rich splicing factor SR45a-like*, a pre-mRNA splicing protein that plays a crucial role during the heat and salt stress responses in plants (Ling, Mahfouz and Zhou, 2021; Li et al., 2021). The potential regulation of such genes by *TraesCS4D01G207500.1*, therefore, offers an explanation as to why its expression under control conditions is significantly associated with an accession's degree of early thermotolerance.

In this work, three stress-associated hub genes hypothesized to control thermotolerance were identified, before confirming a significant association between hub gene RPKM and normalized loss in mean dry biomass, in several landrace accessions. Two of these validated hub genes are almost identical to a thermotolerance gene already characterized in wheat, however, upon further investigation of the hubs, *TraesCS4D01G207500.1* appears to regulate their expression – perhaps explaining the similarity in the relationship between hub gene RPKM and early thermotolerance shown for each of these three hubs. Unlike its *Arabidopsis* orthologue (*AtHSP70-1/Hsc70-1*), however, *TraesCS4D01G207500.1* appears to positively regulate thermotolerance. As well as putatively regulating the expression of the other hubs, it appears that *TraesCS4D01G207500.1* may be able to confer increased early thermotolerance via likely regulation of the expression of a suite of other HSPs, a stress-responsive splicing factor, and Hsfs previously shown to improve thermotolerance in wheat. In addition to this, six other stress-associated master-regulator hub genes were also identified which may serve as good targets for thermotolerance improvement, but could not be validated in the present work. If the findings of recent work (Lu et al., 2022) apply here, expression of these validated hub genes may not only serve as markers for early thermotolerance, but also as markers for tolerance to heat stress at anthesis – potentially allowing breeders to make predictions about heat stress-related yield losses from a very early developmental stage. The present work,

therefore, presents an exciting step forward towards the production of thermotolerant wheat varieties, able to tolerate periods of high temperatures both early and late in development, and provides breeders new validated targets to aid them in this goal.

2.5. Conclusions

This chapter has introduced the YoGI landrace panel as an important resource for wheat breeders, due to its extensive novel genetic and phenotypic diversity. This chapter has also shown how the landrace panel can be used successfully to aid the production of more stress tolerant wheat varieties, as in the present work the expression of three stress-related hub genes were shown to be significantly associated with the thermotolerance of landrace accessions during vegetative development. The present work not only validates these genes as predictive markers for use in breeding programmes, but also suggests *TraesCS4D01G207500.1* expression is able to determine an accession's degree of thermotolerance due to potential action as a master regulator over the expression of an array of HSPs and Hsfs, including the other validated hub genes which show remarkable similarity to a characterized thermotolerance wheat gene.

3. Identification of Candidate Master Regulators of the Response to Early Heat Stress in Climate-adapted Wheat Landraces via Transcriptomic and Co-expression Network Analyses

3.1. Introduction

The damaging effect of heat stress exposure on *Triticum aestivum* L. (bread wheat) yields is well known, with reductions between 3 and 6% being observed for every degree increase above the crop's optimal growth temperature of 20°C (Chowdhury and Wardlaw, 1978; Kobza and Edwards, 1987; Wardlaw et al., 1989; Nagai and Makino, 2009; Ray et al., 2013; Zhao et al., 2017; Tian et al., 2018), with such heat-induced yield losses being evidenced in recent field trials (Riaz et al., 2021; Roychowdhury et al., 2023; Wang et al., 2023). These kinds of yield losses are likely to become more common in the coming years as a result of climate change and global warming, as, according to Intergovernmental Panel on Climate Change (IPCC) predictions, an increase in global mean surface temperatures of between 0.3 and 4.8°C, compared to the prior century, are expected by 2100 (Collins et al., 2013), whilst other models predict more rapid global temperature increases, with such levels being reached by the year 2060 (Wigley and Raper, 2001; Murphy et al., 2004; De Costa, 2011). This is particularly worrying considering that global wheat consumption in 2021/2022 reached almost 800 million metric tonnes and currently accounts for 20% of the globe's annual calorie consumption (Pfeifer et al., 2014; Food and Agriculture Organization of the United Nations et al., 2018; United States Department of Agriculture - Foreign Agricultural Service, 2023), meaning the lives, and livelihoods, of billions around the world depend on the success of the yields of this single crop.

Not only are yearly average global temperatures rising, but seasonal temperature patterns are likely to shift over the coming years, with warmer springs already being increasingly reported; for example, ten of the 13 springs to have occurred since 2010 make up the warmest springs ever recorded, with spring 2022 ranking 6th on this list (NOAA National Centers for Environmental Information, 2022a). This trend towards increasingly warm spring months saw much of Western, Southern and Central Europe experience record temperatures in May 2022, whilst Southern states of the USA experienced their fourth warmest May since records began in 1895 (NOAA National Centers for Environmental Information, 2022a, 2022b). Not only are average temperatures during the spring months rising globally, but unseasonal periods of extreme temperatures are expected to occur more frequently as the climate continues to change (Easterling et al., 2000; Thornton et al., 2014; Haokip, Shankar and Lalrinngheta, 2020). Evidence of such events have been observed recently, with the USA, Italy and Türkiye all experiencing periods of elevated temperature, above 30°C, in May 2020 (NOAA National Centers for Environmental Information, 2020), whilst parts of the American Midwest, such as major spring wheat producing state Minnesota, saw temperatures reach 38°C (NOAA National Centers for Environmental Information, 2018).

This is a pressing issue for much of the Northern Hemisphere, besides South Asia and the Middle East, as, in many western countries, spring wheat is often sown during March and April. The springtime sowing of seeds in these regions means warmer spring months, and increasingly prevalent periods of extreme temperatures, coincide with the early vegetative development of spring wheat crops in regions of high production, such as the USA, Canada and the United Kingdom. These countries produced over 80 million tonnes of wheat combined in 2021 (Food and Agriculture Organization of the United Nations, 2023) and so play crucial roles in global food security. Therefore, it is essential that spring wheat crops in such countries are protected against the increasingly likely threat of heat stress during early development.

The first step towards achieving this is to gain an improved understanding of both thermotolerance and the response to heat stress during early vegetative development. Having previously identified a candidate master-regulator and three validated genetic markers for early basal thermotolerance (**Chapter 2**), the preset work now builds on this previous experiment, this time aiming to understand the transcriptional response to early heat stress in spring habit wheat landraces and identify candidate hub genes, which may regulate this response using weighted gene co-expression network analysis (WGCNA). Together, these works provide a comprehensive examination of early heat stress exposure in bread wheat, generating insights into how these processes may be regulated transcriptionally, and identifying genes which may be responsible for this regulation.

A handful of studies have examined the effect of heat stress on the transcriptome of wheat during vegetative development (Qin et al., 2008; Jin et al., 2020; Liu et al., 2015), however this type of analysis paired with subsequent network analysis is less common, despite this approach enabling the identification of a small number of promising candidate genes potentially playing large regulatory roles in the stress response, reducing the time spent laboriously screening all of the identified stress-responsive genes. Similar combined approaches have been used in other contexts, however, such as to identify regulators of thermotolerance during vegetative development in wheat (Girousse et al., 2018; Mishra et al., 2021); response to heat and cold stresses, and basal thermotolerance in rice (Wang et al., 2022; Zeng et al., 2022; Boulanger et al., 2023); response to combined heat, drought, and salinity stresses in Brachypodium (Shaar-Moshe, Blumwald and Peleg, 2017); and drought stress response in sugarcane (Tang et al., 2023),whilst **Chapter 6** will use this approach to study the response to drought stress in wheat. However, there are no similarly exploratory examples of this approach being used to study the heat stress response in wheat, as yet. Although one study has utilized similar approaches to identify genes that may be regulated by pre-existing candidate genes under heat stress, by examining the effect of heat stress exposure on knockout mutants and wild-type plants (Tian et al., 2022), this study represents the first exploratory example of WGCNA utilization to identify hub genes which may act to coordinate the heat stress response in climate-adapted bread wheat landraces.

3.2. Materials and Methods

3.2.1. Plant Growth Conditions

The seeds used in the present work were from plants which derived from at least three generations of selfing. 13 accessions previously shown to be distinctively tolerant or susceptible under heat stress (**Chapter 2**, **Table S3.1**) were sown in Levington Advance Seed & Modular F2S compost mixed with Aggregate Industries Garside Sands 16/30 sand in an 80:20 ratio, which was treated with CaLypso insecticide (Bayer CropScience Ltd., 0.083ml mixed with 100ml water, applied to each litre of compost). The heat stress treatment used in the present work was identical to that used previously (**Chapter 2**). Plants were placed into a Percival AR-75L growth cabinet with 18h day length, and respective day/night temperatures of 22°C and 16°C until the three-leaf stage. At this point, four replicates of each accession were transferred to a separate Percival AR-75L growth cabinet and exposed to 35°C/30°C (day/night) for 14 days, with all other conditions being the same as in the control cabinet. 2cm leaf tissue samples taken at the three-leaf stage and after 14 days of heat stress exposure, were frozen in liquid nitrogen and stored at -80°C prior to RNA extraction.

3.2.2. RNA Extraction, Sequencing and Mapping

Leaf tissue samples weighing less than 100 mg were used for total RNA extraction via the E.Z.N.A Plant RNA Kit (Omega Bio-Tek, GA, USA) including a DNase treatment, according to

the manufacturer's protocol. Both NanoDrop ND-1000 Spectrophotometer (Thermo-Fisher Scientific, MA, USA) and Qubit 4 Fluorometer (Life Technologies, CA, USA) were used for quantification of RNA concentration, while Agilent Technology 2100 Bioanalyzer (Agilent Technologies, CA, USA) was used to assess RNA quality. Samples were deemed to be acceptable for use in subsequent analysis if their RNA Integrity Number (RIN) value was >7. To help control the effect of the environment on the transcriptome, prior to sequencing, acceptable RNA from at least 3 replicate plants per accession, per condition (pre- or post-heat stress) was pooled, whilst biological replication for each treatment was provided by the different accessions. Samples were stored at -80°C and shipped on dry ice to Novogene (Cambridge, United Kingdom) for sequencing using the Illumina Novaseq 6000 platform (Illumina, CA, USA) with a 150bp paired-end sequencing strategy. Raw reads were trimmed using Trimmomatic v0.39 (Bolger, Lohse and Usadel, 2014) by removing leading and trailing low quality or N bases (below quality 3), minimum length 36 bp and sliding window 4:15. FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to assess the quality of the data, then Salmon v0.8.1 (Patro et al., 2017) was used to map trimmed reads to the *Triticum aestivum* reference genome v1.1 (IWGSC RefSeq v1.1, http://ftp.ensemblgenomes.org/pub/plants/release-46/fasta/triticum_aestivum/). Salmon transcripts per million (TPM), counts and lengths were inputted into R (version 4.1.2.; R Core Team, 2021) using TxImport (Soneson, Love and Robinson, 2015) for further analysis. Raw sequence read data can be found in the SRA data library under accession number GSE232367.

3.2.3. Transcriptomic and Differential Expression Analyses

26 pooled RNA samples from 13 accessions (before and after heat stress, for 13 accessions) were used for transcriptomic analysis. After importing transcriptome data into R using TxImport, the Principal Component Analysis (PCA) function of DESeq2 (version; 1.36.0; Love, Huber and Anders, 2014) was first used to explore count data from RNA-Seq. Genes with fewer than 10 non-zero entries were then removed, leaving 75,732 genes for differential expression analysis (DEA; **Supplementary Data S3.1**). DEA was carried out using the DESeq2 package (version 1.36.0; Love, Huber and Anders, 2014) in R, whereby an additive model was used to identify genes differentially expressed between tolerant and susceptible accessions, as well as between pre- and post-stress samples. Genes which showed a log2FC above/below 1.5/-1.5 and a FDR-adjusted *p*-value (Benjamini and Hochberg, 1995) below, or equal to, 0.05 for either of these comparisons were deemed to be tolerance or response differentially expressed genes (DEGs), respectively. The adaptive shrinkage function was employed in the ranking of genes to shrink the log fold-change estimates of genes with low counts or high dispersion (Stephens, 2017). Due to extremely low numbers of tolerance DEGs identified in the DEA, only response DEGs were studied further.

3.2.4. GO Term Enrichment Analysis

To identify gene ontology (GO) terms significantly enriched amongst upregulated and downregulated response DEGs identified via DEA, GO enrichment analysis was conducted. An approach, used previously (Borrill et al., 2019; Andleeb, Knight and Borrill, 2023), was adopted to transfer GO terms to the v1.1 annotation, from the IWGSC RefSeqv1.0 genome annotation, as GO terms are only available for the v1.0 annotation. The list of genes for which GO terms can be transferred can be found in Andleeb et al. (2023). IWGSC v1.0 GO terms were read into R using the base R function readRDS() for analysis, after being retrieved from: https://opendata.earlham.ac.uk/wheat/under_license/toronto/Ramirez-Gonzalez_etal_2018-06025-Transcriptome-Landscape/data/TablesForExploration/FunctionalAnnotation.rds.

GO terms that upregulated and downregulated response DEGs are annotated with were then collated into two groups, before the agriGO Singular Enrichment Analysis tool (Du et al., 2010;

Tian et al., 2017) was used to conduct a Fisher's exact test on both groups of GO terms, with the GO terms of all genes included in DEA serving as background. 0.05 was the *p*-value threshold; Hochberg (FDR) was the multi-test adjustment method (Benjamini and Hochberg, 1995), and 5 was the minimum number of mapping entries threshold. Significantly enriched GO terms had an FDR-adjusted *p*-value < 0.05. AgriGO's DAG Drawer tool was also used to generate DAG trees for significantly enriched GO terms.

3.2.5. Network Construction and Module Detection

A single co-expression network was constructed via the WGCNA R package (Langfelder and Horvath, 2008, 2012), using TPM data provided by Salmon. No samples were removed after clustering, but 19,965 genes were removed due to too many zero values: leaving 87,580 genes from 26 samples (13 accessions, before and after heat stress exposure) for network construction. The blockwiseModules() function conducted blockwise network construction according to the function's default parameters, except the following: network type = signed hybrid, maximum block size = 5000, soft threshold power = 8 (advised by the package's authors for this number of samples, as no soft threshold power exceeded a reasonable scale-free topology fix index of 0.8), minimum module size = 30, merge cut height = 0.25. After module detection, edge and node files were created using the "exportNetworkToCytoscape()" function with a threshold of 0.1; filtering out weak connections between genes (nodes). Results of sample clustering, scale-free topology fit index as a function of the soft-thresholding power, and mean connectivity as a function of the soft-thresholding power can be found in **Figure 3.1**. Gene expression data after sample clustering and processing via WGCNA, and network construction data are available on Github: https://github.com/andreaharper/HarperLabScripts/.

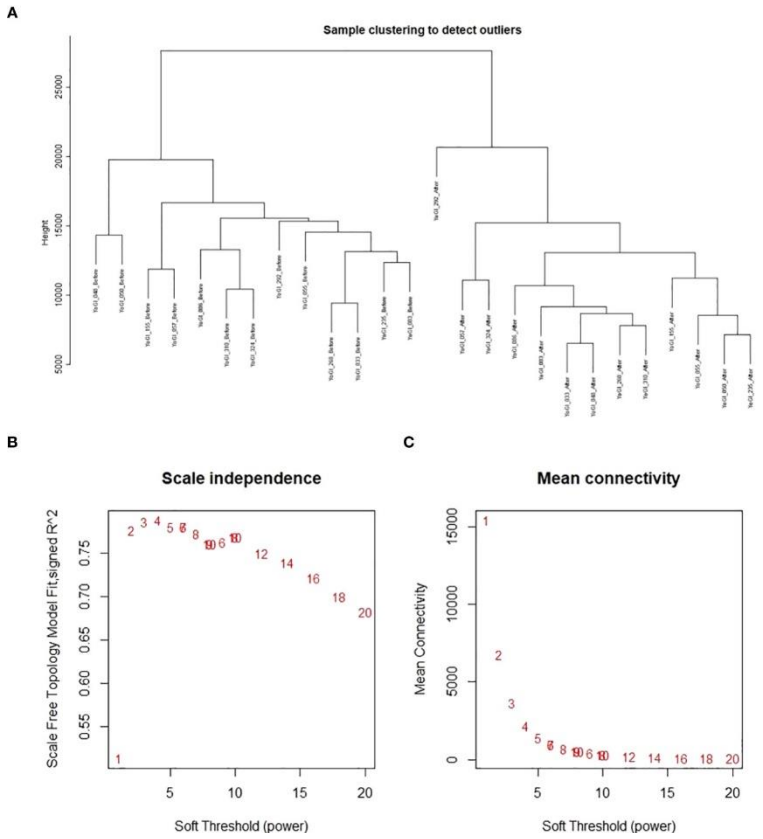

Figure 3.1: Analyses performed by WGCNA prior to co-expression network construction. Results of sample clustering **(A)**, scale-free topology fit index as a function of the soft-thresholding power **(B)**, and mean connectivity as a function of the soft-thresholding power **(C)**.

3.2.6. Identifying Stress-associated Modules

To understand the likely functions of genes within each module, GO enrichment analysis was conducted using the same approach as outlined above. Here, however, GO terms associated with genes within a module were collated and submitted to the agriGO Singular Enrichment Analysis tool, with the GO terms of all genes included in the network serving as background. All other parameters were the same as described above.

As well as this, DEG enrichment analysis was also conducted to identify which modules in the co-expression network contained a significantly larger proportion of response DEGs than expected, and thus may be particularly associated with the heat stress response. To test whether a module was significantly enriched in response DEGs (observed proportion of DEGs above 8.94%), a one-proportion Z-test was used. Modules were deemed to be significantly enriched in DEGs if p was < 0.05.

3.2.7. Network Visualization and Hub Identification

Degree (connection) scores were calculated for each gene, via either the Cytoscape network analyser tool (Assenov et al., 2008), after first visualizing network modules in Cytoscape (version 3.9.1.; Shannon et al., 2003), or via counting the number of connections to and from each gene in the module's WGCNA edge file using the table() function in R. The script used to calculate degree scores in R is available on GitHub (https://github.com/andreaharper/HarperLabScripts/). Visualization and analysis in Cytoscape were used to identify hub genes in the majority of the modules, however the edge counting method in R was used to calculate degree scores for genes in particularly large modules (containing more than ~2000 genes), which often cannot easily be loaded, viewed and analysed in Cytoscape. For the largest modules, the R package vroom (version 1.6.3.; https://vroom.r-lib.org) was used to read Cytoscape edge files into R for analysis.

Hub genes were selected for further analysis based on their high degree scores, significant levels of differential expression, and annotated functions with potential regulatory roles. In cases where multiple genes within a module shared the highest degree score, or the highest degree-scoring genes were found not to be differentially expressed under heat stress, the highest-scoring DEG was deemed to be the hub gene. These well-connected DEGs were selected for further enquiry as they were deemed to be more likely to act as coordinators of the transcriptional response to heat stress than well-connected genes that were not differentially expressed. Where the putative function of the most well-connected DEG suggested no involvement in either the control of gene expression (be that directly as a transcription factor, or more indirectly via involvement in signalling pathways), or in the heat stress response/thermotolerance (for example, as a heat shock protein; HSP), other DEGs with similar degree scores, which were predicted to play such roles based on their annotation, were favoured as the hub gene and candidate master-regulator. If no such well-connected DEGs within a module were likely involved in such processes, the most well-connected DEG was deemed to be the module's hub gene. Uncharacterized hub genes were studied further, as they represented novel candidates for master-regulators of the heat stress response. Orthologues of hub genes, and genes they were connected to, were identified via EnsemblPlants (Yates et al., 2022).

3.2.8. qRT-PCR

cDNA was obtained from the RNA extracted for each one of the biological replicates of the 13 landraces (4 biological replicates, 2 treatments) of the mRNA-Seq experiment. The reaction was carried out using the ImProm-II™ Reverse Transcription System (Promega) using the manufacturer's instruction, 1 µl of Oligo(dT)16 (5 µM) (Eurogentec Ltd, Camberley, UK) and 1µl of each RNA sample. Quantification was performed with a Nanodrop 2000 and after that,

the cDNA of the 4 biological replicates were pooled in equimolar concentrations. qRT-PCR was performed for the genes TraesCS1B02G384900, TraesCS3B02G409300 and TraesCS4D02G212300 and tubulin using the primers described in Supplementary Data Sheet 7 and the iTaq Universal SYBR Green Supermix (Bio-Rad), adding 200ng of cDNA and 0.1 µM of each primes. The qRT-PCR protocol was set on QuantStudio™ 7 Pro Real-Time PCR System (ThermoFisher) as follows: 95 °C for 4.5 min, 40 cycles of 95°C for 15s and 60°C for 15s. The melting curve was performed by initially heating in a 4.5°C/s ratio up to 95°C and maintaining for 10s reducing the temperature to 3.44°C/s up to 60°C and heating in a 0.15°C/s to 95°C kept for 10s with fluorescence measurement in the last step of the PCR and melting curve. The relative expression between after- and before-heat samples was calculated using the delta-delta Ct method using tubulin as the reference.

3.3. Results

3.3.1. Transcriptome Sequencing and Quantification

An average of 49,798,391 reads were obtained from each sample (minimum of 39,938,210 and maximum of 60,591,112) with an average of 92.5% of reads with Q30 and a GC content of 54.9. After trimming, an average of 2,888,493 reads were kept for each sample. An average of 71.5% of the trimmed reads mapped against the wheat genome. Raw data and Salmon outputs are publicly available in the Gene Ontology Repository (accession number: GSE23236). DESeq2 was used to variance-stabilize counts from all 26 samples, before the 500 most variable genes were assessed via principal component analysis (PCA; **Figure 3.2a**). PC1 and PC2, combined, explained 37% of the total variance, with clear distinction between samples taken before and after heat stress exposure being apparent on PC1 (which explained 27% of the observed variation). PC2 explained a smaller proportion of the total variation (10%) and provided some separation between samples, likely relating to variation in each accession's geographical point of origin.

3.3.2. Identification of DEGs and Comparative Transcriptomic Analysis

To identify genes which may be involved in the heat stress response and basal thermotolerance, DEA was employed via DESeq2 (Love, Huber and Anders, 2014). The analysis identified 7827 genes which were significantly differentially expressed before and after heat stress exposure (response DEGs; **Supplementary Data S3.2**), as well as 93 genes which were differentially expressed between tolerant and susceptible accessions (tolerance DEGs; **Supplementary Data S3.2**). Of the response DEGs, 5384 were significantly upregulated after heat stress exposure, whilst 2443 were significantly downregulated (**Figure 3.2b**). 41 tolerance DEGs were expressed at significantly higher levels in tolerant accessions, whilst 52 tolerance DEGs were expressed more in susceptible accessions. The total number of tolerance DEGs was almost 100-fold less than the total number of response DEGs, and so response DEGs became the main point of inquiry in the subsequent analyses.

To understand the likely functionalities of the genes differentially expressed under heat stress, and to examine the differences in gene functionalities between these groups, GO enrichment analysis on the two DEG groups was conducted **(Supplementary Data S3.3)**. GO terms related to DNA damage and replication (for example; "DNA integrity checkpoint" (GO:0031570), "DNA damage checkpoint" (GO:0000077) and "DNA replication" (GO:0006260)) were significantly enriched amongst upregulated DEGs, as was the term "protein refolding" (GO:0042026). There was also an abundance of terms related to cell wall processes (for example; "Cell wall assembly" (GO:0070726) and "Plant-type cell wall organization or biogenesis" (GO:0071669)), as well terms related to both cellulose (for example; "Cellulose microfibril organization" (GO:0010215) and "Cellulose biosynthetic

process” (GO:0030244)) and lignin (for example; “Lignin metabolic process” (GO:0009808) and “Phenylpropanoid metabolic process” (9.7e-05)) synthesis and organization.

However, amongst downregulated DEGs, terms related to photosynthesis were significantly enriched, for example: “Photosynthesis” (GO:0015979), “Photosynthesis, light reaction” (GO:0019684) and “Photosynthetic electron transport in photosystem II” (GO:0009772). Terms related to the drought response were also significantly enriched, for example: “Response to water” (GO:0009415) and “Trehalose biosynthetic process” (GO:0005992), as were terms related to the general stress response, for example: “Response to stress” (GO:0006950), “Response to oxidative stress” (GO:0006979) and “Response to stimulus” (GO:0050896). Similarly, terms potentially related to the salinity response, for example: “Ion transport” (GO:0006811), “Cation transport” (GO:0006812), “Ion homeostasis” (GO:0050801) and “Sodium ion transport” (GO:0006814) were also significantly enriched amongst downregulated DEGs.

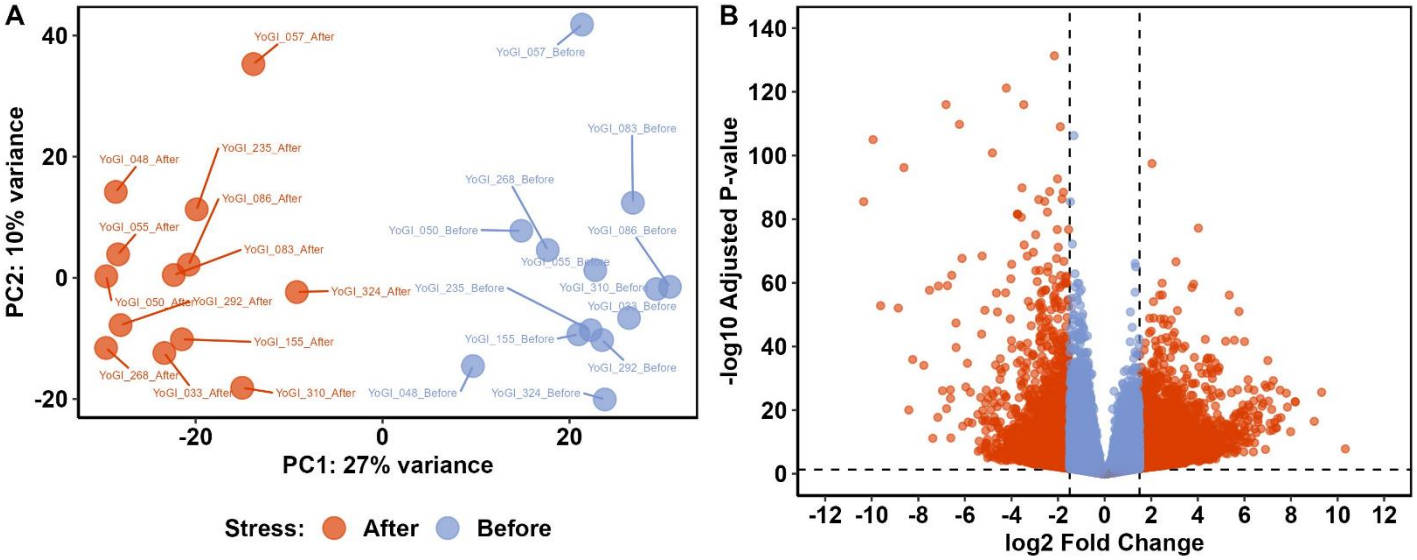

Figure 3.2: Comparative transcriptomic analysis identified a shift in the wheat transcriptome after exposure to early heat stress. Principal component analysis (PCA) of variance-stabilised counts from all 26 samples **(A)** showed clear separation between the two groups on PC1. Differential expression analysis identified 7827 DEGs with significantly different expression before and after exposure to early heat stress **(B)**. Dashed lines indicate DEG thresholds: vertical lines represent the log2FC thresholds of ±1.5, whereas horizontal lines represent the *p*-value threshold of 0.05. DEGs which met these criteria are beyond these threshold lines, coloured red.

3.3.3. Identifying Stress-Associated Modules

The co-expression network was consisted of 73 modules, housing 87,580 genes (**Supplementary Data S3.4)**. Modules within the co-expression network ranged in size from 36 to 26,420 genes, whilst mean and median module size were 1120 and 310 genes, respectively.

Modules significantly enriched in the “response to heat” (GO:0006951), “response to temperature stimulus” (GO:0009266) or “response to stress” (GO:0006950) GO terms likely contain genes involved in the response to heat stress. 11 modules were significantly enriched in these, or other stress-associated, GO terms (**Table 3.1**), with the turquoise and yellow

modules being significantly enriched, specifically, in the “response to heat” GO term. Although it was enriched in the “response to water” (GO:0009415) GO term, the black module may also contain genes involved in responding to elevated temperatures, as drought stress often occurs simultaneously with heat stress. The most significantly enriched GO term, and any significantly enriched stress-associated GO terms, in each module can be seen in **Table S3.2**.

To further explore which modules may be associated with the heat stress response, modules significantly enriched in response DEGs were identified via DEG enrichment analysis. 11 modules were significantly enriched in response DEGs ($p < 0.05$), with a one-proportion Z-test identifying that the proportion of DEGs in these modules was significantly greater than the expected proportion of 8.94% (**Table 3.2**). Amongst these modules, the observed proportions of DEGs ranged from 11.4% (turquoise) to 39.9% (pink).

Table 3.1: 11 modules were significantly enriched in GO terms related to the stress response, according to GO enrichment analysis by the AgriGO v2.0 Singular Enrichment Analysis tool (Du et al., 2010; Tian et al., 2017). The modules enriched in such GO terms are listed, as well as the most significantly-enriched GO term, and the stress-associated GO term they were also enriched in, respectively. In the instances where stress-associated GO terms were the most significantly enriched term in a module, only that term is given.

Module	Enriched GO Term	FDR-adjusted *p*-value
Black	Regulation of RNA biosynthetic process	6.9E-24
	Response to water	0.02
Brown	Amino sugar catabolic process	9E-15
	Response to oxidative stress	1.3E-10
Darkgreen	Multi-multicellular organism process	9.5E-13
	Response to stress	0.001
Darkolivegreen	Sexual reproduction	0.0001
	Response to oxidative stress	0.008
Green	Response to oxidative stress	1.2E-09
Lightcyan	Nucleosome organization	3.4E-09
	Response to stress	0.03
Pink	Photosynthesis	5.8E-18
	Cellular response to stimulus	0.001
Red	Response to biotic stimulus	3.6E-14
	Response to stress	1.7e-05
Salmon	Protein phosphorylation	5.9E-08
	Response to stress	0.002
Turquoise	Translation	9E-128
	Response to heat	0.02
Yellow	Cellular protein localization	1.3E-29
	Response to heat	0.02

Table 3.2: 11 modules were significantly enriched in DEGs, as they contained a significantly higher proportion of DEGs than expected, should the total number have been distributed across modules according to their size (8.94%). These modules are listed, as well as the number of genes in each module, the percentage of these genes which were observed to be DEGs, the mean log2 fold-change of the DEGs within each module, and the *p*-value result from the one-proportion Z-test.

Module	Number of Genes	Observed Percentage of DEGs	Mean log2FC of DEGs	*p*-value
Black	2415	17.8	-2.37	1.82E-52
Blue	6821	14.2	2.54	6.34E-52
Darkgrey	676	29	-2.42	6.61E-75
Darkseagreen4	96	22.9	2	7.95E-07
Darkslateblue	155	25.8	-1.92	9.22E-14
Floralwhite	169	27.2	-2.13	4.10E-17
Grey60	1360	21	-2.19	1.07E-54
Pink	2202	39.9	-2.4	0
Plum	58	34.5	-2.94	4.62E-12
Thistle1	128	13.3	-2.53	0.04
Turquoise	26,420	11.4	2.33	6.15E-45

3.3.4. Hub Gene Identification

Within these stress-associated modules, determined either due to an enrichment of stress-associated GO terms (**Table 3.1**) or an enrichment of DEGs (**Table 3.2**), well-connected DEGs were identified as hub genes which may act to coordinate the transcriptional response to early heat stress. These hub genes are seemingly involved in a range of processes, from thermotolerance, to stress hormone signalling and photosynthesis (**Table 3.3**). However, three of these hub genes, in particular (*TraesCS1B02G384900*, *TraesCS3B02G409300*, and *TraesCS4D02G212300*; **Figure 3.3**), were deemed to be the most promising candidates, not only potentially regulating the transcriptional heat stress response (like the other hub genes), but also of the physiological heat stress response – thanks not only to their own likely function, but the likely functions of the genes they are connected to in their respective modules. Both *TraesCS1B02G384900* and *TraesCS3B02G409300* may determine the expression of potentially superfluous genes, as well as the expression of stress hormone signalling repressors and photosynthesis genes, respectively. *TraesCS4D02G212300*, however, may coordinate the expression of a vast suite of Heat shock-proteins (HSPs), small Heat Shock Proteins (sHSPs) and stress-responsive transcription factors.

3.3.5. qRT PCR validation

qRT-PCR confirmed the patterns of expression for *TraesCS3B02G409300* (*t*-test, $t(24)=5.09$, $p<0.0001$, $n=26$) and *TraesCS4D02G212300* (*t*-test, $t(19.83)=-6.56$, $p<0.0001$, $n=26$), which were found to be down- and up-regulated respectively after heat stress, supporting the role of these genes in activating the early heat stress response. However, the down-regulation of *TraesCS1B02G384900* was unable to be confirmed by qRT-PCR (*t*-test, $t(23.59$, $p=0.98$, $n=26)$.

Table 3.3: Hub genes identified in stress-associated modules may be strong candidates for master-regulators of the heat stress response, based on their high number of connections to other genes within stress-associated modules. Each hub gene's module membership and log2FC are given, as well as their identity and putative function.

Module	Hub Gene	Log2-FC	BLAST Hit	Putative Function	Reference
Black	*TraesCS1B02G384900*	-2.98	*T. aestivum mitogen-activated protein kinase kinase kinase 18-like*	ABA signal transduction	(Matsuoka et al., 2015)
Blue	*TraesCS4D02G212300*	3.29	*T. aestivum 17.9 kDa class I heat shock protein-like (LOC123097951)*	Thermotolerance	(Chauhan et al., 2012)
Brown	*TraesCS4B02G118900*	2.2	*T. aestivum peroxidase 4-like*	ROS Homeostasis, and Lignification	(Fernández-Pérez et al., 2015)
Darkgreen	*TraesCS2D02G589600*	1.65	*TaGSTU153*	Uncharacterized	(Wang et al., 2019)
Darkgrey	*TraesCS1D02G205700*	-1.74	*T. aestivum probable protein phosphatase 2C 47/AtPP2CG1*	Abiotic stress response	(Liu et al., 2012b)
Darkolivegreen	*TraesCS4D02G364400*	-3.82	*T. aestivum transcription factor GHD7-like*	Nitrogen utilization, and Regulation of flowering time	(Zheng et al., 2019; Wang et al., 2021b)
Darkseagreen4	*TraesCS5B02G145800*	2.38	*T. aestivum chalcone isomerase-like protein 2*	Falvonoid synthesis	(Waki et al., 2020)
Darkseagreen4	*TraesCS5D02G145400*	1.62	*T. aestivum chalcone isomerase-like protein 2*	Falvonoid synthesis	(Waki et al., 2020)
Darkslateblue	*TraesCS7D02G333900*	-1.67	*T. aestivum phosphoenolpyruvate carboxylase Tappc1bD*	Photosynthesis and respiration	(Mazelis and Venneslan d, 1957)
Floralwhite	*TraesCS1A02G298600*	-2.03	*T. aestivum WRKY transcription factor WRKY24-like*	Stress hormone signalling repression	(Zhang et al., 2015)
Green	*TraesCS5D02G268900*	1.88	*TaGSFT81*	Uncharacterized	(Wang et al., 2019)
Grey60	*TraesCS2A02G357800*	-1.67	*T. aestivum calmodulin-binding receptor-like cytoplasmic kinase 3*	Calcium signalling	(Zeng et al., 2015)
Lightcyan	*TraesCS1B02G221100*	-2.34	*T. aestivum silicon efflux transporter LSI3-like*	Silicon homeostasis	(Yamaji et al., 2015)
Pink	*TraesCS3B02G409300*	-2.52	*T. aestivum protein EARLY RESPONSIVE TO DEHYDRATION 15-like*	ABA signalling repression	(Kariola et al., 2006)
Plum	*TraesCS4D02G298300*	-4.18	*Aegilops tauschii subsp. strangulata Bowman-Birk type trypsin inhibitor*	Protease inhibition	(Gitlin-Domagalska, Maciejewska and Dębowski, 2020)
Red	*TraesCS7A02G147300*	5.15	*T. aestivum bidirectional sugar transporter SWEET15-like*	Regulation of cell viability under stress	(Seo et al., 2011)

Salmon	*TraesCS1B02G164200*	-1.59	*T. aestivum hypersensitive-induced response protein 1-like*	Hypersensitive response	(Zhou et al., 2010)
Thistle1	*TraesCS5A02G454200*	-1.81	*T. aestivum chlorophyll a-b binding protein of LHCII type 1-like*	Photosynthesis, and ABA signalling	(Liu et al., 2013)
Turquoise	*TraesCS1D02G061400*	1.94	*T. aestivum pentatricopeptide repeat-containing protein At1g09900-like*	Uncharacterized	
Yellow	*TraesCS4B02G339800*	4.2	*T. aestivum sugar transporter ERD6-like 4*	Response to water, and Sugar transport	(Kiyosue et al., 1998)

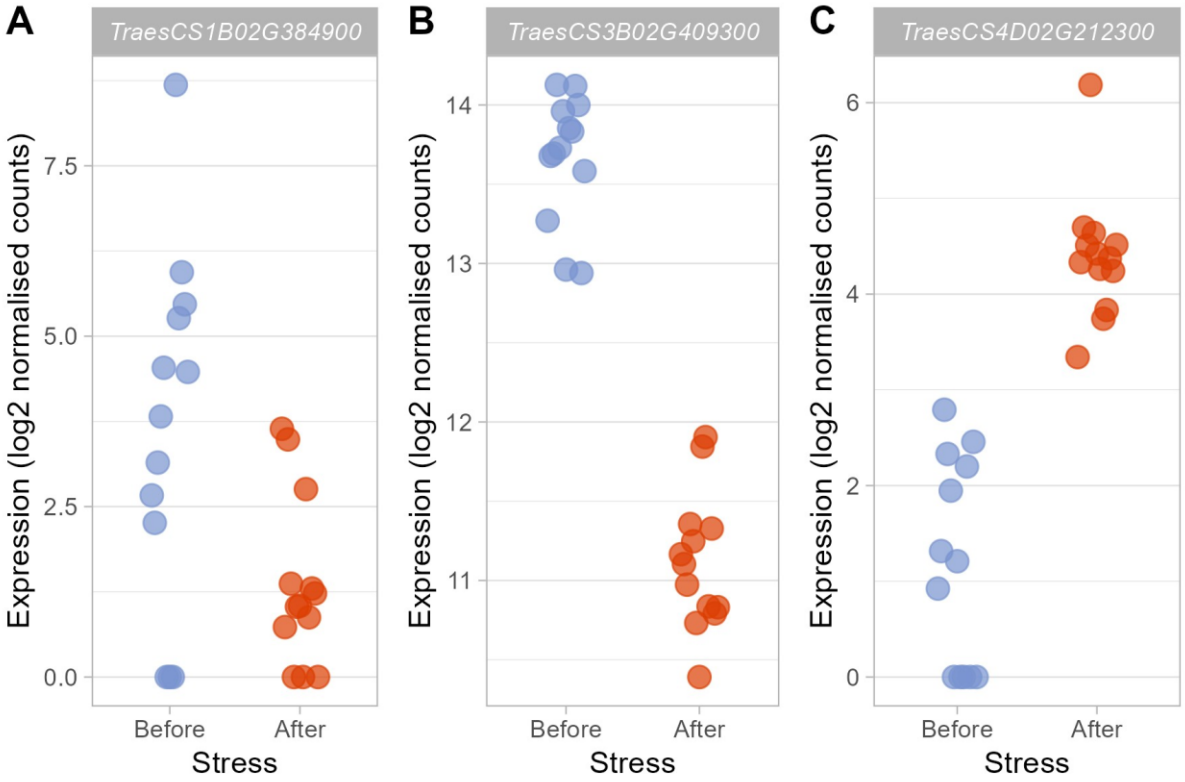

Figure 3.3: Candidate master-regulators of the heat stress response were differentially expressed after heat stress exposure. Those hub genes deemed to be particularly promising based on their membership within stress-associated modules, their putative function, and the putative functions of the DEGs they were connected to showed varying responses to heat stress. *TraesCS1B02G384900* **(A)** and *TraesCS3B02G409300* **(B)** were significantly downregulated (log2FC = -2.98 and -2.52, respectively), whereas expression of *TraesCS4D02G212300* **(C)** was significantly upregulated (log2FC = 3.29).

3.4. Discussion

3.4.1. Heat Stress Causes Widespread Changes in the Wheat Transcriptome

The present work demonstrates that the expression profiles of almost 8000 genes in the spring wheat transcriptome are significantly altered by exposure to early heat stress; 5384 and 2443 genes being significantly upregulated and downregulated, respectively. Amongst these groups of DEGs, genes with different functionalities were significantly enriched. Perhaps predictably given their importance as part of the heat stress response, genes involved in protein refolding were enriched amongst upregulated DEGs (Wang et al., 2004; Kotak et al., 2007; Al-Whaibi, 2011; Mogk, Bukau and Kampinga, 2018; Tian et al., 2021). As well as disrupting protein homeostasis, periods of elevated temperature will also cause single- and double-stranded breaks in DNA, whilst also halting the progression of the replication fork (Velichko et al., 2012; Kantidze et al., 2016; Han et al., 2021). The need to protect cells against such heat-induced DNA damage is a key part of the heat stress response, shown previously to increase thermotolerance in Arabidopsis (Han, Park and Park, 2020), and evidenced by the enrichment of GO terms related to DNA replication and repair amongst upregulated DEGs. Also observed was the enrichment of GO terms related to cell wall processes and lignin biosynthesis – likely evidence of the cell wall remodelling known to occur in plants during periods of high temperature (Yang et al., 2006; Lima et al., 2013; Le Gall et al., 2015; Wu, Bulgakov and Jinn, 2018; Pinski et al., 2021), with lignin synthesis being identified as an important thermotolerance mechanism in rice (Cai et al., 2020).

Amongst downregulated DEGs, however, many terms related to photosynthesis, and photosystem II (PSII) in particular, were significantly enriched. PSII is particularly vulnerable to damage by heat stress (Yamamoto, 2016; Wang et al., 2018a; Hu, Ding and Zhu, 2020), therefore the abundance of these genes amongst downregulated DEGs suggests a partial shutdown of PSII, and thus, a reduced photosynthetic rate under heat stress. Interestingly, terms related to the general stress response were also enriched amongst down-regulated DEGs, suggesting these genes play no role in the tailored heat stress response. Perhaps related to this, terms related to the response to drought and salinity were also enriched amongst downregulated DEGs, including the orthologue of AtPP2CG1, which responds to abscisic acid and positively regulates salt stress tolerance in Arabidopsis (Liu et al., 2012). It may be possible, therefore, that these genes are downregulated to increase transcriptional capacity for genes involved directly in the heat stress response. Similar widespread down-regulation of drought- and salinity-responsive genes under heat stress has not been extensively described previously in similar works in wheat (Qin et al., 2008; Rangan, Furtado and Henry, 2020; Azameti et al., 2022; Lee et al., 2022).

This perhaps speaks to the similarity of the different abiotic stresses, as although drought, salinity, freezing, and heat stresses all cause damage to protein structure and functionality, and cell membrane stability, there exist stress-specific cellular environments under drought, salinity and freezing stresses that are not observed in well-watered plants exposed to high temperatures – such as desiccation, ion imbalance and ice crystal formation. It is those genes involved in responding to these specialized cellular environments, therefore, that are likely to be superfluous under heat stress and, subsequently, are also likely to be downregulated. The same cannot be said for many of the genes involved in responding to heat stress, however, as these genes are largely involved in mitigating the effects common amongst all the abiotic stresses, particularly damage to proteins and membranes. The relatively large cross-over potential of such genes in responding to different abiotic stresses, as a result of the similarities in cellular damage caused by these stresses, is evidenced by the observations of key heat stress-responsive gene families, such as HSPs, acting to enhance tolerance to drought and

salinity stresses, as well as heat, in other species (Gao et al., 2012; Li et al., 2016; Zhai et al., 2016; Guo et al., 2020a; Jiang et al., 2020; Rahman et al., 2022; Do et al., 2023).

3.4.2. *TaMAPKKK18-like* and *TaERD15-like* may coordinate a Transcriptional Shift away from Growth and the Response to Abiotic Stresses other than Heat

Given that the downregulation of genes likely involved in responding to abiotic stresses other than heat seems to be a substantial constituent of the transcriptional heat stress response in wheat landraces, it was interesting that two of the hub genes identified in the co-expression network were connected to a large number of such downregulated DEGs in their respective modules, with one itself likely playing a role in the cold stress response and cold tolerance. The black module contained almost twice as many DEGs as expected (expected number = 216, observed number = 429, p = 1.82E-52), and was enriched in the "Response to abiotic stimulus" and "Response to water" GO terms (FDR-adjusted p = 0.004 and 0.019, respectively). *TraesCS1B02G384900*, *TaMAPKKK18-like*, was downregulated under heat stress (log2FC = -2.98, **Figure 3.3a**), and identified as a hub gene within the black module, being connected to 428 DEGs (100% of the remaining DEGs in the module). Meanwhile, the pink module was identified as particularly associated with the heat stress response as it was significantly enriched in DEGs (expected number = 197, observed number = 879, p = 0), whilst also being enriched in the GO terms "photosynthesis" and "cellular response to stimulus" (p = 1.5E-33 and 0.001, respectively). The hub gene in the module was *TraesCS3B02G409300*, *T. aestivum EARLY RESPONSIVE TO DEHYDRATION 15-like* (*TaERD15-like*). *TaERD15-like* was also found to be downregulated under heat stress (-2.52, **Figure 3.3b**), and was connected to 845 of the 879 remaining DEGs in the module (96%) – all of which were also downregulated.

TaMAPKKK18-like's orthologue (identified via EnsemblPlants; Yates et al., 2022) in Arabidopsis, *AtMAPKKK18*, is a key part of ABA-mediated signal transduction, as it acts to phosphorylate proteins in an ABA-dependent manner (Matsuoka et al., 2015). This kinase activity can determine leaf senescence, growth and stomatal dynamics, as overexpression of the gene led to smaller plants and increased leaf senescence of rosette leaves (Matsuoka et al., 2015), whilst knockout mutants showed more vigorous root growth, as well as increased stomatal aperture (Mitula et al., 2015) – suggesting a link with water use, and subsequently, the drought response. *TaERD15-like*'s orthologue in rice, *OsERD15*, is known to be both expressed more in cold-tolerant varieties, and also to be induced during cold stress exposure (Sperotto et al., 2018; Rativa et al., 2020). Rice and wheat ERD15 proteins are relatively poorly characterized, however in Arabidopsis, they are known to be integral players in the response to abiotic stress, mainly drought and cold, as they act as negative regulators of ABA signalling (Kariola et al., 2006; Aalto et al., 2012). Besides a likely role responding to cold stress, which would be unrequired under high temperatures, a potential duty repressing ABA signalling may also explain the downregulation of the hub gene here, due to the key roles ABA plays during the heat stress response, including increasing antioxidant activity and sucrose metabolism, as well as up-regulating the expression of HSPs and Hsfs (Li et al., 2020).

The likely involvement of both genes in ABA signal transduction, therefore, perhaps explains their identification as hub genes within their respective modules – as the expression of many genes in the wheat transcriptome will respond to this integral signal. *TaMAPKKK18-like*, however, may also be able to have far-reaching effects on gene expression thanks to connections to a suite of heat-responsive transcription factors and signalling proteins: 14% of the DEGs *TaMAPKKK18-like* was connected to in the module were transcription factors (from gene families such as: MYB, WRKY, DREB, ERF and Hsf), whilst *TaMAPKKK18-like* was also connected to 17 differentially-expressed JAZ proteins – key repressors of JA-signalling and

JA-induced gene expression (Santner and Estelle, 2007; Kazan and Manners, 2012; Wager and Browse, 2012; Sasaki-Sekimoto et al., 2014).

Within their respective modules, *TaMAPKKK18-like* and *TaERD15-like* were connected to a large number of DEGs which appear to be involved in responding to abiotic stresses other than heat. For instance, connected to *TaMAPKKK18-like* in the black module were: homoeologues *TraesCS1A02G423800* (-2.25) and *TraesCS1B02G455900* (-2.51), *T. aestivum late embryogenesis abundant 14-A-like* genes, whose orthologue in Arabidopsis increased salt tolerance when overexpressed (Jia et al., 2014); *TraesCS6B02G268100* (-3.57) and its homoeologue *TraesCS6D02G238200* (-4.4), *T. aestivum AP2 domain CBF (CBFI)*, are likely involved in the cold response (Medina et al., 1999); *TraesCS6D02G332500* (-2.58), *T. aestivum cold-shock CS120*, is also likely involved in the response to cold stress thanks to shared sequence identity with regions of cold-response genes in Arabidopsis, such as *AtRAB18* (Lång and Palva, 1992; Lang et al., 1994; Mantyla, Lang and Palva, 1995; Puhakainen et al., 2004), and *TraesCS1D02G263200* (-1.87), *T. aestivum ERF019-like*, which encodes an ethylene-responsive transcription factor whose orthologue in Arabidopsis improves drought tolerance and water use, through reduced stomatal aperture and transpiration, when overexpressed (Scarpeci et al., 2017). Similarly, *TaERD15-like* was connected to *TraesCS4B02G332700* (-2.32), *TraesCS4B02G332800* (-2.68), *TraesCS4D02G329500* (-2.21) and *TraesCS5A02G503800* (-2.91) – copies of *T. aestivum ABA-inducible PHV A1-like*, also known as *HVA1* or *WCOR615*. The barley gene, *HVA1*, has been found to increase drought and salinity tolerance when overexpressed in rice and wheat (Xu et al., 1996; Sivamani et al., 2000; Rohila, Jain and Wu, 2002; Chandra Babu et al., 2004; Bahieldin et al., 2005; Chen et al., 2015). *TaERD15-like* was also connected to *TraesCS5A02G503900* (-1.58), *T. aestivum cold-responsive LEA/RAB-related COR (Wrab17.1)*, another COR protein which has been shown to respond to ABA and cold stress (Tsuda et al., 2000), and may play a role in the biotic stress response (Gaoshan et al., 2018).

Despite both appearing to be involved in determining the downregulation of superfluous drought- and cold-responsive genes under early heat stress, *TaMAPKKK18-like* and *TaERD15-like* may also play key roles in the regulation of other genes involved in separate processes. For instance, reduced expression of *TaMAPKKK18-like* under heat stress may also activate stress hormone signalling, thanks to the co-downregulation of several ABA and JA signalling repressors such as: *TraesCS7A02G201200* (-5.43), *TaTIFY 11e-like*, encoding a likely repressor of jasmonate responses due to its membership in the JAZ family (Santner and Estelle, 2007; Kazan and Manners, 2012; Wager and Browse, 2012; Sasaki-Sekimoto et al., 2014); and *TraesCS3A02G347500* (-2.66), *T. aestivum WRKY24-like*, whose orthologue in rice is a negative regulator of GA and ABA signalling (Zhang et al., 2015). The downregulation of such signalling genes, therefore, may allow key stress hormones to accumulate in plant tissue under heat stress and act as part of the heat stress response (Li et al., 2020).

Similarly, *TaERD15-like* is itself a likely repressor of ABA signalling downregulated under early heat stress, however, here, it was connected to a large number of genes which seemingly play roles in photosynthesis, largely as part of PSII – the most heat-labile part of the photosynthetic apparatus (Yamamoto, 2016; Wang et al., 2018a; Hu, Ding and Zhu, 2020). Nine of these downregulated genes: *TraesCS1A02G403300* (-2.79), *TraesCS1B02G432700* (-2.73), *TraesCS1D02G411300* (-2.66), *TraesCS2A02G204800* (-1.87), *TraesCS2B02G220100* (-1.53), *TraesCS5B02G463100* (-3.06), *TraesCS5D02G329200* (-2.61), *TraesCS5D02G464900* (-3.9), *TraesCS7D02G276300* (-2.53), encode Chlorophyll a-b binding proteins, which form antenna complexes in PSII and act to absorb sunlight, transferring excitation energy to PSII to power photosynthetic electron transport (Jansson,

1994, 1999). Under intense heat stress, PSII light-harvesting complexes fall off of thylakoid membranes, subsequently reducing the efficiency of electron transfer, which results in reduced photosynthesis (Janka et al., 2013; Mathur, Agrawal and Jajoo, 2014; Hu, Ding and Zhu, 2020). 16 other DEGs connected to the hub were constituent parts of the heat-labile PSII reaction centre, all of which were also downregulated under heat stress. The downregulation of these genes, as well as the nine Chlorophyll a-b binding protein genes, suggests inactivation of PSII under heat stress. As well as being connected to genes which are part of PSII, *TaERD15-like* was also connected to *TraesCS4A02G177500* (-2.15), *T. aestivum ribulose bisphosphate carboxylase/oxygenase activase A, chloroplastic-like* – otherwise known as *TaRca2* (Caruana, Orr and Carmo-Silva, 2022). The TaRca2 isoforms are the most heat-labile of the Rubisco activase proteins, meaning, during periods of heat stress, less functional protein is available to remodel the active site of Rubisco to release tightly-bound inhibitors, leading to a reduced photosynthetic rate (Salvucci, Portis and Ogren, 1985; Bhat et al., 2017; Degen, Orr and Carmo-Silva, 2021).

The damaging effect of heat stress exposure on PSII activity is well known (Yamamoto, 2016; Wang et al., 2018a; Hu, Ding and Zhu, 2020), however the present work suggests *TaERD15-like* may be playing a central role in this inactivation, as it was connected to a large number of downregulated PSII genes. Downregulation of *TaERD15-like*, therefore, may be a preventative tactic taken by plants to limit the build-up of damaged photosynthesis proteins under heat stress – a wise tactic considering that this is often toxic to cells (McClellan et al., 2005; Gil et al., 2017).

3.4.3. sHSP Hub Gene May Promote the Heat Stress Response via Up-regulation of Thermoprotectants and Stress-responsive Transcription Factors

The blue module was enriched in DEGs (expected number = 610, observed number = 966, *p* = 6.34E-52), but not in any GO terms related to the stress response. The most well-connected gene in this module, *TraesCS4D02G212300*, *T. aestivum 17.9 kDa class I heat shock protein-like* (LOC123097951), was also upregulated under heat stress (3.29, **Figure 3.3c**). The small HSP (sHSP) hub gene also shares remarkable sequence identity (97%) with *TaHSP26*; a sHSP located in the chloroplast, whose expression is induced by heat stress exposure (Chauhan et al., 2012; Khurana, Chauhan and Khurana, 2013). *TaHSP26* has been known to be involved in thermotolerance for over two decades with Joshi et al. (Joshi et al., 1997) finding that the gene was expressed in thermotolerant recombinant inbred lines, but not susceptible ones. More recently, further evidence for the gene's role in increasing thermotolerance has been provided, as when the gene was expressed in *Arabidopsis*, PSII activity, photosynthetic pigment production, biomass and seed yield under heat stress were all higher than that of WT plants (Chauhan et al., 2012). The present work suggests that the similar gene, and fellow sHSP, *TraesCS4D02G212300* (herein referred to as the "sHSP hub gene"), may act as a key coordinator of the response to early heat stress.

The sHSP hub was connected to 952 of the 966 remaining DEGs in the module, 60 of which were annotated as HSPs. For example: *TraesCS1D02G284000* (2.99), *TaHSP70d*, is a known thermotolerance gene (Hu et al., 2018); *TraesCS2A02G033700* (2.44), *T. aestivum heat shock protein 90-1*, shows sequence identity (76%) to *AtHSP81.4*; whilst *TraesCS1A02G340100* (4.87), and homeologues *TraesCS3B02G308100* (5.82) and *TraesCS3D02G273600* (3.6), encode *T. aestivum* chaperone protein ClpB1-like, and share sequence identity (71%, 72% and 72%, respectively) with *AtHSP101* (*ClpB1*) – a gene whose expression is known to respond to heat stress, and whose protein aids protein refolding under high temperatures, and facilitates the deaggregation of toxic ubiquitylated protein aggregates via interaction with the proteasome (Queitsch et al., 2000; Hong and Vierling, 2001; Tonsor et al., 2008; McLoughlin et al., 2019). The sHSP hub gene was also connected to six genes:

TraesCS1B02G294300 (4.02), *TraesCS1A02G285000* (2.93), *TraesCS3B02G390700* (4.32), *TraesCS3D02G351900* (3.85), *TraesCS3D02G352400* (2.0) and *TraesCS4A02G098600* (3.58), which share 78% sequence identity with *AtHSC70-1* – a repressor of thermotolerance in Arabidopsis (Tiwari, Khungar and Grover, 2020). The upregulation of these six genes, and the previous observation, in **Chapter 2**, that increased expression of a wheat orthologue of *AtHSC70-1* can be used as a marker for increased thermotolerance suggests these genes may play positive roles in both thermotolerance and the heat stress response in *T. aestivum*. The majority of the 60 HSPs connected to the hub were fellow sHSPs, a group of proteins known to delay formation of harmful protein aggregates under heat stress and enhance thermotolerance in a wide variety of plant species, such as rice, maize and poplar (Murakami et al., 2004; Kim et al., 2012b; Sun et al., 2012b; Zhou et al., 2012; Chen et al., 2014; Merino et al., 2014; Tian et al., 2021). The upregulation of these genes under heat stress in wheat corroborates these observations, and suggests the hub gene may act as a master-regulator of these crucial protective genes.

The sHSP hub was also connected to a large group of transcription factors known to play key roles in abiotic stress responses in wheat and other species. Expression of homoeologues *TraesCS7A02G270100* (2.05) and *TraesCS7B02G168300* (2.08), *TaHsfB2-3* and *TaHsfB2-4*, respectively, respond to heat stress treatment (Duan et al., 2019), and these genes belong to a family of transcription factors which act to determine the expression of many other stress-responsive gene family members as part of abiotic stress responses, particularly HSPs (Guo et al., 2016b). *TraesCS3A02G281900* (1.62), *T. aestivum probable WRKY transcription factor 65,* shows some sequence identity (74%) to a small region of its Arabidopsis orthologue, *AtWRKY65* – a gene known to increase thermotolerance and repress thermomorphogenesis when acting alongside its homologues (Qin et al., 2022). However, *TraesCS2D02G414300* (2.72), *T. aestivum ethylene-responsive transcription factor ERF105-like*, may act as part of other stress responses. The gene's orthologue in Arabidopsis is involved in promoting freezing tolerance and cold acclimation (Bolt et al., 2017), suggesting an action as part of the generalized stress response in wheat, as opposed to a tailored response to cold stress. Similarly, the hub is connected to other genes which have previously been described as playing roles in response to stresses other than heat, but their upregulation, and connection to the hub gene, in the present work suggests they may act as part of the general stress response: *TraesCS4B02G176700* (2.03), *TaWRKY19*, has been shown to regulate abiotic stress tolerance when overexpressed in Arabidopsis – leading to increased salt, drought and freezing tolerance, likely via the up-regulation of stress-responsive genes such as *DREB2A*, *RD29A*, *RD29B* and *Cor6.6* (Niu et al., 2012), whilst *Aegilops tauschii subsp. strangulata ethylene-responsive transcription factor 1-like* (*TraesCS4B02G200200* ; 1.8), has been shown to prevent disease progression and regulate the expression of genes involved in the biotic stress response (Lorenzo et al., 2003). Likewise, *TraesCS5D02G148800* (4.58), *TaNAC29*, increases salt and drought tolerance when overexpressed in Arabidopsis (Huang et al., 2015), whereas overexpression of the Arabidopsis namesake of *TraesCS5A02G510100* (2.86) *T. aestivum zinc finger protein CONSTANS-LIKE 4-like*, *AtCOL4*, led to reduced ABA sensitivity and increased salinity tolerance (Min et al., 2015). The upregulation of such genes in the present work suggests a shared role as part of the general stress response, unlike the downregulated drought-, salinity- and cold-responsive genes connected to *TaMAPKKK18-like* and *TaERD15-like* which likely act as part of the tailored response to these stresses. The hub's connection to this suite of upregulated transcription factors, as well as its connection to 60 fellow upregulated HSPs and sHSPs suggests a new function for the poorly-characterized sHSP hub gene as a potential activator of the heat stress response.

Although the role of sHSPs, and TaHSP26 in particular, in acquired thermotolerance and the response to heat stress is widely accepted to be the prevention of protein misfolding and

aggregation of heat labile proteins, recent work suggests that this group of proteins may also act to regulate the activity of signalling proteins, which, in turn, improves thermotolerance by impeding processes such as cell death (Guihur et al., 2020). It may be possible, therefore, that the sHSP hub gene identified in the present work regulates the heat-responsive expression of the genes it is connected to in the co-expression network *via* effects on the activity of these signalling proteins; however, further work is required to determine the exact mechanism by which the sHSP hub gene may indeed regulate the expression of these genes in response to heat stress.

3.5. Conclusions

The present work demonstrates that early heat stress exposure causes large shifts in the wheat transcriptome, with almost 8000 response DEGs being identified, whilst the likely functionalities of genes being upregulated and downregulated suggests a shift away from growth and development, to stress response and damage mitigation. This work also identified a widespread downregulation of genes potentially involved in responding to other abiotic stresses, likely due to the fact that the cellular conditions these genes respond to are not present under heat stress. Pairing these DEA results with the co-expression network then identified heat-associated modules, within which were several promising candidates which may act as regulators of the transcriptional and physiological early heat stress response. Downregulation of two of the most promising candidates under early heat stress (*TaMAPKKK18-like* and *TaERD15-like*) may act to downregulate the expression of these superfluous stress-responsive genes, whilst a sHSP hub gene may activate the expression of HSPs and fellow sHSPs, as well as transcription factors known to play key roles in various abiotic stress responses, including the response to heat. This work, therefore, represents a vital step towards the creation of more thermotolerant wheat varieties, and provides key new insights into the transcriptional response of wheat to early heat stress, as well as candidate genes which may regulate this response.

4. Exposure to Early Heat Stress Causes Novel Flower Emergence Time Response but has no Effect on Yield under Glasshouse Conditions

4.1. Introduction

After identifying markers of early thermotolerance in **Chapter 2**, and using a selection of accessions, with a range of early thermotolerance levels, for comparative transcriptomic analysis in **Chapter 3**, the present work aimed to understand whether early thermotolerance could be used as a marker to predict yield after exposure to early heat stress. Much of the work examining the effect of heat stress on wheat yields has exposed plants to heat stress at, or around, grain filling and anthesis (Dias and Lidon, 2009; Pradhan and Prasad, 2015; Vignjevic et al., 2015; Balla et al., 2019; Qaseem, Qureshi and Shaheen, 2019; Djanaguiraman et al., 2020; Schittenhelm et al., 2020; Shenoda et al., 2021) – stages of wheat development deemed to be the most vulnerable to damage by elevated temperatures. There are far fewer examples, however, of yield traits being examined after the application of heat stress earlier in development (Rahman and Wilson, 1978; Matsunaga et al., 2021). Matsunaga et al. (2021) employed a stress regime similar to that used in the present work whereby plants were exposed to elevated temperatures during early development, before the stress was removed until maturity. Unlike the present work, however, the authors only examined the effect that this stress treatment had on one thermotolerant wheat variety, whereas the present work aimed to examine how exposure to, and then removal of, early heat stress would affect the yields of multiple landrace accessions with a range of thermotolerance levels, and determine whether early thermotolerance was an indicator of yield after exposure to early heat stress.

Similarly, the work by Matsunaga et al. (2021) did not explore how flowering time was effected by exposure to, and then removal of, early heat stress. The present work however, aimed to test the hypothesis that this stress treatment may cause delayed flower emergence, based on the results of the comparative transcriptomic analysis in **Chapter 3**. This analysis revealed that a large number of putative flowering-promoting genes were housed within a single module in the co-expression network, and were all downregulated after exposure to 14 days of heat stress at the three-leaf stage (**Table 4.1**) – suggesting that exposure to this period of early heat stress may lead to delayed flowering or flower emergence as a result of this downregulation. After examining the effect this heat stress treatment had on flower emergence time in the same landrace accessions used in **Chapter 3**, the present work then aimed to determine whether the heat-induced expression responses of any of the identified differentially expressed genes (DEGs) could be used as markers to predict this response.

4.2. Materials and Methods

4.2.1. Flower Emergence Time and Yield Experiment Plant Growth Conditions

The accessions used for validation of early thermotolerance predictive markers in **Chapter 2**, plus an isogenic control (YoGI_350/Paragon), were used to test the effect of early heat stress exposure on flower emergence time and yield. The heat stress treatment used in the present work was the same as described in **Chapters 2** and **3**. However, here, eight replicates per condition were used, whilst 14 days after the three-leaf stage, all plants were moved to glasshouse control conditions (day length: 16/8h, 20°C/14°C (day/night)) until maturity. As in **Chapters 2** and **3**, although air temperature was controlled within the experiment, the effect that this had on soil temperature is unknown, as this was not measured. Any phenotypic responses seen, therefore, can only be confidently associated with changes in air temperature.

4.2.2. Flower Emergence Time Data Collection and Analysis

Flower emergence was deemed to have occurred when the first flower was visible on any of the plant's heads. The number of days to flower emergence from 14 days after the 3-leaf stage (the point when plants were moved from growth cabinets to the glasshouse) was then

determined, before outliers were identified and removed from the dataset using the Tukey method, whereby all values more than 1.5 inter-quartile ranges (IQRs) away from the first and third quartile are removed (Tukey, 1977). Mean values for each accession in each condition were then calculated. The number of days to flower emergence under each condition were then compared for each accession as the normalized difference in number of days to flower emergence (stressed mean days divided by control mean days) was calculated. To determine whether the means in each conditions were significantly different, a two-sample *t*-test was performed.

4.2.3. Yield Data Collection and Analysis

Mature plants were allowed to dry completely before above-ground biomass was harvested. Shoots and heads were separated and weighed, before seed mass per head was measured, and the number of seeds per head were counted. Plant-level traits (shoot biomass weight, total head weight, total biomass weight, total seed weight and total seed number) were calculated for each plant. Outlier detection, using the Tukey method described above, was then conducted to identify outlier replicate plants per accession, per condition for each of these traits. Subsequently, mean seed weight (MSW), thousand grain weight (TGW), harvest index (HI) and head weight proportion (total head weight divided by total biomass weight) were calculated from the remaining data. Because plants often produce multiple heads, head-level traits, such as individual head weight, seed weight per head, and seed number per head were also examined to understand how these traits varied across the heads of each plant. Due to the variation in productivity between primary, secondary, tertiary (and so on) heads per plant, outlier detection was conducted, as described above, to identity outlier heads across all replicates of an accession within a condition. From the remaining data, MSW per head and relative seed weight per head (seed weight per head divided by head weight) were calculated. Mean values for these plant- and head-level traits were then calculated per accession, per condition. Accession mean values between treatment groups were then compared via two-sample *t*-test.

4.2.4. Testing Relationship between Early Thermotolerance and Yield Traits

To determine whether those accessions which showed high levels of thermotolerance during early development (i.e. low normalized loss in mean dry biomass scores) also experienced lesser effects on yield after exposure to, and then removal of, early heat stress, Pearson's linear correlation analysis was conducted in R. Correlations between each accession's normalized loss in mean dry biomass scores and their respective normalized difference in yield and maturity traits (i.e. heat stress mean yield trait divided by control mean of yield trait) were tested.

4.2.5. Testing Relationship between Flower Emergence Time and Yield Traits

To determine whether there was any relationship between the normalized difference in flower emergence time between stress treatments, and the normalized difference in yield traits between stress treatments, Pearson's linear correlation analysis was conducted in R.

4.2.6. Determining whether Expression Responses of DEGs were Markers of Flowering Emergence Time after Early Stress

In **Chapter 3**, 7827 genes were deemed to be significantly differential expressed after 13 of the 15 accessions used for early thermotolerance predictive marker validation in **Chapter 2** were exposed to early heat stress, compared to their expression before the stress was applied – transcriptional responses which may determine flower emergence time later in development.

To test whether the transcriptional response of each DEG could be used as a marker to predict flower emergence time after exposure to, and then removal of, early heat stress, regression analysis was conducted between each of these 13 accessions' normalized difference in mean number of days to flower and their log2-fold-change for each DEG. Before log2-fold-change values were calculated, 0.001 was added to each normalized count value (removing zeros to prevent subsequent errors), and these values were then log2-transformed. R (version 4.1.2.)

packages “purrr” (version 1.0.1.) and “broom” (version 1.0.4.) were then used to conduct these regression analyses in a loop. Summary statistics (adjusted R^2 and *p*-values) were extracted for each gene and collated in a table. Genes were deemed to be predictive markers of flowering time under early heat stress if adjusted $R^2 > 0.55$ and $p < 0.05$. Genes were deemed to be particularly promising markers if their putative function suggested a role in flowering or signal transduction.

4.3. Results

4.3.1. The Effect of Early Heat Stress on Yield and Maturity Traits

Across all accessions, exposure to, and the removal of, early heat stress had no significant effect (*t*-test, $p > 0.05$) on any of the plant-level or head-level yield/maturity traits measured (**Table 4.2**). Individually, however, some accessions showed significant differences in these traits between conditions – for example, total head weight, total biomass, total seed weight, total seed number, total head number, harvest index, head weight proportion, individual head weight, seed weight per head, seed number per head and head weight by seed weight were all significantly affected by exposure to, and removal of, early heat stress in YoGI_048. In contrast, however, YoGI_078 showed no significant differences for any of the plant-level or head-level traits. Mean and standard deviation values shown by each accession for these traits are available in **Supplementary Data S4.1**, whilst normalized differences are available in **Table 4.3**, and results of the two-sample *t*-tests conducted to determine whether mean values between conditions differed significantly are available in **Supplementary Data S4.2**.

4.3.2. Determining Whether Tolerance to Early Heat Stress Exposure Equates to Lesser Yield Losses

None of the correlations between early thermotolerance (determined in **Chapter 2**) and normalized difference in yield and maturity traits were deemed to be significant. Results of the Pearson’s linear correlation analyses can be found in **Table 4.4**.

4.3.3. The Effect of Early Heat Stress on Flower Emergence Time

Across all accessions, the mean number of days to flower emergence shown by plants exposed to control conditions throughout their development (46.7 days) was significantly less than the mean number of days to flower emergence shown by plants which experienced exposure to, and then removal of, early heat stress (57 days, *t*-test: $p = 0.014$). Individually, all of the accessions showed delayed flower emergence after exposure to, and then removal of, early heat stress, with this delay being statistically significant for 13 of the 16 accessions screened (**Table 4.5**). Among these 13 accessions, normalized difference in number of days to flower emergence ranged from 1.13 (13% delay, 10.3 days) to 1.56 (56% delay, 24.6 day delay). The three accessions which did not show significantly delayed flower emergence were; YoGI_062 (5% delay, 1 day delay, $p = 0.33$); YoGI_083 (14% delay, 3.9 day delay, $p = 0.12$), and the isogenic control, YoGI_350 (4% delay, 6.3 day delay, $p = 0.39$). There was also no correlation between early thermotolerance and length of flower emergence delay, as tested by Pearson’s linear correlation analysis (**Table 4.4**).

4.3.4. Determining Whether Flower Emergence Time after Early Stress Exposure affects Yield Traits

Normalized difference in mean number of days to flower emergence was significantly correlated with the normalized differences in both total biomass weight, and shoot biomass weight (**Table 4.6**). No significant correlation was found between normalized difference in mean number of days to flower emergence, and the other measured yield traits, however. Results of the Pearson’s linear correlation analyses can be found in **Table 4.6**.

4.3.5. Identifying Predictive Markers of Flower Emergence Delay under Early Heat Stress

To test the relationship between the fold-change of all heat-responsive DEGs identified in **Chapter 3** and flower emergence delay, regression analyses for all DEGs were performed. Across all 7827 DEGs, the mean adjusted R^2 and *p*-values were 0.02 and 0.46, respectively, whilst the expression responses of 38 genes met the criteria to be classed as predictive markers (**Table S4.1**). Of these 38, 10 genes were members of the co-expression network pink module – the module containing many down-regulated putative flowering-promoting genes. The expression response of seven genes from the 38 identified markers (**Figure 4.1**) were deemed to be particularly promising markers of delayed flower emergence after exposure to, and then removal of, early heat stress, given their putative involvement in flowering and/or the floral transition.

Table 4.1: A large number of genes putatively involved in promoting the onset of flowering were co-expressed, housed within the same co-expression network module, and showed significantly downregulated expression after exposure to early heat stress in **Chapter 3**. The downregulation of these genes led to the hypothesis that early heat stress exposure may lead to delayed flower emergence.

Gene	Log2FC	BLAST Hit	Orthologue	Reference
TraesCS2A02G132300	-2.21	*T. aestivum protein RICE FLOWERING LOCUS T 1-like*	*AtTSF*	(Yamaguchi et al., 2005; Lee et al., 2019)
TraesCS2A02G173500	-1.87	*T. aestivum nuclear transcription factor Y subunit B-10-like*	*AtNF-YB2*	(Kumimoto et al., 2008; Sato et al., 2019)
TraesCS2A02G337300	-2.31	*T. aestivum 14-3-3-like protein GF14-B*	*OsGF14-B*	(Taoka et al., 2011)
TraesCS2A02G359400	-2.23	*T. aestivum nuclear transcription factor Y subunit B-3-like*	*OsHD5*	(Kumimoto et al., 2008; Sato et al., 2019)
TraesCS2B02G154800	-2.88	*T. aestivum protein RICE FLOWERING LOCUS T 1-like*	*AtTSF*	(Yamaguchi et al., 2005; Lee et al., 2019)
TraesCS2B02G288300	-2.28	*T. aestivum flowering-promoting factor 1-like protein 4*	*OsFPFL4*	(Guo et al., 2020b)
TraesCS2B02G344600	-2.46	*T. aestivum 14-3-3-like protein GF14-B*	*OsGF14-B*	(Taoka et al., 2011)
TraesCS2B02G378700	-2.1	*T. aestivum nuclear transcription factor Y subunit B-3-like*	*OsHD5*	(Kumimoto et al., 2008; Sato et al., 2019)
TraesCS2D02G134200	-3.66	*T. aestivum protein RICE FLOWERING LOCUS T 1-like*	*AtTSF*	(Yamaguchi et al., 2005; Lee et al., 2019)
TraesCS2D02G180700	-1.66	*T. aestivum nuclear transcription factor Y subunit B-10-like*	*AtNF-YB2*	(Kumimoto et al., 2008; Sato et al., 2019)
TraesCS2D02G325600	-2.44	*T. aestivum 14-3-3-like protein GF14-B*	*OsGF14-B*	(Taoka et al., 2011)
TraesCS2D02G358300	-1.97	*T. aestivum nuclear transcription factor Y subunit B-3-like*	*OsHD5*	(Kumimoto et al., 2008; Sato et al., 2019)
TraesCS3A02G434400	-1.6	*T. aestivum MADS-box transcription factor 51*	*OsMADS51*	(Kim et al., 2007)
TraesCS6D02G030500	-5.27	*T. aestivum flowering-promoting factor 1-like protein 4*	*OsFPFL4*	(Guo et al., 2020b)
TraesCS7D02G213000	-2.24	*T. aestivum WCO1*	*OsHD1*	(Shaw et al., 2020; Takagi, Hempton and Imaizumi, 2023)

Table 4.2: Across all accessions screened, exposure to, and then removal of, early heat stress had no significant effect on any of the yield traits measured. Mean values for each trait, across all accessions in each condition, are listed as well as standard deviations (±) for each of these means. Mean values between conditions were compared via two-sample *t*-test. The *p*-value results from these tests are also listed.

Trait	Control Mean	Heat Mean	*p*-value
Shoot Weight (g)	2.13±1.25	2.26±1.45	0.27
Total Head Weight (g)	1.45±0.35	1.52±0.36	0.32
Total Biomass (g)	3.61±1.42	3.78±1.61	0.17
Total Seed Weight (g)	1.05±0.35	1.09±0.35	0.56
Total Seed Number	28.74±10.09	28.41±8.89	0.99
MSW (g)	0.04±0.01	0.04±0.01	0.44
TGW (g)	37.34±6.69	38.55±7.04	0.41
Head Number	1.51±0.69	1.71±0.77	0.12
Harvest Index	31.59±12.21	31.43±11.41	0.63
Head Weight Proportion	0.43±0.12	0.43±0.12	0.7
Individual Head Weight (g)	1.09±0.46	1.05±0.5	0.39
Seed Weight per Head (g)	0.8±0.42	0.77±0.43	0.39
Seed Number per Head	21.08±9.55	19.51±9.17	0.42
MSW per Head (g)	0.04±0.01	0.04±0.01	0.33
Proportion Seed Weight per Head	0.7±0.17	0.7±0.12	0.6

Table 4.3: Accessions showed varying responses in yield to early heat stress exposure. Normalized difference values (heat mean divided by control mean) for each trait are listed for each accession, whilst significant differences between means from the two conditions (as identified by two-sample *t*-test) are signified according to the following: $p \leq 0.05$ = *, $p \leq 0.01$ = **, $p \leq 0.001$ = ***, $p \leq 0.0001$ = ****.

Accession Name	Shoot Weight	Total Head Weight	Total Biomass	Total Seed Weight	Total Seed Number	MSW	TGW	Head Number	Harvest Index	Head Weight Proportion	Individual Head Weight	Seed Weight per Head	Seed Number per Head	MSW per Head	Proportion Seed Weight per Head
YoGI_033	1.18	0.35	0.77	0.06	0.06	0.89	0.89	1.25	0.09	0.48	0.30	**0.05*** **	0.05	0.89	0.15
YoGI_048	0.83	**0.92** **	**0.87***	**1.01*** **	**0.83*** **	1.17	1.18	**0.69*** **	**1.07** **	**1.08***	**1.41***	**1.48** **	**1.12** **	1.17	**0.96** **
YoGI_050	0.38	0.69	0.52	0.66	0.69	0.95	0.95	1.00	1.27	1.31	0.71	0.67	0.71	0.95	0.98
YoGI_055	1.41	1.07	**1.23***	1.26	1.21	1.04	1.04	0.71	1.06	0.91	1.52	1.68	1.60	1.07	1.38
YoGI_057	1.16	1.46	1.28	1.72	2.57	0.72	0.72	1.45	1.34	1.14	0.91	**1.11***	**1.58** **	0.70	1.19
YoGI_062	**1.24*** **	**0.93** **	**1.08**** **	**0.98** **	**0.79***	1.28	1.28	1.75	0.93	0.87	**0.59**** **	**0.60**** **	**0.48**** **	1.26	1.07
YoGI_078	1.32	1.10	1.16	0.98	0.86	1.21	1.21	1.38	0.82	0.89	0.80	0.72	0.62	1.18	0.88
YoGI_083	0.32	0.95	0.48	1.10	0.81	1.40	1.39	0.38	**2.31***	**1.98***	3.27	**4.01** **	**2.55** **	1.53	1.19
YoGI_086	0.96	1.81	1.23	3.85	**4.02***	**1.11*** **	**1.11*** **	0.86	**3.11***	1.47	2.31	4.77	4.37	**1.08** **	2.18
YoGI_155	1.31	0.90	1.11	0.94	1.18	0.86	0.87	0.86	0.85	0.82	1.06	1.09	1.09	0.87	**1.09***
YoGI_235	2.60	2.18	2.41	2.13	1.50	1.42	1.42	0.50	0.93	0.95	**3.61***	3.63	**2.57***	1.43	0.98
YoGI_268	0.92	0.47	**0.66***	0.33	0.48	**0.70** **	**0.70** **	1.38	0.48	0.69	0.32	0.21	0.31	**0.69***	0.69
YoGI_292	**1.06***	**1.15***	**1.10** **	1.13	**0.81***	1.39	1.39	**0.50***	0.99	1.02	2.21	2.23	1.61	1.38	0.98
YoGI_310	1.45	**0.84***	1.13	**0.75***	1.16	**0.65***	**0.65***	0.50	**0.64** **	**0.72*** **	1.87	1.66	2.36	**0.69** **	0.89
YoGI_324	0.14	0.96	0.29	1.11	0.73	1.42	1.43	0.32	3.73	3.00	3.71	5.09	2.31	1.54	1.22
YoGI_350	**4.65***	1.46	2.84	1.44	1.96	0.75	0.75	1.75	0.51	0.52	**0.88** **	**0.90*** **	1.15	**0.84***	1.19

Table 4.4: There was no correlation between early thermotolerance and the normalized difference (heat mean divided by control mean) of any of the measured yield traits, or the normalized difference in mean number of days to flower emergence. Results of each Pearson's linear correlation analysis are listed.

Normalized Difference in Trait	Correlation Coefficient	*p*-value
Individual Head Weight	0.38	0.17
Seed Weight per Head	0.29	0.45
Seed Number per Head	0.19	0.49
MSW per Head	0.008	0.98
Proportion Seed Weight per Head	0.05	0.85
Shoot Weight	-0.36	0.18
Total Head Weight	0.14	0.62
Total Biomass	-0.11	0.7
Total Seed Weight	0.06	0.83
Total Seed Number	0.06	0.83
Total MSW	-0.05	0.87
Total TGW	-0.05	0.86
Head Number	-0.04	0.88
Harvest Index	0.09	0.75
Head Weight Proportion	0.31	0.26
Normalized Difference in Number of Days to Flower Emergence	-0.37	0.17

Table 4.5: Exposure to, and then removal of, early heat stress led to delayed flower emergence in all of the accessions screened. This delay was significant for 13 of the 16 accessions, as determined by a two-sample *t*-test. The mean number of days to flower emergence from 14 days after the three-leaf stage, in both conditions, are listed, as well as the standard deviation (±) for each mean. The normalized difference in mean number of days to flower emergence (heat mean divided by control mean), and the *p*-value result of the two-sample *t*-test for each accession are also provided.

Accession Name	Control Mean Days to Flower Emergence	Heat Mean Days to Flower Emergence	Normalized Difference in Mean Days to Flower Emergence	*p*-value
YoGI_033	31.38±3.38	40.00±0	1.27	0.0002
YoGI_048	30.67±1.75	39.29±0.76	1.28	0.0001
YoGI_050	39.83±3.25	45.83±2.14	1.15	0.0036
YoGI_055	28.14±0.69	38.00±0.82	1.35	0.0001
YoGI_057	37.17±3.43	55.57±10.05	1.50	0.0014
YoGI_062	19.00±1.41	20.00±2.28	1.05	0.3305
YoGI_078	32.38±3.38	48.83±5.71	1.51	<0.0001
YoGI_083	27.14±3.48	31.00±5.21	1.14	0.1214
YoGI_086	35.63±4.87	42.25±5.87	1.19	0.0277
YoGI_155	33.63±2.88	44.14±5.55	1.31	0.0004
YoGI_235	43.86±2.54	68.43±3.21	1.56	<0.0001
YoGI_268	80.57±8.5	90.86±4.38	1.13	0.0147
YoGI_292	103.38±7.31	121.00±5.63	1.17	0.0002
YoGI_310	29.50±2.45	36.38±3.11	1.23	0.0002
YoGI_324	33.57±3.87	42.29±2.98	1.26	0.0005
YoGI_350	141.57±9.45	147.86±16.28	1.04	0.3944

Table 4.6: For the majority of the yield traits, there was no correlation with the normalized difference in mean number of days to flower emergence. A significant relationship was observed between the length of flower emergence delay after early heat stress exposure, and shoot biomass weight and total biomass weight, however. Results of each Pearson's linear correlation analysis are listed, with significant associations highlighted in bold.

Normalized Difference in Trait	**Correlation Coefficient**	***p*-value**
Individual Head Weight	0.08	0.78
Seed Weight per Head	0.05	0.87
Seed Number per Head	0.04	0.88
MSW per Head	0.09	0.74
Proportion Seed Weight per Head	-0.01	0.97
Shoot Weight	**0.57**	**0.03**
Total Head Weight	0.3	0.28
Total Biomass	**0.6**	**0.02**
Total Seed Weight	0.06	0.83
Total Seed Number	0.03	0.9
Total MSW	0.22	0.43
Total TGW	0.24	0.4
Head Number	-0.2	0.48
Harvest Index	-0.02	0.95
Head Weight Proportion	-0.1	0.73

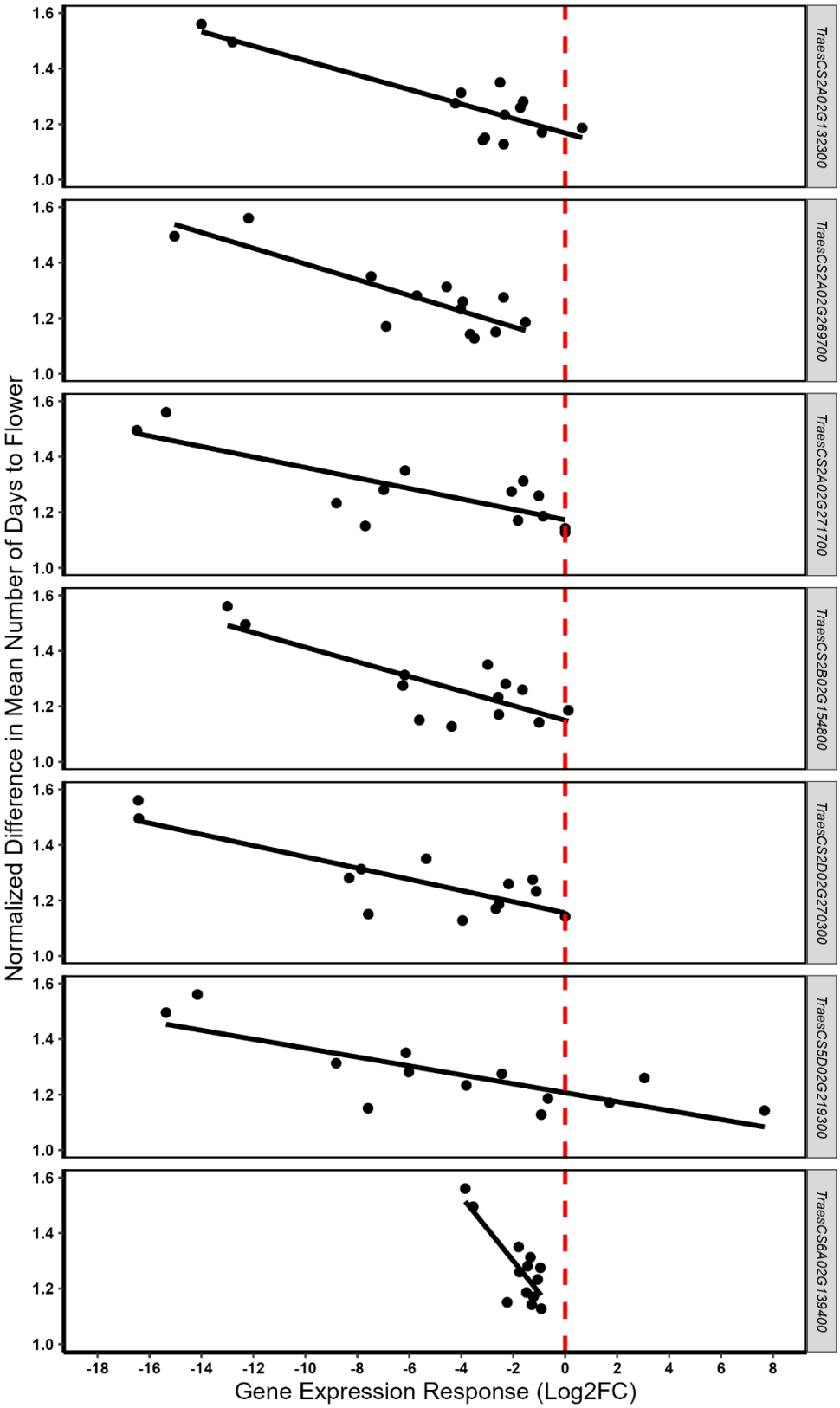

Figure 4.1: Identification of flower emergence delay markers. Linear regression analysis identified seven particularly promising genes, whose transcriptional response to early heat stress exposure could be used as markers to predict the length of the observed flower emergence delay. In all cases, those accessions which showed the most severe downregulation of the gene also tended to show the longest delay.

4.4. Discussion

4.4.1. Early Heat Stress Exposure Caused Novel Flower Emergence Delay

Differential expression and co-expression network analyses in **Chapter 3** identified that a large number of genes putatively involved in promoting flowering were housed within the same module, and were all downregulated significantly after exposure to heat stress (**Table 4.1**). This led to the hypothesis that exposure to heat stress during early vegetative development may subsequently cause delayed flower emergence, due to the downregulation of these genes.

The vast majority of accessions indeed showed significantly delayed flower emergence after exposure to, and then removal of, early heat stress, with the largest delay being 24.6 days. This observation is at odds with prior work in Arabidopsis which found that heat stress exposure accelerates flowering, whilst periods of cold tend to delay flowering (Lee et al., 2013; Balasubramanian et al., 2006), and wheat (Rahman et al., 2009; Nahar, Ahamed and Fujita, 2010; Hakim et al., 2012; Hemming et al., 2012; Hossain and da Silva, 2012; Hossain et al., 2012, 2013). Further, a meta-analysis of historical flowering date data from over 400 plant species found that flowering times accelerated by between four and six days, per single degree centigrade increase in atmospheric temperature (Jagadish et al., 2016), suggesting this response to prolonged periods of increased temperature is shared amongst flowering plants.

Despite these data seeming to contradict the reported reproductive growth patterns under heat stress in Arabidopsis and wheat, there are some critical differences between these examples and the present work which explain this discrepancy. Here, plants were exposed to an intense burst of heat stress for a two week period, before being moved back to optimal conditions – whereas, in the published works, plants were exposed to constant, less intense heat stress (~25°C) throughout their development. There is no published work which exposes plants to intense heat stress for a short period of time during early development, before removing the stress and examining flower emergence time, and subsequently no observations of such a stress treatment causing delayed flower emergence – making the present work completely novel.

Although the majority of the accessions screened showed significantly delayed flower emergence after exposure to, and then removal of, early heat stress, the length of this delay varied across the small panel, whilst three accessions (including the isogenic control) showed statistically insignificant delays in flower emergence time. Under control conditions, variation in flowering time between accessions or varieties is often a result of different combinations of alleles in photoperiod sensitivity *Ppd-1* genes, varying levels of expression of the vernalisation requirement (*VRN*) and earliness per se (Eps) genes, or copy number variation in members of these gene families (Slafer, 1996; Snape et al., 2001; Distelfeld, Li and Dubcovsky, 2009; Díaz et al., 2012; Langer, Longin and Würschum, 2014; Arjona et al., 2020). This sort of basal variation, however, is unlikely to adequately explain the observed variation in flower emergence delay after exposure to, and then removal of, early heat stress – a trait obviously affected by exposure to the stress, and therefore likely controlled by transcriptional or epigenetic changes in response to the elevated temperature (Riboni et al., 2014). Differences in flowering time under different environmental conditions have been shown to be regulated by such changes; for instance, the downregulation of flowering-promoting genes under drought stress led to delayed flowering in rice (Galbiati et al., 2016), whilst floral repressor *FLC* is epigenetically silenced in response to a period of prolonged cold during vernalisation, subsequently allowing flowering to occur (Bastow et al., 2004). To test whether the observed flower emergence delay could be explained by such transcriptional responses to early heat stress, data from **Chapter 3** were utilized in the present work.

4.4.2. Yield Traits were Largely Unaffected by Exposure to Early Heat Stress under Glasshouse Conditions, but the Effect Varied between Accessions

Firstly, it is worth noting that although large normalized difference values were observed for many of the yield traits by almost every accession, the majority of these differences were not deemed to be statistically significant by two-sample *t*-test. This is likely a result of the relatively high variability seen for these data, with large standard deviations being observed across the panel of accessions (**Supplementary Data S4.1**). Perhaps future work examining the effect of early heat stress exposure on yield traits could use a much higher number of replicates per accession, per condition, in an attempt to reduce such variation.

Across all accessions, none of the 15 yield traits measured in the present work were significantly affected by exposure to, and then removal of, early heat stress (**Table 4.2**). However, certain agriculturally-relevant traits of individual accessions were significantly affected – for example, YoGI_062 and YoGI_086 showed significantly reduced seed number after exposure to, and then removal of, early heat stress, relative to the control, whereas YoGI_268 showed significantly reduced total biomass, MSW and TGW. This sort of significant yield loss was expected, as the damage to vegetative tissue caused by early heat stress exposure (**Chapter 2**) likely impeded normal growth and development after the stress was removed. Similar reductions in agriculturally-relevant traits have been seen when wheat plants were exposed to elevated temperatures during vegetative development; for example, exposure to 30/23°C (day/night) until ear emergence led to reduced spikelet number per ear (Rahman and Wilson, 1978), whilst more recent work utilized historical climate and crop data to simulate (accounting for varietal change over time) that as the climate warmed over the 25 years under study, biomass and grain yield both decreased at three sites in China (Liu et al., 2010).

However, despite the damaging effect of early heat stress on wheat seedlings, some accessions showed significant increases in several yield traits – for example, YoGI_048, YoGI_292 and YoGI_310 all showed significantly increased total head weight, whilst YoGI_048, YoGI_055 and YoGI_292 showed significantly increased total biomass. Increased biomass production and shoot weights under heat stress may be expected, as a result of the observed significant flower emergence delay after early heat stress exposure in the majority of the accessions screened. A significant positive correlation was found between the length of flower emergence delay and these traits, meaning the accessions which showed the shortest delay also tended to produce less biomass under heat stress, relative to the control, and vice versa (**Table 4.6**). This would be expected, given that previous work has found that stress exposure generally causes wheat plants to move more rapidly through their development in order to produce seeds as quickly as possible (Rahman et al., 2009; Nahar, Ahamed and Fujita, 2010; Hakim et al., 2012; Hossain and da Silva, 2012; Hossain et al., 2012, 2013), resulting in less vegetative biomass production as a result of less time spent in vegetative development. However, the majority of accessions screened in the present work showed significantly delayed flower emergence, and thus spent a longer amount of time in vegetative development, meaning they spent more time producing larger, or a greater number of, leaves – hence the increased biomass production under heat stress by these accessions.

This increased biomass production may also explain some of the observed increases in other yield traits, as this greater amount of vegetative tissue likely meant plants were able to accumulate more sugars to fuel grain production, thanks to a higher photosynthetic capacity. Such a relationship between biomass and grain yield has previously been observed in wheat (Gaju et al., 2016), as when the data generated by Gaju et al. (2016) is used for Pearson's correlation analysis, a significant positive correlation is found between biomass weight and grain yield (correlation coefficient = 0.87, p = 3.59E-10). Similarly, when the raw data from all the samples in the present work are used for Pearson's correlation analysis, a significant positive correlation is found between total biomass weight and total seed weight (correlation coefficient = 0.33, p = 6.45E-07), suggesting high levels of vegetative biomass may indeed contribute to the higher yields observed here.

Further work screening the effect of early heat stress exposure on the thermotolerant wheat line Norin 61, also saw similar trends to those observed in the present work (Matsunaga et al., 2021). The authors found that exposure to 38/18°C (day/night) from the two-leaf stage until tillering, followed by exposure to control conditions 22/18°C (day/night) until maturity (a stress treatment not dissimilar from that used in the present work) caused harvest index and thousand kernel weight to significantly increase relative to control plants, whilst no significant detrimental effect was observed for other agriculturally-relevant traits, such as grain yield, grain weight per spike, grain number per spike and total grain number. Interestingly, although the authors did not measure flowering time, they found that this stress treatment had no effect on biomass production, whilst longer periods of heat stress caused significantly reduced total biomass as well as reduced grain yield, grain weight per spike and grain number per spike – providing further support for the hypothesis that an increased amount of biomass is able to support higher yields, whereas heat-induced biomass losses may incur yield penalties, as there is less vegetative tissue available for photosynthesis to support grain development.

It is also likely that the lack of significant yield losses may be due to the fact that the present work was conducted in the glasshouse, as opposed to the field. In the present work, plants were grown under glasshouse conditions until they were fully mature and dried ready for harvest – however, the significantly delayed flower emergence seen for the majority of accessions may have led to reduced yields in the field, as crops are often harvested at, or around, a fixed time point. The delayed flower emergence of stressed plants, therefore, will likely cause delayed grain formation and a reduction in the time available for seed maturation and grain filling before harvest, as plants are developmentally stunted – effectively reducing yield. Later harvesting may mitigate this issue, however disruption to the crop cycle would likely lead to downstream complications for farmers; for example, unpredictable harvest dates may mean the wheat crop cycle becomes desynchronized from those of other crops, whilst a later harvest date may incur additional costs as a result of increased nutrient, pesticide or irrigation application. Therefore, it would be interesting for future work to determine whether exposure to, and then removal of, early heat stress, and the subsequent delay in flower emergence, in the field causes yield losses, or whether similar trends as observed in the present work would again be seen – similarly, this work would also allow the “agribusiness” effects of unpredictable harvest dates (as a result of delayed flower emergence and likely stunted development) to be seen.

To determine whether those accessions which tended to show improved yield after exposure to, and removal of, early stress were also those deemed to be thermotolerant in **Chapter 2**, the relationship between normalized loss in mean dry biomass and the normalized differences (heat mean relative to control mean) in the yield traits described above were tested via Pearson’s linear correlation analysis. No significant relationship was observed between early thermotolerance and these yield traits, suggesting an accession’s ability to tolerate heat stress during early development has no bearing on how its yield will be effected later in development. This was perhaps unexpected given that early heat stress exposure caused significantly reduced growth in wheat seedlings (**Chapter 2**), likely as a result of the physiological and molecular damage discussed at length in **Chapter 1**. Therefore, although there was no correlation between the definition of thermotolerance used in the present work (little difference in the dry weights of wheat seedlings exposed to heat stress and control conditions), and yield traits after exposure to, and then removal of, early heat stress, this does not necessarily mean that seedling thermotolerance cannot be used to predict the effect of early heat stress on yield. Instead, perhaps there would have been a correlation between these factors if seedling thermotolerance was defined in a different way; such as, by the membrane thermostability, antioxidant enzyme activity, or photosynthetic rate under heat stress of these seedlings. Further work is required to screen such traits in these accessions, and also to determine whether such traits show a correlation with the yield effects of early heat stress exposure observed in the present work. Also, further work is required to determine whether the

relationship between early thermotolerance and yield effects observed in the present work is also observed under field conditions.

4.4.3. Early Heat Stress DEGs connected to *TaERD15-like* are Markers for Flower Emergence Delay

Work in **Chapter 3** identified that the downregulated hub gene, *TraesCS3B02G409300* (*TaERD15-like*), was connected, in the co-expression network, to a large group of genes which appear to promote flowering and the floral transition (**Table 4.1**). Like *TaERD15-like*, this group of genes were all also downregulated under early heat stress, suggesting exposure to this stress may cause a flowering delay as a result of the downregulation of such genes. The subsequent finding that exposure to, and then removal of, early heat stress indeed caused significantly delayed flower emergence in the majority of the landrace accessions gave credence to this hypothesis. Then, to determine whether the expression changes in other DEGs may cause this phenotype, regression analysis was employed. There was a significant relationship between gene expression change under early heat stress exposure, and flower emergence delay, across 13 of the 16 screened accessions (those used for differential expression analysis in **Chapter 3**), for 38 genes – 10 of which were housed within the pink module in the co-expression network, and were connected to the hub gene, *TaERD15-like*.

Amongst these 10 genes were several which are particularly promising candidates for coordinators of the floral delay. For example, the expression responses of *TraesCS2A02G132300* (R^2 = 0.71, *p* = 0.0002), and its homeologue *TraesCS2B02G154800* (R^2 = 0.61, p = 0.001) were identified as predictive markers of delayed flower emergence, and likely play a role in promoting flowering, as the homeologues are known as *T. aestivum protein RICE FLOWERING LOCUS T 1-like*. Their namesake in rice, *RFT1*, is a major floral activator under long day conditions (Komiya et al., 2008; Komiya, Yokoi and Shimamoto, 2009), with overexpression leading to extremely early flowering (Pasriga et al., 2019), and natural mutations in *RFT1* being accountable for flowering time variation under long-day conditions (Ogiso-Tanaka et al., 2013). However, their orthologous rice gene (identified via EnsemblPlants; (Yates et al., 2022), *OsFTL12*, acts to repress heading date thanks to antagonistic function against RFT1 and Hd3a (another major floral activator), as the protein binds to other members of the florigen activation complex, competing with Hd3a, to form the florigen repression complex (Zheng et al., 2023) – providing a question as to what the function of these homeologues are in wheat. In the present work, those accessions which showed the most severe downregulation of these homeologues also showed the longest flower emergence delay (**Figure 4.1**), suggesting the function of these genes may more closely reflect that of their namesake, *RFT1*, than their orthologue, *OsFTL12*. Furthermore, the downregulation of *RFT1* has already been linked with delayed flowering under stress in rice, as work by Galbiati et al. (2016) showed that drought stress exposure led to delayed flowering in rice, likely as a result of the drought-responsive downregulation of flowering-promoting genes, including *RFT1* – mirroring the relationship between transcriptional response and flower emergence delay observed for *RFT1*'s namesakes in the present work.

Further support that the homeologues likely act to promote flowering comes via the function of their Arabidopsis orthologue, *TWIN SISTER OF FT* (*TSF*). This gene is a crucial regulator of the floral transition, with mutation leading to delayed flowering under short day conditions, whilst overexpression led to early flowering (Yamaguchi et al., 2005). More recent work (Lee et al., 2019) not only confirmed these results, but also suggested that *TSF* acts early in development to determine the fate of the inflorescence meristem, with the inflorescence meristem maintaining vegetative shoot identity in *ft-10 tsf-1* mutants at the early bolting stage, before subsequently flowering much later in development. The importance of *TSF* in the floral homeotic transformation at the shoot apex of the main inflorescence was also shown, with this transformation being inhibited completely when *FT* and *TSF* were mutated, as the main inflorescence was reverted to a vegetative shoot. Therefore, the results of the regression analysis, paired with the functions of the homeologues' rice namesake and Arabidopsis orthologue, suggests that downregulation of these genes under early heat stress exposure

may subsequently lead to delayed flower emergence via inhibition of the floral transition in the shoot apex of the inflorescence, and the inflorescence meristem – effectively halting stressed plants in vegetative development for longer than those under control conditions.

Another downregulated gene, connected to the *TaERD15-like* hub gene, identified as a predictive marker for delayed flower emergence in the present work was *TraesCS2A02G269700*, *T. aestivum flowering-promoting factor 1-like protein 4* (R^2 = 0.68, *p* = 0.0003). Like the homeologues discussed previously, *TraesCS2A02G269700* also likely acts to promote flowering, as it's orthologous gene in rice, *Os04g0282400* (*OsFPFL4*), acts to control floral development, with RNA interference (RNAi) of the gene delaying flowering (Guo et al., 2020b). Again, those accessions which showed the most severe downregulation of *TraesCS2A02G269700* also showed the longest flower emergence delay (**Figure 4.1**), further suggesting that the gene acts to promote flowering and that its downregulation under early heat stress exposure may have subsequently led to delayed flower emergence.

Despite being connected to over a quarter of the flower emergence delay markers identified in the present work, including the genes discussed previously, the expression response of the hub gene, *TaERD15-like*, itself was not deemed to be a predictive marker for delayed flower emergence, despite its expression response being significantly, but relatively weakly, associated with flower emergence delay (R^2 = 0.32, *p* = 0.026). Although *TaERD15-like* may regulate the expression of these flower emergence markers under early heat stress, other factors are also likely to contribute towards the control of their expression under these conditions, meaning changes in *TaERD15-like* expression, alone, are likely insufficient to regulate the expression of the genes which seemingly control this phenotype.

Taking the homeologous flower emergence markers as an example, it is known that the addition of activating H3K9 histone modifications around the *RFT1* locus, particularly at the 5' UTR, is positively correlated with increased expression of *RFT1* (Komiya et al., 2008), whilst the flowering delay seen in *lvp1* mutants has been linked with reduced expression of *RFT1* thanks to decreased levels of the activating H3K36me2/3 modification at the locus (Sun et al., 2012a). Meanwhile, other work has characterized a gene regulatory network, identifying several positive (*OsMADS50* and *Ehd1*) and negative (*Hd1*, *phyB* and *Ghd7*) regulators which act to determine flowering time under long day conditions in rice, via regulation of *RFT1* expression (Komiya, Yokoi and Shimamoto, 2009). As well as regulation by various other proteins and epigenetic marks, Komiya et al. (2008) also found that *RFT1* expression increases when another major floral activator, *Hd3a*, is mutated. Similarly, expression of the homeologues' Arabidopsis orthologue, *TSF*, is known to be positively regulated by CONSTANS, which is itself under the control of the circadian clock (Yamaguchi et al., 2005). These authors also found that expression of *TSF* was repressed by high levels of *FLC* expression, whilst vernalization removed this repression thanks to the downregulation of *FLC* expression, and suggest that phyB may also negatively regulate *TSF* expression, as *TSF* expression was elevated in *phyB* mutants. These findings, therefore, may provide an insight as to why *TaERD15-like* was not identified as a marker for delayed flower emergence, despite being connected to such markers in the co-expression network. As although this connection suggests *TaERD15-like* may regulate the expression responses of these genes, and their connection in the co-expression network suggests they are significantly co-expressed, the hub gene is likely one of many factors which act to determine the expression of these putative floral activators, so its own expression response, alone, may not be able to explain the expression response of such genes, and therefore cannot explain the observed delay in flower emergence after exposure to, and then removal of, early heat stress.

4.4.4. Expression Response of Heat-responsive DEGs Act as Predictive Markers of Flower Emergence Delay Length

Besides DEGs connected to the hub gene in the pink module, several other DEGs are also promising candidates for coordinators of the floral delay seen in the present work. For example, the downregulated gene *TraesCS6A02G139400*, *T. aestivum cold-responsive*

protein kinase 1-like, housed in the black module, was also identified as a significant marker of delayed flower emergence (R^2 = 0.63, p = 0.0008). Again, those accessions which showed the greatest level of downregulation of the gene were those which showed the longest delay in flower emergence time after exposure to, and then removal of, early heat stress (**Figure 4.1**) – suggesting the gene plays a positive role in floral development. *TraesCS6A02G139400*'s orthologue in Arabidopsis, *AtCRPK1*, is a cold-activated plasma membrane protein which plays a key role in cold signal transduction, via phosphorylation of 14-3-3 proteins (Liu et al., 2017). Phosphorylation of 14-3-3 proteins allows them to translocate to the nucleus, where they interact with C-repeat-binding factor (CBF) proteins, key players in the cold response and freezing tolerance, forming a protein complex which promotes their 26S proteasome-mediated degradation. 14-3-3 proteins, however, also play prominent roles in flowering in many species such as Arabidopsis, rice, wheat and barley (Mayfield et al., 2007; Jaspert, Throm and Oecking, 2011; Li, Lin and Dubcovsky, 2015), forming a florigen activation complex with FT, or FT homologs, and a series of other proteins, which then activates the expression of various genes, and subsequently promotes the floral transition. However, the formation of this complex occurs in the cytoplasm, before then translocating to the nucleus to regulate gene expression (Taoka et al., 2011; Li, Lin and Dubcovsky, 2015). Therefore, with reduced expression of *TraesCS6A02G139400* under early heat stress, a reduced level of 14-3-3 phosphorylation and nuclear translocation would be expected – leaving more unphosphorylated 14-3-3 protein in the cytoplasm for florigen activation complex formation, and the subsequent promotion of flowering. However, perhaps the 14-3-3 protein targets, phosphorylated by *TraesCS6A02G139400*, play a negative role in flowering. This has been observed in rice, with *OsGF14c* overexpression leading to late flowering as a result of increased cytoplasmic retention of florigen Hd3a via interaction with OsGF14c (Purwestri et al., 2009), whilst overexpression of a wheat 14-3-3 gene in Arabidopsis similarly led to delayed flowering, suggesting it plays a repressive role in the floral transition (Li et al., 2013). Further work with *TraesCS6A02G139400* TILLING and/or overexpression lines would allow the gene's effect on flowering time after early heat stress exposure to be better understood, whilst also providing insight into how, or if, the phosphorylation of 14-3-3 proteins, and their subsequent interactions with florigens, is responsible for the delayed flower emergence phenotype after exposure to, and then removal of, early heat stress.

Two homoeologues, *TraesCS2A02G271700* (R^2 = 0.6, p = 0.0011) and *TraesCS2D02G270300* (R^2 = 0.65, p = 0.0006), were also identified as markers of delayed flower emergence, as accessions which showed the most severe downregulation of the genes again also tended to show the longest delay in flower emergence (**Figure 4.1**). The genes, known as *T. aestivum transcription factor BHLH6-like*, are the wheat orthologues of the rice transcription factor *OsbHLH006* (*OsRERJ1*). In rice, the gene coordinates the response to phosphate starvation, with its expression being induced significantly by phosphate deficiency, whilst overexpression of the gene led to enhanced phosphate starvation responses, likely via the upregulation of other genes involved in the response to phosphate starvation, as well as the production of longer root hairs (He et al., 2021). Besides this, expression of the gene is also known to respond to jasmonic acid (JA), suggesting the transcription factor plays a role in JA-mediated processes – for example, expression of the gene under biotic stress was hampered in JA-deficient mutant lines (Miyamoto et al., 2013), whilst expression of anti-sense *RERJ1* mRNA caused resistance to JA and overproduction of *REJ1* mRNA resulted in shoot growth inhibition (Kiribuchi et al., 2004). As well as inhibiting shoot growth, JA signalling also appears to play a repressive role in the floral transition in several species, including Arabidopsis and wheat (Diallo et al., 2014; Zhai et al., 2015; Wang et al., 2017), with Diallo et al. (2014) finding that, in wheat, MeJA application led to delayed flowering via the downregulation of key flowering-promoting genes *TaVRN1* and *TaFT1*. JA also functions in the response to heat stress, with jasmonates accumulating after heat stress, as well as after combined heat and light stress in Arabidopsis (Clarke et al., 2009; Balfagón et al., 2019), whilst plants containing mutations in key JA signalling and biosynthesis genes showed reduced thermotolerance and tolerance to combined heat and light stress, respectively (Clarke

et al., 2009; Balfagón et al., 2019). Similarly, more recent work has suggested that stress to the endoplasmic reticulum mediates the early stages of the heat stress response in rice, and that elevated JA accumulation under heat stress precedes, and thus may coordinate, this response (Sandhu et al., 2021). Together, these works therefore suggest that the accumulation of JA as part of the early heat stress response may subsequently lead to delayed flowering.

However, despite the JA accumulation that likely occurs under early heat stress exposure in the present work, the homoeologues were downregulated, unlike their rice orthologue, *OsbHLH006* (*OsRERJ1*), whose expression is induced by JA. This downregulation suggests the homoeologues' function may also be the opposite of that described for *OsRERJ1*, and they therefore act to promote the physiological processes which are repressed by JA – such as flowering. This is a hypothesis supported by the results of the linear regression marker identification analysis, as well as the observation that basic helix-loop-helix (bHLH) transcription factors, such as the homoeologues, activate the expression of key Arabidopsis floral activator, *CONSTANS*, and that overexpression of four of these transcription factors led to early flowering (Ito et al., 2012). Clearly, little is known about the function of the homoeologous markers in wheat, with their expression response in the present work seemingly being a direct contradiction to that of their orthologue in rice. Therefore, further work is required to gain a better understanding of their function; examining the accumulation of JA in wheat landrace accessions under early heat stress will provide insights as to whether their expression is regulated by the hormone, whilst work with TILLING mutant lines containing mutations in these genes will allow their effect on flower emergence time, and on the expression of flowering-promoting genes, to be more clearly determined.

Perhaps a much clearer link between JA signalling and the observed flower emergence delay is provided by another marker identified in the present work; *TraesCS5D02G219300*, *T. aestivum protein TIFY 10c-like*. The marker's orthologue in rice, *OsTIFY10c*/*OsJAZ8*, is a known repressor of JA signalling (Yamada et al., 2012), like other members of the protein family (Chini et al., 2007; Thines et al., 2007; Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu, Figueroa and Browse, 2011; Wang et al., 2017). JAZ proteins carry out this repression under low JA conditions, as they bind to transcription factors and prevent the expression of JA-inducible genes – in the presence of JA, however, JAZ proteins are bound by the hormone and targeted for degradation via the 26S proteasome pathway, subsequently allowing free transcription factors to bind target sequences and activate the expression of JA-inducible genes, passing on the JA signal (Wager and Browse, 2012). Therefore, downregulation of a JA signalling repressor, such as *TraesCS5D02G219300*, under early heat stress will likely subsequently allow transduction of the JA signalling pathway, causing the phenotypes discussed previously – namely, delayed flowering. This was indeed observed in the present work, as those accessions which showed the longest flower emergence delay showed the most severe downregulation of *TraesCS5D02G219300* (**Figure 4.1**), whilst the gene was actually upregulated after heat stress exposure in some of those accessions which showed the shortest flower emergence delays. In recent times, this link between JAZ proteins and flowering time has been made, with mutations to several *JAZ* genes resulting in significantly delayed flowering in Arabidopsis, whilst increased expression of *JAZ* genes led to accelerated flowering in Arabidopsis and tomato (Thatcher et al., 2016; Major et al., 2017; Guo et al., 2018; Yu et al., 2018b; Oblessuc et al., 2020; Liu et al., 2021).

However, given that JA likely accumulates under early heat stress (as discussed previously), it would be expected that the expression of *JAZ* genes would be induced as part of the feedback loop controlling their expression, and JA signalling (Chung et al., 2008). In the present work, however, *TraesCS5D02G219300* expression is downregulated. Further work is therefore required to better understand to what extent JA accumulates under early heat stress, and if such hormonal accumulation is correlated with the expression of the putative JA-responsive genes discussed here. These results would then also provide insights into the relationship between JA accumulation under early heat stress, and delayed flower emergence

observed in the wheat landraces after exposure to, and then removal of, early heat stress – a relationship of seeming importance, given the identification of several putative JA-responsive genes as markers of flower emergence delay in the present work. As with the other genes discussed here, mutation or overexpression of *TraesCS5D02G219300* would allow more robust conclusions to be formed about the gene's effect on flowering time.

4.5. Conclusions and Future Work

The present work aimed to identify what effect exposure to, and then removal of, early heat stress would have on agriculturally-relevant yield traits and flower emergence time, whilst also determining whether early thermotolerance could be used to predict any effect on yield. Across all accessions, exposure to early heat stress had no significant effect on yield traits, whilst these traits did differ significantly between conditions for some accessions. This variation between accessions, however, was not related to their varying degrees of early thermotolerance, which could not be used to predict the effect of early heat stress on any of the yield traits. However, exposure to, and the removal of, early heat stress was found to significantly affect flower emergence time, causing a significant delay in the vast majority of the accessions screened – a novel finding which contradicts the conventional understanding that increased temperature throughout development promotes flowering. This delay may be the result of the downregulation of putative flowering-promoting genes, as well as the downregulation of genes involved in environmental and jasmonic acid signal transduction, as the expression response of these genes (amongst others) to early heat stress were identified as significant predictive markers of the length of flower emergence delay. A significant relationship was observed between this flower emergence delay and biomass weight at yield, further suggesting that early heat stress exposure, and the subsequent downregulation of these genes, impeded the floral transition, subsequently increasing the amount of time spent in vegetative development.

In the identification of the novel flower emergence phenotype seen in the present work, this thesis perhaps poses more questions than it is able to answer. As mentioned earlier, elucidating whether modern elite varieties show the same phenotypic response when exposed to early heat stress in the field is crucial to understand whether crop growth cycles and "agribusiness" considerations are affected as a result of this putative prolonged vegetative development. The identification of candidate gene expression markers, whose expression is changed under early heat stress, which may coordinate this phenotypic response is promising, with these markers being valuable targets for breeders aiming to manipulate, or nullify this phenotypic response. Preventing this downregulation under early heat stress may be key in preventing the seeming prolongation of vegetative development, and delayed flower emergence – this could be achieved by CRISPR-mediated promotor region modification (disturbing transcription factor binding sites, this preventing heat-mediated repression).

Modifications to the promoter regions of these genes relies on an understanding of what transcription factors are controlling the heat-mediated downregulation of expression – however, this is, as yet, unknown. Further work is required to understand this, however, due to the fact that different accessions show different levels of gene expression marker downregulation (and subsequently, different length of flower emergence delay), sequence variation in regulatory regions likely underlies this variation in gene expression change, and can be identified and targeted by breeders for modification. Similarly, once such sequence variation has been identified, these alleles can be used in marker-assisted breeding and crosses with short-delay accessions possessing such variation – further means by which wheat breeders could ensure the absence of this phenotype in HYVs.

Further screening of the YoGI landrace panel to determine how this phenotypic response varies across the panel would identify more candidate accessions to play a role as the short-delay parent in such crosses and breeding programmes, whilst the study of the regulatory regions of the gene expression markers in more accessions would increase confidence in the

alleles associated with a lack of heat-responsive gene expression change, and, subsequently, a short flower emergence delay – ensuring the alleles identified are robust markers for use in marker-assisted breeding.

5. Using Open-source Gene Expression Data to Identify Candidate Master-regulators of the Drought Response

5.1. Introduction

Researchers have recognized that a key way of protecting future wheat crops from the effects of drought stress may be to better understand how the drought response is regulated, with the hope that this knowledge can be used to develop more tolerant cultivars. This is reflected in the vast amount of work conducted in recent years to determine how the wheat transcriptome changes in response to drought stress, and subsequently to identify which genes may be involved in mitigating the effects of water scarcity (Aprile et al., 2009; Kadam et al., 2012; Placido et al., 2013; Reddy et al., 2014; Begcy and Walia, 2015; Liu et al., 2015; Ajigboye et al., 2017; Ma et al., 2017; Chaichi et al., 2019; Iquebal et al., 2019; Mao et al., 2020; Chu et al., 2021; Dugasa et al., 2021; Nouraei et al., 2022; Rasool et al., 2022; Vuković et al., 2022; Xi et al., 2023).

With the advent of open-access publishing and improved data availability practices, data generated by such transcriptomic work has been increasingly re-analysed by other researchers to extract new insights either missed or not fully explored in the original publication. One of the approaches employed to utilize these open-access data in recent years has been co-expression network analysis, with multiple datasets often being pooled prior to co-expression network construction, before hub genes potentially controlling key traits are identified. This approach has been used successfully to study homeologous gene expression and homeologue loss, as well as pistillody-stamen development in wheat (Takahagi, Inoue and Mochida, 2018; Alabdullah et al., 2019; Chen et al., 2020), whilst also being used to identify candidate regulators of thermotolerance, and abiotic and biotic stress responses in rice and barley (Amrine, Blanco-Ulate and Cantu, 2015; Zhu et al., 2019; Luo et al., 2022; Muvunyi et al., 2022; Boulanger et al., 2023).

During the completion of the present work there were no published examples of this approach being employed to study the drought response in wheat, however one such study has since been published (Lv et al., 2020). In this work, the authors identified three modules associated with the drought response, with 12 genes within these modules being identified as likely regulators of drought resistance mechanisms and thus targets for breeders in marker-assisted selection programmes, in the development of more drought tolerant cultivars. However, the authors used only differentially expressed genes to construct their co-expression network via weighted gene co-expression network analysis (WGCNA; Langfelder and Horvath, 2008, 2012) – an action explicitly advised against by the package's authors, as excluding non-DEGs from co-expression network construction can invalidate the scale-free topology assumption of the analysis and lead to the formation of a small number of highly correlated modules. Additionally, the authors did not examine the effect that manipulating the activity or expression of the identified hub genes would have on drought tolerance, nor on the physiological or transcriptomic drought responses. Instead, they validated that the expression of these genes was indeed drought-responsive via quantitative real-time PCR (qPCR). Therefore, although the work by Lv et al. (2020) was the first example of open-source expression data being used to construct a co-expression network and study the drought response in wheat, the approach used could be further optimized based on WGCNA's own criteria, whilst additional experiments, with mutants or overexpression lines, could have more conclusively proved that the identified hub genes were indeed master-regulators of the drought response.

Due to COVID-19-enforced pandemic restrictions, it was not possible to gather transcriptome data from YoGI panel landrace accessions before and after exposure to drought stress. The

aim of the present work, therefore, was to use open-source gene expression data from studies which exposed plants to drought stress, and stress hormone treatments, to identify candidate master regulators of the drought stress response. To do this, WGCNA was employed to construct a co-expression network, before hub genes in modules containing genes associated with the stress-response were identified and TILLING lines containing nonsense or missense mutations in these genes were screened to determine how drought tolerance was affected. As a consequence of the discussed shortcomings in the methodology of the work by Lv et al. (2020), the present work aimed to provide a more robust and thorough example of how open-source gene expression data can be used to identify candidate master-regulators of the drought response in wheat, whilst also identifying promising TILLING mutant lines for use in breeding programmes to develop drought-tolerant cultivars.

5.2. Materials and Methods

5.2.1. Gathering Data and Pre-processing

Microarray expression datasets from works studying gene expression under drought stress or stress hormone treatment were gathered from the NCBI Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo) by searching using key terms such as “wheat”, “*Triticum aestivum*”, “drought”, “abiotic stress” and “stress”. For datasets to be considered for meta-analysis, their experimental design must have used at least two biological replicates per condition, and any samples from wild relatives or engineered lines (for example, KO mutants, recombinant inbred lines (RILs) or transgenic lines) included in the dataset were excluded from the analysis. Microarray datasets were selected for use in the present work as opposed to RNA-seq datasets, as only one RNA-seq dataset available on NCBI GEO met the above criteria (at the time the work was conducted). Only datasets which used the Affymetrix GeneChip® Wheat Genome Array platform (GPL3802) were considered for analysis, in an attempt to reduce inter-array correlation artefacts. The array contains 61,290 probe sets (61,115 probe sets after control probes are removed), representing 55,052 transcripts in the wheat transcriptome. After filtering datasets according to these criteria, the final meta-dataset contained 57 arrays from 5 experimental setups [4 investigating gene expression response to drought stress (GSE31759, GSE70443, GSE30436, GSE42214), and 1 investigating the response to various stress hormone treatments (GSE103430)]. CEL files from these datasets were read using the BioConductor “affy” package (Gautier et al., 2004) in R statistical programming environment platform (R Core Team, 2021), before gene expression was normalized using the Robust Multi-array Average (RMA) method (Irizarry et al., 2003). RMA-normalized meta-dataset used for all subsequent analyses can be found in **Supplementary Data S5.1**.

5.2.2. Identification of Drought-responsive Probes

A two-tailed *t*-test was used to determine whether probes showed significantly different expression in the treatment condition (drought, or stress hormone) compared to the control condition, within the expression data of each study in the meta-dataset. Those probes which showed a *p*-value < 0.05, and a log2FC greater/less than 1.5/-1.5 were deemed to be differentially expressed. The number of differentially expressed genes (DEGs) in each study was determined using these criteria. Those probes which were DEGs in at least one of the studies that exposed plants to drought stress were deemed to be drought DEGs, whereas those probes which were DEGs under hormone treatment were deemed to be DEGs for that specific hormone; for example, an ABA DEG.

5.2.3. Gene Ontology Term Enrichment Analysis of DEGs

To understand the functionalities of drought-responsive genes, gene ontology (GO) terms significantly enriched amongst upregulated and downregulated DEGs were identified via the agriGO Singular Enrichment Analysis tool (Du et al., 2010; Tian et al., 2017). Some probes were significantly upregulated in one study, but significantly downregulated in another – in these instances, probes were included in both the upregulated and downregulated groups for GO enrichment analysis. The names of probes present in each group were collated and submitted to the agriGO Singular Enrichment Analysis tool, before a Fisher's exact test was performed for each group, using all the GO terms associated with the probes within the Affymetrix GeneChip® Wheat Genome Array as background; 0.05 as the *p*-value threshold; Hochberg (FDR) as the multi-test adjustment method (Benjamini and Hochberg, 1995), and 5 as the minimum number of mapping entries threshold. A GO term was considered enriched in a module when its FDR-adjusted *p*-value was < 0.05. AgriGO's internal database of GO terms associated with each probe in the Affymetrix GeneChip® Wheat Genome Array was used during GO analyses, meaning only probe names had to be submitted as the input for analysis.

5.2.4. Weighted Gene Co-expression Network Construction and Module Detection

The WGCNA package (Langfelder and Horvath, 2008, 2012) was used to construct a co-expression network using normalised expression data. None of the probes had missing/zero values, or mean values below 1, so all probes were included in the network construction. The blockwiseModules() function conducted blockwise network construction according to the function's default parameters, except the following: maximum block size = 5000, soft threshold power = 9 (the first power to exceed a scale-free topology fit index of 0.8), minimum module size = 30, merge cut height = 0.25. After module construction, edge and node files were created using the "exportNetworkToCytoscape()" function with a threshold of 0.1; filtering out weak connections between nodes.

5.2.5. Module GO Term Enrichment Analysis

To identify those modules containing genes involved in the stress response, GO terms significantly enriched in each module were identified via use of the agriGO Singular Enrichment Analysis tool. To do this, names of probes present in each module were collated and submitted to the tool, which used all the GO terms associated with the probes within the Affymetrix GeneChip® Wheat Genome Array as background. The parameters used were the same as described above for DEG GO enrichment analysis.

5.2.6. DEG Enrichment Analysis

After the identification of DEGs, the proportion of probes which were differentially expressed under different conditions were calculated. These proportions were then used to determine the expected number of DEGs (for different conditions) in each module, based on the size of the module. For example, if 10% of all probes were drought DEGs, it would be expected that 10% of all probes in each module would be drought DEGs. A one-proportion Z test was then used to calculate whether the observed proportion of DEGs in each module was significantly greater than the expected proportion. Those modules which possessed a significantly higher proportion of DEGs than expected were deemed to be enriched in DEGs, and particularly associated with the condition (drought or stress hormone treatment) in question.

5.2.7. Network Visualization and Hub Gene Identification

To identify hub genes, degree (connection) scores were calculated for each gene within a module, either using the Cytoscape (version 3.9.1.; Shannon et al., 2003) network analyser tool (Assenov et al., 2008), or by counting the number of connections to and from each gene in the WGCNA edge file, using the table() function in R. The script used to calculate degree scores in R is available on GitHub: https://github.com/andreaharper/HarperLabScripts/.

Cytoscape was used to visualize modules, and for hub gene identification in the majority of cases, however particularly large modules are often difficult to load, view and analyse in Cytoscape. In these cases (modules containing ~2000 genes or more), R was used to calculate degree score instead. Those genes in a module with the highest degree scores (most connections) were identified as the central hubs. The blue module was found to be significantly enriched in the "response to water" (GO:0009414) GO term, but was also amongst the largest in the co-expression network. The module, therefore, likely contains genes involved in diverse processes – so, to focus on the response to water, a subnetwork was created using genes annotated with the "response to water" (GO:0009414) GO term within the module as guide genes. It was thought that by only examining the connections to and from these genes, the subsequently identified hub gene would be a better candidate regulator of the drought response, than the hub gene of the entire, much larger, module. In most cases, hub genes were identified as the most well-connected gene within a module or subnetwork, however, if another well-connected gene was a particularly promising candidate, due either to its likely function or expression pattern in the meta-dataset, then that gene was selected as the hub gene for further exploration.

5.2.8. BLASTing Probe Sequences to Wheat Transcriptome

GenBank accession sequences associated with each probe from the Affymetrix GeneChip® Wheat Genome Array were BLASTed against the published IWGSC RefSeq v1.0 high confidence CDS models (International Wheat Genome Sequencing Consortium (IWGSC), 2018) in BLAST2GO (Götz et al., 2008). IWGSC RefSeq v1.0 high confidence CDS models are available for download at: https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Annotations/v1.0/. For a BLAST hit to be deemed significant, the e-value threshold was set at 0.001, whilst the percentage identity threshold was 70%. 33,805 probes had a significant BLAST hit, leaving 27,848 probes without one. BLAST results can be found in **Supplementary Data S5.2**. In the case of a probe of interest not attaining a significant blast hit from BLAST2GO, the GenBank accession sequence associated with each probe was blasted manually against the v1.0 reference transcriptome with lower threshold values, to identify the gene with which it shared some significant sequence identity. When referring to probes, homologous gene names are given followed by the probe name in brackets. If a gene name does not accompany a probe name, it is due to a lack of significant sequence similarity with any transcript in the v1.0 annotation, as determined by the two described BLASTing methods.

5.2.9. TILLING Mutant Acquisition

TILLING mutant lines for candidate hub genes were sourced from wheat-tilling.com (**Table S5.1**). To identify lines with mutations in candidate hub gene sequences, the BLAST scaffold search function was used, with the GenBank accession sequence associated with each candidate probe being the input. This identified the corresponding gene sequence (BLAST hit with highest similarity), and subsequent mutant lines carrying insertions in this gene. Mutant lines were selected if the insertion carried by that line caused a nonsense mutation, or a missense mutation with a SIFT score of 0. Only mutant lines in the hexaploid Cadenza background were acquired. Cadenza wild-type seeds were also sourced to be used as controls. Sequence information provided for TILLING mutants comes from a single M_2 plant, however the seeds received have been produced via several rounds of self-pollination and are subsequently M_4 or M_5 generation. Since the identification of these TILLING mutants, the described method can no longer be used as the website has changed. Now, TILLING mutant lines for a gene of interest are identified by searching for the gene in EnsemblPlants (https://plants.ensembl.org/index.html), before navigating to the "variant table" section, where all the information provided previously on the old website is present.

5.2.10. TILLING Mutant Line Drought Tolerance Screen

It was hypothesized that mutation to these hub genes, via TILLING, would either reduce basal drought tolerance, or impede the drought response – subsequently leading to changes in drought tolerance. Because a large number of TILLING mutant lines were acquired, the likely effect of the mutation they carried (i.e. their drought tolerance) was initially screened to identify those lines which may be the most promising for subsequent analysis and backcrossing. This screen was completed, initially, without genotyping to gain a crude understanding of their drought tolerance. However, leaf tissue samples were taken so that genotyping could be completed later to confirm the presence of the target mutation, in the case that backcrossing failed. To screen drought tolerance, the growth of TILLING mutants under drought and control conditions was compared to the growth of wild-type Cadenza under these conditions.

Seeds were sown in Levington Advance Seed & Modular F2S compost mixed with Aggregate Industries Garside Sands 16/30 sand (80:20 ratio), treated with CaLypso insecticide (Bayer CropScience Ltd., 0.083ml mixed with 100ml water, applied to each litre of compost). Two seeds were sown per 7-inch pot, with surplus plants being removed at the one-leaf stage to leave one plant per pot. At the three-leaf stage, a leaf sample was taken and used for DNA extraction and genotyping. Plants were grown under controlled glasshouse conditions (16 h daylight; 20 °C /15 °C day/night), and were watered daily until the three-leaf stage. From this point, six replicates of each line were not watered for ten days (drought condition), whilst the remaining six replicates of each line continued to be watered daily (control condition). After this period of ten days, plants in the drought condition were watered daily for three further days, to serve as a recovery period. During these three days, plants in the control condition continued to be watered daily. 13 days after the three-leaf stage was reached, each plant was harvested by cutting the stem at the soil surface. Fresh biomass measurements were taken by weighing the harvested biomass, whilst dry biomass measurements were taken by drying the harvested biomass at 65˚C for two days before weighing. Soil moisture content (SMC%) was recorded using an ML3 Thetaprobe Soil Moisture Sensor with an HH2 Moisture Meter (Delta-T Devices, Cambridge, United Kingdom) to quantify the severity of the drought stress treatment at the three-leaf stage, 10 days after the three-leaf stage (end of drought period for stressed plants) and 13 days after the three-leaf stage (after drought recovery for stressed plants). The probe was inserted into the soil to its full depth before moisture levels were recorded. Mean SMC% of conditions, at each time point, were compared via one-way ANOVA.

5.2.11. TILLING Mutant Backcrossing and Drought Tolerance Screening of Non-backcrossed Mutants

Although the initial screen of TILLING mutant lines indicated which mutation had an effect on the drought response or drought tolerance, TILLING mutants likely contain background mutations at loci besides the gene of interest due to the random, and global, nature of the mutagenesis provided by ethyl methanesulfonate (EMS). Backcrossing to the wild-type mutant background variety reduces these background mutations, whilst genotyping the locus of interest ensures the desired mutation has not been lost as part of the backcrossing process. To reduce the amount of background mutations, whilst maintaining the desired mutation in the gene of interest, TILLING mutant plants confirmed to contain the mutation of interest (via the genotyping process outlined below) were backcrossed to wild-type Cadenza plants through one generation, before selfing offspring of these crosses and growing to maturity (producing BC_1S_1 seed). TILLING mutant plants were used as the donor parent, whilst Cadenza wild-type plants were used as the recurrent parent in the backcross.

It was hoped that the drought tolerance of confirmed BC_1S_1 mutants could be compared to wild-type Cadenza and out-segregant plants, via the drought tolerance screen as described above. As above, tissue samples were taken during the experiment and plants were

genotyped. However, genotyping these plants revealed there were insufficient numbers of BC_1S_1 mutant plants for any of the crosses to enable a robust and statistically valid experiment, and analysis, to be carried out. Instead, some promising lines from the initial drought tolerance screen were genotyped to confirm the presence of the target mutation in these plants, and eliminate any non-mutants from the dataset. These data were then be used to determine whether confirmed mutants from these lines showed different levels of drought tolerance, compared to Cadenza. Comparisons could not be made to out-segregant plants, as genotyping revealed there were insufficient numbers for statistically-robust analysis to be carried out for the TILLING mutant lines screened.

5.2.12. DNA Extraction

To confirm a plant's genotype, DNA was first extracted from small leaf tissue samples taken during the seedling stage, and stored at -20°C. Frozen tissue was homogenized by placing a metal bead in each Eppendorf and grinding to a fine powder using a TissueLyser. This powder was then re-suspended in 500µL of DNA extraction buffer (200mM Tris, 240mM NaCl, 25mM EDTA and 1% (weight/volume) SDS) and 75µL of chloroform, before being vortexed for five minutes. Samples were then centrifuged at 14,000 rpm for ten minutes, before the supernatant was taken and mixed (1:1 ratio) with 100% isopropanol. Samples were then centrifuged at 14,000 rpm for ten minutes. The supernatant was then removed, before the remaining sample was mixed with 500µL of 70% ethanol, followed by a brief vortexing. Samples were then centrifuged at 14,000 rpm for five minutes, before the ethanol was removed and open Eppendorf tubes were inverted and allowed to dry for 60 minutes. The remaining sample was then re-suspended in 100µL of 1x TE buffer (10mM Tris-HCl (pH 8), 1mM EDTA), before vortexing and allowing samples to sit at room temperature for 20 minutes. Samples were stored at -20°C prior to further use.

5.2.13. Primer Design and Genotyping

Due to the homeologous nature of the wheat genome, primers may bind to, and amplify, homeologous genes from all three genomes. To avoid this, and ensure only the homeologue of interest would be amplified by the polymerase chain reaction (PCR), primers were designed to overlap with regions of homeologue-specific sequence variation upstream and downstream of the mutation locus in the gene. Designing primers in this way means they contain these regions of distinct sequence variation, and so should only bind to, and amplify, the homeologue of interest during PCR. To identify these regions of homeologue-specific sequence variation, transcript sequences for all three homeologues were sourced and aligned in MEGA (version 11; Tamura, Stecher and Kumar, 2021), before primer sequences spanning these regions were identified. Once primers were designed, their properties were assessed to determine GC content, melting temperature, and potential self-complementarity issues such as hairpin formation and self-dimerization (Kibbe, 2007). Final primers were diluted to 10µM for use in PCRs. Primer information can be found in **Table S5.2**.

Once DNA was extracted from samples and suitable primers had been designed, PCR was used to amplify a fragment from the gene of interest, before sequencing could take place to confirm the presence or absence of the point mutation. Due to its increased fidelity and low error rate, compared to Taq polymerase, New England Biolabs Q5® High-Fidelity DNA Polymerase was used for PCR amplification. This reduced the likely occurrence of amplification errors that could be misinterpreted as mutations upon sequencing. Each PCR reaction contained: 1µL DNA, 5µL 5x Q5 reaction buffer, 0.5µL 10mM dNTPs, 1.25µL of each 10µM forward primer and 10µM reverse primer, 0.25µL Q5® High-Fidelity DNA Polymerase, 5µL GC enhancer, 5.75µL nuclease-free water. The PCRs followed the same basic thermocycling conditions (**Table S5.3**), with the annealing step being optimized to confer

specific binding and amplification, and the extension time being optimized for the fragment size. Annealing temperatures and extension times for each PCR can be found in **Table S5.2**.

After PCR amplification of target loci, PCR products were purified prior to sequencing using the Qiagen QIAquick ® PCR & Gel Cleanup Kit, following the manufacturer's instructions. 2.5µL of purified product was then mixed with 5µL of nuclease free water, and 2.5µL of (either forward, or reverse) 10mM primer and sent to Eurofins for LightRun Tube Sanger sequencing. Upon receipt of the sequencing results, the presence or absence of the target point mutation (as defined in **Table S5.1**) in each amplified locus was determined. Those samples which contained the point mutation were defined as mutants, whilst those that did not were excluded prior to subsequent analysis.

5.2.14. Hub Gene Validation Data Processing and Analysis

After samples from the initial drought tolerance screen had been genotyped, those plants identified as non-mutants were excluded from the dataset. The remaining dry biomass measurements were used to calculate mean trait scores for each line in each condition, from which normalized loss in mean dry biomass scores, between drought and control conditions, were calculated. These scores were used as measures of drought tolerance, and were calculated according to the equation below.

$$Normalized\ loss\ in\ mean\ dry\ biomass = 1 - \left(\frac{Mean\ drought\ dry\ biomass\ weight\ (g)}{Mean\ control\ dry\ biomass\ weight\ (g)}\right)$$

For the mutant lines genotyped from the initial screen, a greater biomass loss than observed in Cadenza signified that mutation to the hub gene may reduce drought tolerance, and vice versa if a lesser biomass loss than observed in Cadenza was shown.

5.3. Results

5.3.1. Identification and Functional Analysis of Drought-responsive Genes

A total of 2916 probes were found to be significantly differentially expressed in at least one study within the meta-dataset. In total, 988 probes were significantly upregulated in at least one study within the meta-dataset, whilst 2007 were significantly downregulated at least once. This meant that 79 probes showed different expression responses in different studies; being significantly upregulated in one, and significantly downregulated in another.

22 GO terms were significantly enriched amongst upregulated DEGs, with the vast majority being associated with different parts of the photosynthetic reaction (for example; "photosynthesis, light harvesting" (GO:0009765), "photosynthesis, light reaction" (GO:0019684), and "photosynthesis" (GO:0015979)). Meanwhile, 100 GO terms were significantly enriched amongst downregulated DEGs, with many, again, being associated with photosynthesis, including the three examples given for upregulated DEGs. Also enriched amongst downregulated DEGs were terms related to the response to stimuli other than water deprivation , for example; "response to inorganic substance" (GO:0010035), "response to high light intensity" (GO:0009644), "response to other organism" (GO:0051707), and "response to biotic stimulus" (GO:0009607). Enriched GO terms amongst DEGs can be found in **Supplementary Data S5.3**.

5.3.2. Identification of Drought-associated Modules in the Co-expression Network

The WGCNA package (Langfelder and Horvath, 2008, 2012) was used to construct a weighted gene co-expression network in R, using the open-source expression meta-dataset after pre-processing. This package groups probes with similar expression patterns across all samples

into modules via the average linkage hierarchical clustering of RMA-normalized expression values. The analysis was able to assign 59,700 out of 61,290 probes (97.4%) to 41 modules. The remaining 1590 probes were assigned to the grey module, which is a pseudo-module containing all probes which could not be assigned to any of the other modules as they do not have strong expression correlations with other probes. Module size ranged from 34 to 16,565 probes, whilst the mean and median number of probes per module were 1456 and 320, respectively.

To determine which modules were significantly associated with drought tolerance or the stress response, GO term enrichment analysis was conducted using the agriGO Singular Enrichment Analysis tool. Those modules enriched in GO terms related to water use, drought response, stress response or responding to stimuli likely contain genes involved in the stress response, or which may determine a plant's degree of drought tolerance. Seven out of 42 modules were found to be significantly enriched in such GO terms (**Table 5.1**), with the blue module being the only module to be enriched in a GO term directly related to the drought response ("response to water deprivation", FDR = 0.022). Three of these seven modules were significantly enriched in the GO term "response to stress", with one other being significantly enriched in the related term "cellular response to stress". Other commonly enriched terms were those related to stimulus response, namely "response to stimulus", "response to chemical stimulus", "response to abiotic stimulus" and "cellular response to stimulus", were found in six of these seven stress-associated modules.

To gain further insight into which modules may be particularly associated with drought tolerance or the stress response, the number of differentially expressed genes (DEGs) under drought stress in each module was calculated. To be deemed a DEG, the probe must have shown a log2FC greater/less than 1.5/-1.5, and a *p*-value less than 0.05, between drought and control conditions within at least one study in the meta-dataset. This resulted in 2916 DEGs from a total of 61,115 probes (4.77%). Then, to determine whether modules were enriched in drought DEGs, a one-proportion Z test was used to test whether the observed proportion of drought DEGs in each module was greater than the expected proportion (4.77%). This resulted in 20 modules containing a significantly higher proportion of drought DEGs than expected (**Table 5.2**). Only two modules enriched in stress-associated GO terms were also enriched in drought DEGs (grey60 and orange).

Table 5.1: GO terms significantly enriched in modules deemed to be associated with drought tolerance or the stress response. The most significantly enriched GO term for each of these modules is listed, as well as examples of stress-associated GO, and terms related to regulatory processes which affect gene expression or protein production. FDR-adjusted *p*-values are given in brackets after each GO term.

Module	***GO Term***
Blue	Cellular Nitrogen Compound Metabolic Process (2.3E-17)
	DNA Packaging (2.2E-08)
	Response to Water Deprivation (0.022)
Brown	Peroxidase Reaction (2E-18)
	Response to Oxidative Stress (1.6E-12)
	Response to Stress (0.015)
Darkgrey	Galactose Metabolic Process (7.8E-05)
	Response to Stimulus (0.0057)
	Response to Stress (0.0068)
Grey60	Cellular Nitrogen Compound Metabolic Process (3.3E-05)
	Cellular Response to Stress (0.016)
	Response to Abiotic Stimulus (0.028)
Orange	Embryonic Development Ending in Seed Dormancy (7.1E-05)
	Response to Stress (0.00079)
	Response to Abiotic Stimulus (0.0027)
Pink	Macromolecule Localization (1.4E-08)
	Cellular Response to Stimulus (1.6E-06)
	Cellular Response to Chemical Stimulus (0.00013)
Steelblue	Cellular Response to Stimulus (0.025)
	Regulation of Biological Process (0.047)
	Nucleotide Binding (0.015)

Table 5.2: 20 modules had a higher proportion of drought DEGs than expected (4.77%). These modules are listed, as well as the number of genes in each module, the percentage of these genes which were observed to be DEGs, and the *p*-value result from the one-proportion Z-test.

Module	Number of Genes	Observed Percentage of DEGs	*p*-value
Darkgreen	272	11.4	1.46E-07
Darkmagenta	55	54.6	1.66E-67
Darkorange	150	16	5.47E-11
Darkred	318	9.43	4.76E-05
Darkturquoise	207	7.25	0.047
Grey60	804	9.08	4.92E-09
Lightcyan	1121	8.83	8.84E-11
Lightsteelblue1	34	11.76	0.028
Lightyellow	513	6.63	0.024
Mediumpurple3	39	12.82	0.009
Midnightblue	1158	6.65	0.001
Orange	181	12.71	2.72E-07
Orangered4	41	14.63	0.002
Paleturquoise	76	22.37	3.05E-13
Purple	2191	7.07	2.09E-07
Red	2648	8.91	7.51E-24
Royalblue	437	13.04	2.43E-16
Saddlebrown	101	11.88	0.0004
Sienna3	55	36.36	2.05E-28
Tan	1883	8.71	5.25E-16

5.3.3. Drought-associated Hub Gene Identification and Initial Drought Tolerance Screen of TILLING Mutants

GO term and DEG enrichment analyses identified several stress-associated modules, before the hub genes in these modules were deemed to be candidate master-regulators of the drought response (**Table 5.3**). To identify those hub genes from these modules which may be the most promising candidates, their putative functions were assessed – with those that seemingly play a role in the stress response being deemed particularly promising. Finally, the initial drought tolerance screen of TILLING mutant lines was used to identify which of the promising candidates may be best for further exploration. Information on the TILLING mutant

lines screened for drought tolerance, and the predicted effect of the mutations on drought tolerance, is available in **Table S5.1**.

Drought stress caused a significant reduction in SMC% (**Figure 5.1a**), as mean SMC% differed significantly between the treatment groups 10 days after the three-leaf stage, but not at the three-leaf stage, before the drought treatment had started (ANOVA: p = <2E-16 and 0.089, respectively). Mean SMC% did differ significantly between conditions at harvest (after plants in the drought condition had experienced a three-day recovery period) – however, although statistically significant, this difference was only slight (mean control SMC% = 48.1, mean drought SMC% = 50.5; ANOVA: p = 0.041) and so likely had no effect on plant growth. In total, 16 TILLING mutant lines were screened for drought tolerance without genotyping. Normalized loss in mean dry biomass ranged from 0.13 to 0.62, whilst wild-type Cadenza showed normalized loss in mean dry biomass of 0.50 (**Figure 5.1b**). Only one mutant line, Cadenza0393, showed larger biomass losses than Cadenza (0.62). The most drought tolerant mutant line was Cadenza1687, showing a normalized loss in mean dry biomass of 0.13. Using this information, and knowledge about hub genes' putative functions, and the putative functions of the genes they are connected to within their respective modules, two hub genes were identified as particularly promising candidate master-regulators of the drought response: *TraesCS4D01G050400* (Ta.963.2.A1_at) was identified as the hub gene within the blue module "response to water" GO term subnetwork and whose TILLING mutant line (Cadenza0423) showed a normalized loss in mean dry biomass of 0.25 in the initial screen; and *TraesCS7D01G347300* (Ta.29814.1.S1_at), which was identified as the hub gene within the DEG-enriched purple module and whose TILLING mutant (Cadenza1687) was the most drought tolerant in the screen.

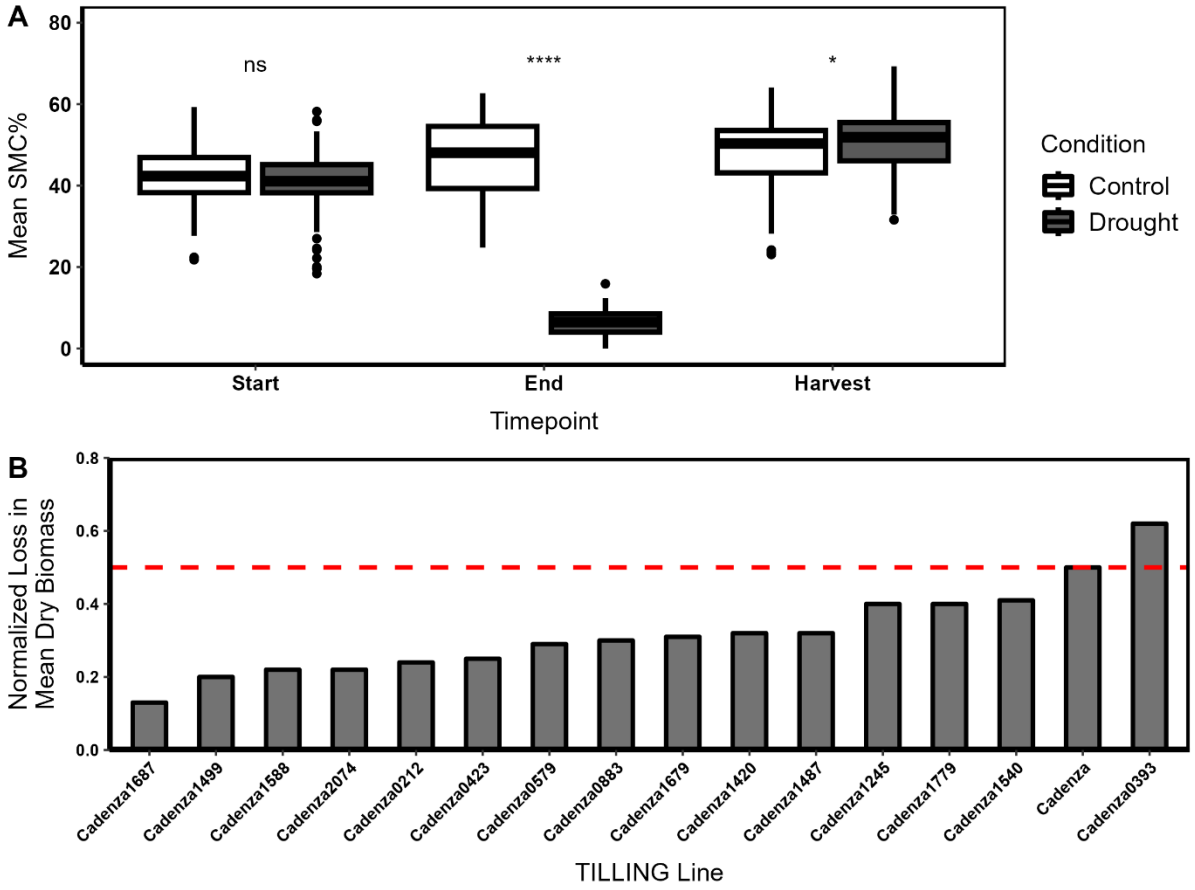

Figure 5.1: A) Drought stress treatment caused a significant reduction in SMC%. Mean SMC% at the three-stage (pre-drought) did not differ significantly between conditions (ANOVA: $p = 0.089$), whilst at ten days after the three-leaf stage mean SMC% of plants in the drought condition was significantly lower than that of plants in the control condition ($p < 2E\text{-}16$). Mean SMC% also varied significantly at harvest (13 days after the three-leaf stage; $p = 0.041$), however this difference was slight. **B) TILLING mutant lines were largely more drought tolerant than wild-type Cadenza.** Normalized loss in mean dry biomass scores ranged from 0.13 to 0.62, with wild-type Cadenza showing a score of 0.5 (indicated by a horizontal red dashed line). All but one TILLING line in the initial drought tolerance screen showed lower normalized loss in mean dry biomass scores than the wild-type Cadenza.

Table 5.3: Hub genes identified in stress-associated modules may be strong candidates for master-regulators of the drought stress response, based on their high number of connections to other genes within stress-associated modules. Each hub gene's module membership and putative function are given.

Module	Hub Probe	Hub Gene	BLAST Hit	Putative Function	Reference
Blue	Ta.963.2.A1_at	*TraesCS4D01G050400*	*T. aestivum probable histidine kinase 4*	Negative regulation of abiotic stress responses and ABA signalling	(Tran et al., 2007; Tran, Shinozaki and Yamaguchi-Shinozaki, 2010; Jeon et al., 2010; Kang et al., 2012)
Brown	Ta.14580.2.S1_at	*TraesCS4B01G340100*	*T. aestivum peroxidase 1-like*	Detoxification of ROS	(Yoshida et al., 2003)
Darkgreen	TaAffx.54965.1.S1_x_at	*TraesCS3A01G299200*	*T. aestivum nuclear matrix constituent protein 1b-like*	Organizing nuclear morphology	(Sakamoto and Takagi, 2013)
Darkgrey	Ta.5829.1.S1_at	*TraesCS4A01G119900*	*T. aestivum outer envelope pore protein 16-2, chloroplastic-like*	ABA signalling	(Pudelski et al., 2012)
Darkmagenta	TaAffx.13392.2.S1_at	*TraesCS6B01G459300*	*T. aestivum wall-associated receptor kinase 2-like*	Response to extracellular environment	(Kanneganti and Gupta, 2008)
Darkorange	Ta.27927.1.A1_s_at	*TraesCS4D01G070700*	*T. aestivum exocyst complex component SEC3A-like*	Exocytosis	(Wu and Guo, 2015)
Darkred	Ta.6713.1.S1_at	*TraesCS4D01G177200*	*T. aestivum mitochondrial import receptor subunit TOM40-1-like*	Protein transport	(Gabriel, Egan and Lithgow, 2003)
Darkturquoise	Ta.3851.1.S1_a_at	*TraesCS5A01G540800*	*T. aestivum coatomer subunit alpha-3-like*	Maintaining Golgi apparatus	(Ahn et al., 2015)
Grey60	TaAffx.110693.1.S1_x_at	*TraesCS1A01G262100*	*T. aestivum pheophytinase, chloroplastic-like*	Chlorophyll breakdown	(Guyer et al., 2018)
Lightcyan	Ta.17378.1.S1_at	*TraesCS2D01G543900*	*T. aestivum ethylene-responsive transcription factor 3*	Drought and salinity tolerance	(Rong et al., 2014)
Lightsteelblue1	Ta.27812.1.A1_at	*TraesCS5B01G330800*	*T. aestivum receptor-like protein kinase FERONIA*	Control of vegetative-reproductive growth transition	(Wang et al., 2020a)

Lightyellow	Ta.13185.2.A1_x_at	*TraesCS2A01G084000*	*T. aestivum phospholipase C (PI-PLC1-2A)*	Stress response	(Wang et al., 2020b)
Mediumpurple3	Ta.4517.2.S1_a_at	NA	*T. aestivum protein FAR1-RELATED SEQUENCE 5-like*	Transcription factor	(Ma and Li, 2018)
Midnightblue	TaAffx.129125.1.S1_at	*TraesCS5B01G099600*	*TaSKP1-9*	Protein degradation	(HajSalah El Beji et al., 2019)
Orange	Ta.30566.2.S1_x_at	NA	*T. aestivum sucrose transport protein SUT4-like*	Sucrose transport	(Weise et al., 2000)
Orangered4	TaAffx.39452.2.S1_at	*TraesCS1D01G237300*	*T. aestivum uncharacterized LOC123181825*	Uncharacterized	
Paleturquoise	TaAffx.54780.1.S1_at	*TraesCS3D01G012800*	*T. aestivum G-type lectin S-receptor-like serine/threonine-protein kinase SD2-5*	Biotic stress response	(Teixeira et al., 2018)
Pink	TaAffx.129139.2.S1_x_at	*TraesCS4D01G115100*	*T. aestivum mRNA-decapping enzyme-like protein*	mRNA expression and turnover	(Vidya and Duchaine, 2022)
Purple	Ta.29814.1.S1_at	*TraesCS7D01G347300*	*T. aestivum peroxidase 1-like*	Detoxification of ROS	(Yoshida et al., 2003)
Red	Ta.11437.1.A1_x_at	NA	*T. aestivum uncharacterized LOC123190427 (LOC123190427)*	Uncharacterized	
Royalblue	Ta.10187.1.A1_at	*TraesCS3A01G161900*	*T. aestivum uncharacterized LOC123060859 (LOC123060859)*	Uncharacterized	
Saddlebrown	Ta.7428.2.A1_a_a	*TraesCS2A01G557500*	*T. aestivum 40S ribosomal protein S25-like*	Translation	(Hertz et al., 2013)
Sienna3	Ta.26280.1.S1_at	*TraesCS3D01G159200*	*T. aestivum uncharacterized LOC123077847 (LOC123077847)*	Uncharacterized	
Steelblue	Ta.6291.1.S1_s_at	*TraesCS3D01G429200*	*T. aestivum protein DEK-like*	DNA supercoiling	(Kappes et al., 2004)
Tan	Ta.20947.1.S1_a_at	*TraesCS5D01G481500*	*T. aestivum 50S ribosomal protein L17, chloroplastic-like*	Translation	(Maurastoni et al., 2023)

5.3.4. Partial Validation of *TraesCS4D01G050400* and *TraesCS7D01G347300*

Although the initial screen suggested mutant lines carrying mutations in these hub genes may show improved drought tolerance (as described above), the genotype of these plants had not yet been determined. After a failed attempt to backcross these mutant lines into the wild-type Cadenza background, to reduce the number of background mutations present, samples from the initial screen were genotyped to confirm their identity and make more robust conclusions about the effect that mutating these hub genes had on drought tolerance. After genotyping, and the removal of non-mutants, Cadenza0423 (*TraesCS4D01G050400*) and Cadenza1687 (*TraesCS7D01G347300*) showed normalized loss in mean dry biomass scores of 0.39 and 0.1, respectively – a marked improvement compared to wild-type Cadenza's score of 0.5. It is worth noting, however, that only two confirmed Cadenza0423 mutant replicates were present in the control condition.

5.3.5. Identifying ABA-associated Modules

956 probes (out of 61,115) were deemed to be ABA DEGs (1.56%), with seven modules housing a significantly higher proportion of ABA DEGs than expected (**Table 5.5**). There was some crossover between drought and ABA DEGs, with 257 genes being differentially expressed under both conditions (**Figure 5.2**). This left 699 genes which were differentially expressed under ABA treatment only. Three modules were enriched in ABA DEGs, but not drought DEGs, suggesting they contain many genes involved in ABA-mediated processes besides the drought response. The darkgrey module was particularly interesting as it contained 98 ABA DEGs, out of 181 total nodes (54%), and was enriched in the GO terms "response to stimulus", "response to stress" and "response to hormone stimulus", whilst not being enriched in drought DEGs. Of the 98 ABA DEGs, 11 were also drought DEGs, whilst 87 showed no expression response under drought stress in the meta-dataset.

5.3.6. ABA-associated Hub Gene Identification

The hub gene in the darkgrey module, *TraesCS4A01G119900* (Ta.5829.1.S1_at), was one of the 87 ABA-only DEGs in the module, being significantly upregulated under ABA treatment, but not under drought stress. The hub was connected to 113 probes within the module; 69 of which were ABA-only DEGs, with all of these DEGs also being upregulated in response to ABA treatment. Although the validation of this hub gene in coordinating the ABA-mediated response to abiotic stresses is beyond the scope of the present work, it may be a promising target for further exploration by others or a valuable breeding target in the production of stress-tolerant varieties.

Table 5.5: Seven modules had a higher proportion of ABA DEGs than expected (1.56%). These modules are listed, as well as the number of genes in each module, the percentage of these genes which were observed to be DEGs, and the *p*-value result from the one-proportion Z-test. Also listed in whether these modules were also significantly enriched in drought DEGs.

Module	Number of Genes	Observed Percentage of DEGs	*p*-value	Also enriched in drought DEGs?
Black	2628	3.96	1.75E-23	No
Darkgrey	181	54.14	0	No
Lightcyan	1121	3.39	3.83E-07	Yes
Purple	2191	9.40	3.96E-193	Yes
Sienna3	55	58.18	5.40E-252	Yes
Tan	1883	3.56	1.31E-12	Yes
Violet	69	5.80	0.002	No

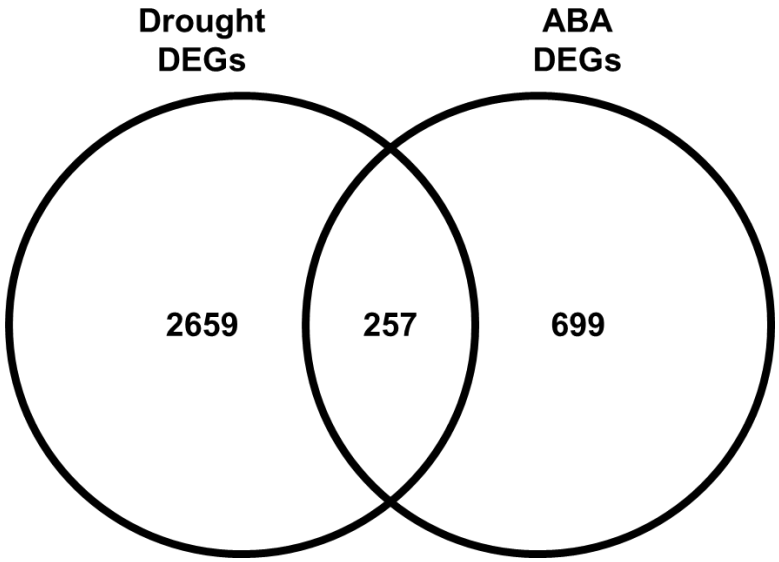

Figure 5.2: Comparing the expression responses of probes under drought and ABA treatments. The metadataset contained 257 probes which were differentially expressed under both drought stress and ABA treatment. 2659 probes were differentially expressed under drought stress only, whilst 699 probes were differentially expressed under ABA treatment only.

5.4. Discussion

5.4.1. Effect of drought stress on gene expression

In total, 2916 probes were deemed to be significantly differentially expressed after drought stress exposure in at least one study within the meta-dataset; 988 being upregulated and 2007 being downregulated. Interestingly, 79 probes showed different transcriptional responses to drought stress exposure between studies in the meta-dataset. This is likely due to discrepancies in the timing of the drought stress application during the plant's development,

or differences in the varieties used between studies. For example, GSE31759 used Chinese Spring whereas GSE30436 used C306 – two varieties with differing levels of drought tolerance, amongst other phenotypic differences.

Drought stress exposure appeared to cause the deprioritization of growth, as a large number of the terms enriched amongst downregulated DEGs pertained to photosynthesis. The damaging effects of abiotic stresses, particularly drought, on photosynthetic capacity is well known and has been observed in rice and barley (Daszkowska-Golec et al., 2019; Gan et al., 2019; Yu et al., 2020a). This reduction in photosynthetic capacity comes as a result of, amongst other things, drought-induced reductions in leaf area, reduced gas exchange (and subsequently CO_2 assimilation) as a result of increased stomatal closure, and impaired function of the photosynthetic apparatus (Zargar et al., 2017). Subsequently, many grasses, such as rice, barley, Miscanthus and *Brachypodium distaychon*, alter their photosynthetic activity via the downregulation of photosynthetic genes, as seen in the present work, when faced with drought stress (Priest et al., 2014; Daszkowska-Golec et al., 2019; De Vega et al., 2021; Liang et al., 2021).

Interestingly, however, GO terms associated with photosynthesis were also significantly enriched amongst upregulated DEGs. In particular, these enriched terms were associated with the light-dependent reaction and light harvesting, whereby solar energy is captured via light-harvesting complexes and transferred to chlorophyll. Key components of these light-harvesting complexes are Chlorophyll a-b binding proteins, transferring excitation energy to photosystem II to power photosynthetic electron transport (Jansson, 1994, 1999). Recent work in pear, *Pyrus bretschneideri*, has identified several members of this protein family whose gene expression was upregulated under drought treatment (Wu et al., 2023), as observed in the present work, whilst work in tobacco showed that overexpression of a cold-responsive light-harvesting complex antenna protein gene from tomato enhanced cold tolerance (Deng et al., 2014) – perhaps suggesting that a similar upregulation of genes involved in light-harvesting may take place in wheat, explaining the enrichment of these GO terms amongst upregulated DEGs.

Similar to observations made when examining the transcriptional response to heat stress (**Chapter 3**), enriched amongst downregulated genes were terms associated with the response to stresses, both biotic and abiotic, other than drought. As hypothesized in that work, such a downregulation may come as a result of different cellular environments caused by each stress – for example, colonization by a pathogen or the formation of ice crystals, will be absent under drought stress conditions, therefore, the genes involved in responding to such cellular perturbations will be unrequired under drought stress. The downregulation of such unrequired genes may, therefore, increase the availability of the transcriptional machinery for expression of those genes that are involved in the tailored drought stress response. However, this comparison must be made with caution, as although similar trends are seen, the present work utilizes data from studies which exposed plants to drought stress later in their development, whereas the work described in **Chapter 3** exposed plants to heat stress much earlier in their development – therefore meaning the effects of stress exposure, on physiology and the transcriptome, are not directly comparable.

5.4.2. *TraesCS4D01G050400* may Negatively Regulate the Drought Response in Wheat

The blue module was deemed to be drought-associated, as it was enriched in the GO term “response to water deprivation” (FDR = 0.0224), despite not being enriched in drought DEGs (expected number of DEGs = 362, observed number of DEGs = 365, $p = 0.44$). The module was also one of the largest formed during co-expression network construction, meaning it

likely contained genes with diverse functions. To identify hub genes which may regulate the expression of genes likely involved in the response to drought, a subnetwork was created using connections to and from all (31) probes in the blue module which possess the enriched GO term “response to water deprivation” – resulting in a subnetwork with 7242 nodes, and 122,493 edges. A similar method of using guide genes of interest to create subnetworks and examine certain processes more closely has been adopted during the study of meiosis in wheat (Alabdullah et al., 2019).

TraesCS4D01G050400 (Ta.963.2.A1_at) was identified as a promising candidate master-regulator of the drought response, as not only was it the most well-connected DEG within the subnetwork, but it’s shared sequence identity with a well-characterized Arabidopsis gene suggests a pivotal role as part of the drought response. The hub was one of 31 probes which possessed the GO term “response to water deprivation”, and was connected to 1592 genes within the subnetwork, including 15 other “response to water deprivation” genes, and 80 drought DEGs. *TraesCS4D01G050400*, *T. aestivum probable histidine kinase 4*, shares sequence identity with *AtAHK4*, the hub gene’s Arabidopsis orthologue (identified via EnsemblPlants; Yates et al., 2022) which is a known negative regulator of ABA signalling and the drought, cold, osmotic and salinity responses (Tran et al., 2007; Tran, Shinozaki and Yamaguchi-Shinozaki, 2010; Jeon et al., 2010; Kang et al., 2012). Interestingly, this probe showed different expression responses to drought between studies in the meta-dataset; being upregulated (insignificantly, log2FC = 1.01, *p*-value = 0.02) under drought stress in GSE31759, whilst being significantly downregulated under drought stress in GSE30436. This is likely due to differences in drought tolerance between the lines used in these studies; GSE31759 used Chinese Spring (not especially drought tolerant), whilst GSE30436 used the drought tolerant line, C306. It can, therefore, be hypothesized that downregulation of *TraesCS4D01G050400* under drought stress leads to improved drought tolerance; a hypothesis supported by the previous work (Tran et al., 2007; Tran, Shinozaki and Yamaguchi-Shinozaki, 2010; Jeon et al., 2010; Kang et al., 2012).

TraesCS4D01G050400 may be able to act as a repressor of the drought response in wheat, thanks to potential regulation over the expression of several drought-responsive genes, as, in the subnetwork, the hub gene was connected to both upregulated and downregulated genes. *TraesCS5A01G057500* (TaAffx.18447.5.S1_s_at), *TabZIP6*, was downregulated after exposure to drought stress in the meta-dataset, whilst transgenic overexpression of the gene in Arabidopsis led to reduced freezing tolerance, due to repression of *COR* genes and *CBFs* (Cai et al., 2018) – suggesting *TabZIP6* may also act as a negative regulator of the drought response in wheat, and its downregulation is a key part of enabling this response. Amongst other downregulated genes the hub gene was connected to were a group which may act to promote stomatal opening, such as Ta.18480.2.S1_at, *T. aestivum probable inactive receptor kinase At1g48480 (LOC123098205*). *At1g48480*, otherwise known as *AtRKL1*, has been shown to be downregulated under drought treatment and hypothesized to act in stomatal opening, upstream of ABA in the signalling pathway (Tarutani et al., 2004). Similarly, the hub gene was connected to *TraesCS7B01G354900* (Ta.5345.2.S1_a_at), *T. aestivum carbonic anhydrase, chloroplastic-like*, whose Arabidopsis orthologue, *AtCA1*, is a known negative regulator of stomatal development and stomatal aperture (Hu et al., 2010; Engineer et al., 2014). Downregulation of the gene in the meta-dataset, however, may suggest that it acts to positively regulate these processes in wheat as stomata close in response to drought stress to prevent transpirational water loss.

As well as deactivating the expression of some genes, downregulation of the hub gene, may also alleviate the repression of some genes, allowing them to be upregulated. This was the case for *TraesCS5B01G062000* (Ta.22091.1.A1_at), *T. aestivum phosphatidylinositol 3-*

kinase, root isoform-like, whose Arabidopsis orthologue, *AtVPS34* (*PI3K*), regulates stomatal closure with overexpression leading to increased stomatal closure (Takahashi et al., 2017; Zhang et al., 2019) – exemplifying that downregulation of the hub gene may not only repress stomatal opening (as described above), but also actively promote stomatal closure. The hub gene was also connected to further upregulated genes which may prevent water loss, as the Arabidopsis orthologues of *TraesCS7A01G278500* (TaAffx.38271.1.A1_at), *T. aestivum fatty acyl-CoA reductase 1-like*, and *TraesCS1A01G341300* (Ta.12753.1.S1_at), *T. aestivum BAHD acyltransferase DCR-like* (*AtFAR1* and *AtPEL3*, respectively), are involved in suberin and cutin biosynthesis, respectively (Panikashvili et al., 2009; Domergue et al., 2010; Rani et al., 2010). Cutin and suberin synthesis are common drought responses and are a key mechanism of drought tolerance, with their synthesis and deposition on cell surfaces preventing water loss (Cameron, Teece and Smart, 2006; Shepherd and Wynne Griffiths, 2006; Seo and Park, 2011; Xue et al., 2017; de Silva et al., 2021; Shukla et al., 2021; Chen et al., 2022; Liu, Wang and Chang, 2022).

As a combined result of the characterized function of the hub gene's *Arabidopsis* orthologue, its expression under drought stress in the meta-dataset, and its connections to multiple genes involved in processes crucial to the drought response, the present work hypothesizes that *TraesCS4D01G050400* acts as a key repressive master-regulator of the drought response in wheat. Although the results of the partial hub gene validation do suggest this may be the case, with TILLING mutant plants containing a missense mutation (SIFT = 0) showing improved drought tolerance compared to the Cadenza control, robust conclusions cannot be confidently made from these data, due to only two mutant replicates being present in the control condition. A further screen of more mutant plants would allow this phenotype to be confirmed, whilst screening the drought tolerance of backcrossed mutants would allow for the true effect of hub gene mutation on drought tolerance to be understood.

5.4.3. *TraesCS7D01G347300* may play an Unexpected Role in the Drought Response, despite being connected to Drought Tolerance Genes

The purple module was not significantly enriched in any stress-associated GO terms, but was significantly enriched in drought DEGs (expected number of DEGs = 105, observed number of DEGs = 155, p = 2.09E-07), suggesting it contained genes involved in the drought response. The hub gene within this module was *TraesCS7D01G347300* (Ta.29814.1.S1_at), *T. aestivum peroxidase 1-like*, the wheat orthologue of the rice gene, *OsPRX15*. Neither gene is very well characterized, however the hub's role as a peroxidase means it is likely involved in the response to oxidative stress, and the detoxification of reactive oxygen species. Although the hub gene was not identified as a DEG in the meta-dataset, its connection to several drought-responsive genes suggests it may act to coordinate their expression.

Several of the drought-responsive genes connected to the hub gene within the purple module may be involved in responding to abiotic stresses, such as drought, salinity and cold. For example, *TraesCS1D01G296500* (Ta.13183.1.S1_x_at), *T. aestivum Wcor18*, was upregulated in the meta-dataset. Expression of *Wcor18* has been found to respond to low temperatures (the gene was expressed at temperatures as low as -15°C), high salinity, drought and ABA treatment in wheat seedlings, leading the authors to suggest it may play a pivotal role in holistic abiotic stress tolerance, and the response to several stresses (Wang et al., 2020c). Similarly, the hub was also connected to the upregulated gene, *TraesCS6B01G383800* (Ta.2787.1.S1_at), *T. aestivum dehydrin (wdhn2)* – a gene known to also respond to drought, salinity, cold and ABA treatment (Zhang et al., 2022a). The authors also found that expression of *WDHN2* in *Escherichia coli* improved tolerance to osmotic stress, high salinity, low temperature and high temperature, suggesting it likely plays a pivotal role in general abiotic stress tolerance in wheat. Another likely drought tolerance gene connected to

the hub gene was *TraesCS1B01G276800* (Ta.30711.1.S1_x_at), *T. aestivum Bowman-Birk type proteinase inhibitor B5-like*. This family of protease inhibitors have been linked with improved drought tolerance in Arabidopsis, with overexpression of the *AtBBI* gene leading to normal growth despite nine days of drought treatment, as well as increased leaf relative water content, compared to control plants (Malefo et al., 2020) – potentially explaining the gene's upregulation in the meta-dataset. More recently, this gene family has been described in wheat, with the expression of some members being shown to respond to biotic and abiotic stresses (Xie, Ravet and Pearce, 2021) – however *TraesCS1B01G276800* was not listed as a member in this work, suggesting the family may contain more than the 57 members identified by the authors.

The hub gene was also connected to upregulated repressors of hormonal signalling, such as *TraesCS3B01G353200* (Ta.4458.1.A1_at), *T. aestivum probable indole-3-acetic acid-amido synthetase GH3.1*, whose Arabidopsis orthologue is *AtGH3.6*/*DFL1*. GH3 proteins are known to be involved in the conjugation of amino acids to auxin, thus biologically inactivating the hormone, as overexpression of *AtGH3.6* led to increased accumulation of these conjugates, without affecting overall auxin levels (Staswick et al., 2005). Upregulation of the gene in the meta-dataset suggests it is likely involved in the response to drought stress, or improved drought tolerance – a hypothesis supported by work on related GH3-genes. For example, *AtWES1* expression was found to be induced after exposure to various abiotic stresses, whilst activation-tagged mutants displaying increased *AtWES1* expression showed improved tolerance to drought, freezing and salinity stresses (Park et al., 2007). Similarly, work in rice found that activated expression of a GH3 gene in *tld-D* mutants subsequently increased the amount of IAA-amino acid conjugates, reducing free IAA and improving drought tolerance, potentially as a result of increased late embryogenesis abundance (LEA) gene expression (Zhang et al., 2009).

However, the hub gene was also connected to several downregulated genes within the module which are seemingly involved in the response to environmental conditions besides water limitation. For example, *TraesCS7A01G122000* (Ta.3395.2.S1_at), *T. aestivum MADS-box transcription factor 5*, is known to respond to phosphorus starvation stress (Shi et al., 2016), whilst the Arabidopsis orthologue (*AtHAK5*) of *TraesCS3D01G439200* (TaAffx.55592.2.A1_at), *Triticum aestivum potassium transporter 5-like*, is a key player not only in potassium ion uptake in low potassium environments (Nieves-Cordones et al., 2010), but is also linked to improved salinity tolerance, as tolerant Arabidopsis accessions showed greater *AtHAK5* expression under control conditions, and a greater degree of upregulation after exposure to salinity stress, compared to the Col-0 control (Sun et al., 2015). It could be argued that downregulation of *TaMADS5* in the meta-dataset suggests it plays a repressive role during the drought response, as well as its role in the response to phosphorus starvation – however, the hub gene's connection to genes involved in responding to abiotic stresses other than drought may be a further example (as discussed above, and in **Chapter 3**) of these unrequired genes being downregulated to increase the transcriptional capacity for the expression of genes involved in the tailored drought response.

Perhaps counter-intuitively, however, given its connection to several genes which likely play key roles in the drought response or drought tolerance, and its role as a peroxidase, non-backcrossed TILLING mutants containing a missense mutation (SIFT = 0) in the hub gene showed markedly improved tolerance to drought stress at the seedling stage, compared to the Cadenza control. Further work is required to determine whether this phenotypic change is a result of the background mutations present in these plants, or if mutation to the hub gene does lead to improved drought tolerance. Similarly, further work is required to confirm whether the hub gene does have regulatory action over the expression of the genes it is connected to

within the co-expression network. Its function as a peroxidase does not make it an obvious candidate for a transcriptional regulator, however it may be possible that it's likely action responding to oxidative stress and detoxifying reactive oxygen species could lead to downstream signalling affecting gene expression (Dalton, Shertzer and Puga, 1999; Turpaev, 2002; Mase and Tsukagoshi, 2021).

5.4.4. Meta-analysis Identified Genes Responsive to ABA but not to Drought

Many of the modules identified as drought-associated were enriched in GO terms such as "response to chemical stimulus", "cellular response to stimulus" and "response to abiotic stimulus", suggesting they contain genes which respond to signals released during abiotic stress, such as stress hormones. Some modules were enriched in these GO terms, without being enriched in drought DEGs, suggesting they contain genes which respond to cellular signals in response to perturbations other than drought stress. For example, modules enriched in signal response GO terms and ABA DEGs, but not in drought DEGs, may contain genes which respond to abiotic stresses such as salinity, heat or cold, amongst other environmental cues, in an ABA-dependent manner. The hub genes within these modules may, therefore, regulate the ABA-mediated responses to such stresses, and although their validation was beyond the scope of the present work, such hub genes may be promising targets for further enquiry into how the transcriptional and physiological responses to other abiotic stresses may be coordinated.

956 probes (1.56%) were identified as ABA DEGs, whilst a one-proportion Z-test identified seven modules which were significantly enriched in these genes. Perhaps predictably, given its importance as a signalling molecule during the response to drought stress (Muhammad Aslam et al., 2022), 257 genes were differentially expressed under both drought stress exposure and exogenous ABA treatment within the metadataset, suggesting their response to drought stress may be ABA-mediated. This meant that 699 and 2659 genes were differentially expressed under ABA treatment only and drought stress only, respectively. The 2659 genes which were only differentially expressed under drought stress likely respond to stress hormone signals besides ABA, or to the cellular environment specifically created by drought stress (of which, ABA signalling is likely to be one of several components) which is more complex than exogenous ABA treatment. The 699 ABA-only DEGs, however, may be involved in other ABA-mediated processes, besides the drought response, with modules containing a large number of such genes potentially being a good source of candidate hub genes which act as master regulators of these processes.

5.4.5. Co-expression Network Analysis Identified a Candidate ABA-mediated Regulator of the Response to Abiotic Stresses besides Drought

As well as identifying hub genes within drought-associated modules, the present work also identified a hub gene within a module particularly associated with the ABA response, and potentially with responses to abiotic stresses other than drought. The majority of the genes within the darkgrey module were ABA DEGs, whilst the module as a whole was significantly enriched in various terms related to the abiotic stress response and response to hormone signalling – both suggesting the module houses genes involved in an ABA-mediated stress response. The module was not significantly enriched in drought DEGs, however, with only 11 of the 98 ABA DEGs also being differentially expressed under drought stress, suggesting the remaining 87 genes may respond to abiotic stresses other than drought in an ABA-dependent manner. The hub gene in this module, *TraesCS4A01G119900* (Ta.5829.1.S1_at), *T. aestivum outer envelope pore protein 16-2, chloroplastic-like*, was itself an ABA DEG. Expression of the hub gene's Arabidopsis orthologue, *AtOEP16-2*, is induced by ABA treatment, whilst *atoep16-2* mutants are hypersensitive to ABA (Pudelski et al., 2012).

The hub was connected to 69 ABA-only DEGs within the module, several of which seemingly play roles in the stress response. Two genes connected to the hub gene encode LEA proteins; Ta.13396.1.S1_at, *A. tauschii LEA6-like*, and *TraesCS1B01G381200* (Ta.727.1.S1_x_at), *TaLEA3*. The expression of both genes was upregulated in response to ABA treatment, but not drought stress. *LEA6* genes in rice have been shown to respond to drought stress and ABA treatment, whilst the wider class of LEA proteins are known to respond to numerous stresses (heat, cold, salt, osmotic and drought) in several species, largely in an ABA-dependent manner (Chourey, Ramani and Apte, 2003; Olvera-Carrillo et al., 2010; Rodríguez-Valentín et al., 2014; Zamora-Briseño and de Jiménez, 2016; Lim, Lim and Lee, 2018; Magwanga et al., 2018; Chen et al., 2019; Shi et al., 2020), suggesting these genes may respond to abiotic stresses other than drought as a result of ABA signalling. *TraesCS5D01G379300* (Ta.14247.1.S1_at) was upregulated under ABA treatment, but not drought stress, and is *T. aestivum salt-induced YSK2 dehydrin 1 (DHN1)*. Like LEAs, dehydrins have been shown to respond to multiple abiotic stresses in several species (Kosová, Vítámvás and Prášil, 2014; Kumar et al., 2014; Hassan et al., 2015; Li et al., 2017; Verma et al., 2017; Edrisi Maryan et al., 2019), however, they largely respond to water shortage or changes in cellular osmotic potential, predominantly during drought, freezing and salinity stress (Kosova, Vitamvas and Prasil, 2007; Kosová, Vítámvás and Prášil, 2014; Yu, Wang and Zhang, 2018). Here, the dehydrin's expression only changes in response to ABA treatment, suggesting the gene may be involved in responding to stresses other than drought, in an ABA-dependent manner.

Similarly, the hub gene was connected to *TraesCS5A01G531300* (Ta.13443.1.S1_at), *TaTAAC1* (an orthologue of *AtFAR5*) – a gene which was upregulated in response to ABA treatment, but not drought stress in the meta-dataset. *AtFAR5* is a salt-responsive gene which generates primary fatty alcohols associated with suberin deposition (Domergue et al., 2010). Increased suberin deposition is known to be controlled by ABA (Chen et al., 2022), and besides playing a preventative role in water loss (as discussed previously), suberin deposition is also induced by exposure to waterlogging, salinity, cadmium and ammonium stresses, suggesting it's build-up also prevents the movement of damaging factors into cells (Krishnamurthy et al., 2009; Líška et al., 2016; Ranathunge et al., 2016; Chen et al., 2022) – for instance, the degree of suberin deposition in rice primary roots was found to be negatively correlated with sodium ion uptake into plant shoots (Krishnamurthy et al., 2009, 2011). The hub was also connected to two genes which may be involved in cold-acclimation or freezing tolerance due to their membership of gene families known to be involved in such processes (Houde et al., 1992; Lång and Palva, 1992; Lang et al., 1994; Mantyla, Lang and Palva, 1995; Karlson et al., 2002; Sasaki and Imai, 2011): *TraesCS6A01G350200* (Ta.123.1.S1_x_at), *T. aestivum cold-shock protein CS120-like*, and *TraesCS5B01G426800* (Ta.2704.1.S1_at), *T. aestivum dehydrin Rab15-like*.

Therefore, the abundance of ABA-only DEGs, seemingly involved in the response to salinity and cold stress, connected to *TraesCS4A01G119900* within the darkgrey module suggests the hub gene may act as a master-regulator of the ABA-mediated response to such stresses. It may be hypothesized that the genes connected to the hub gene, described here, respond to ABA and not to drought stress exposure in the meta-dataset, as the drought stress treatments used may illicit a lesser ABA response than is required to activate their expression – a response threshold perhaps met only during exposure to other abiotic stresses – or, that transcription factors activated under drought stress, but absent under these other stresses, repress expression of these genes under drought stress, despite the accumulation of ABA. Further work is required to understand how the expression of the hub gene, and the genes connected to it in the darkgrey module, respond in wheat plants exposed to different environmental cues – allowing a better understanding of which abiotic stress, if any, these

genes respond to in an ABA-mediated manner to be gained. Further experimental work is also required to confirm whether the hub gene does have ABA-mediated regulatory action over the expression of these genes, as suggested by their connection within the co-expression network, and whether mutations to the hub gene impede these responses.

5.5. Conclusions

In all, the present work represents a thorough use of open source gene expression data to identify candidate master-regulators of the response to drought stress and ABA treatment, via WGCNA. Comparative transcriptomic analysis showed that almost 3000 probes were significantly differentially expressed after drought stress exposure, with genes particularly related to photosynthetic processes being largely downregulated. However, some photosynthesis genes were also significantly upregulated under drought stress, suggesting they play a positive role in the drought response, as observed in other species. The present work identified two hub genes, in modules containing genes involved in the drought response, which may be good candidates for master-regulators of the drought response. Non-backcrossed TILLING lines containing mutations in these hub genes showed improved drought tolerance compared to wild-type Cadenza, however successful backcrossing into a wild-type background is required to reduce the effect of background mutations, and ensure a robust appraisal of the effect of hub gene mutation on drought tolerance can be seen. Similarly, the present work identified modules containing genes associated with the response to ABA treatment, but not the response to drought. Within these modules, one hub gene in particular stood out as a candidate master-regulator of the ABA-mediated response to abiotic stresses besides drought, as it was connected to a large number of genes, differentially expressed after ABA treatment, which likely play roles in the cold and salinity responses. Although further exploration of this hub gene was beyond the scope of the present work, further work could explore the expression of the hub gene, and the genes it is connected to in the module, under various different stresses, to determine whether they act as part of the ABA-mediated response to these perturbations, whilst TILLING lines could be used to determine whether the hub gene coordinates such expression responses. The present work, therefore, identifies several promising candidate hub genes which may coordinate the response to both drought stress and ABA treatment – hub genes which may be valuable targets for breeders in their production of climate-resilient varieties.

6. Transcriptomic and Co-expression Network Analyses on Diverse Wheat Landraces Identifies Candidate Master Regulators of the Response to Early Drought

6.1. Introduction

Triticum aestivum L. (bread wheat) is relied upon by billions of people as a primary source of both calories and protein (Pfeifer et al., 2014; Food and Agriculture Organization of the United Nations et al., 2018). As the global population continues to grow, the number of livelihoods that will be dependent on the success of wheat crop yields is staggering. To meet this demand, therefore, the yields of key crops like wheat need to increase by at least 50% in the coming decades (Godfray et al., 2010; Tilman et al., 2011; Ray et al., 2013). The changing climate poses a major threat to this necessary yield increase, however, with rising global temperatures leading to the depletion of water supplies and periods of intense drought stress (Hansen et al., 2006). Drier growth conditions paired with reduced water supply is of particular concern for the agricultural sector, as it accounts for between 80 and 90% of all freshwater usage, with cereal crop cultivation alone accounting for 27% (Hoekstra and Mekonnen, 2012; Ray et al., 2013; Dunn et al., 2019). In the coming decades, climate change will cause changes in precipitation patterns that may affect wheat-growing regions especially severely, with recent work finding that up to 60% of the current global wheat-growing area may face severe water scarcity by the end of the century, compared to only 15% currently (Trnka et al., 2019). As well as the threat that future drought events pose to wheat crops, drought stress has been causing significant damage around the world for the last few decades, with 161Mha of wheat harvested areas experiencing yield loss through drought between 1983 and 2009, equating to an economic loss of $47 billion (Kim, Iizumi and Nishimori, 2019; Iizumi et al., 2018). Therefore, the cultivation of drought tolerant wheat varieties is of paramount importance, if global wheat crops are to be protected against the effects of water shortage in a climate where water supplies are becoming increasingly scarce.

In the present work, the effect of drought stress exposure during early development on gene expression in spring habit wheat landrace accessions was examined. With spring wheat often being sown during March in the Northern hemisphere, the present work mimics drought stress events that occur during April once plants have germinated and established in fields. Although much of the work concerning the effect of drought stress on wheat growth studies the perturbation's effect on yield (Aprile et al., 2009; Zhang et al., 2018; Kim, Iizumi and Nishimori, 2019; Qaseem, Qureshi and Shaheen, 2019; Senapati et al., 2019; Abou-Elwafa and Shehzad, 2021; Lan et al., 2022; Wan et al., 2022), periods of water shortage are becoming increasingly common during the early stages of spring wheat growth, all around the world. April 2022, for example, was an incredibly dry month for many of the world's largest wheat-producing countries, with almost 50% of the United States experiencing moderate to exceptional drought (NOAA National Centers for Environmental Information, 2022d), whilst large parts of Europe (including major spring wheat-producing nations such as the United Kingdom) experienced a drier month than normal (NOAA National Centers for Environmental Information, 2022c). The pressing nature of this threat to wheat crops is perhaps reflected in the increasing amount of research into the effect of drought stress on the early growth of wheat, over the last few years (Guo et al., 2017; Sallam et al., 2018; Ahmed et al., 2020, 2022; Mahpara et al., 2022; Nardino et al., 2022; Sharma et al., 2022). These works largely identify tolerant cultivars for use in breeding programmes, but do not aim to understand the genetic control of the drought response at this stage of development – something that is relatively understudied, despite its importance (Ajigboye et al., 2017; Vuković et al., 2022; Mao et al., 2020). The need, therefore, to better understand the genetic control of the early drought response in order to aid the production of drought tolerant wheat varieties is already present,

and likely to become more pressing as temperatures increase and precipitation patterns change over the coming decades.

Due to the sheer number of genes involved in complex processes, such as the drought response, identifying those which play the most pivotal roles can be difficult. The use of weighted gene co-expression network analysis (WGCNA), however, identifies groups of genes which are co-expressed across samples, from which candidate master-regulators of these groups of genes can be identified (Langfelder and Horvath, 2008, 2012). Such master-regulators of drought-responsive genes, therefore, are likely to be those which play key roles in the drought response. The approach has been utilized successfully to identify "hub genes" in wheat encoding proteins such as transcription factors, heat shock proteins (HSPs) and regulators of stress hormone signalling (Lv et al., 2020; Du et al., 2022), which act to determine a plant's degree of drought tolerance via their regulation of other drought-responsive genes. The present work employs a similar approach, but is distinct from these works due to its use of wheat landraces: genetically and phenotypically diverse cultivars selected by local farmers to grow successfully in a vast array of climates around the world (Zeven, 1998). **Chapter 2** previously exemplified the genetic diversity of the YoGI landrace panel, before utilizing it to identify candidate master-regulators, and genetic markers, of basal early thermotolerance, whilst **Chapter 3** utilized YoGI landrace accessions to study the transcriptional response to early heat stress. The present work, however, represents a novel study into the use of gene expression data from wheat landraces under drought stress, to identify candidate master-regulators of the transcriptional early drought response.

6.2. Materials and Methods

6.2.1. Selection, Growth, and Sampling of Plants

14 spring habit accessions with a range of drought tolerance levels were used in the present work (**Table S6.1**). Genomic tile plots visualising the A, B, and D genomes for each accession in the YoGI landrace panel (**Chapter 2**) were used to exclude accessions with significant genomic dominance or putative rearrangement, and to ensure all accessions used were hexaploid. Seeds were sown in Levington Advance Seed & Modular F2S compost mixed with Aggregate Industries Garside Sands 16/30 sand (80:20 ratio), treated with CaLypso insecticide (Bayer CropScience Ltd., 0.083ml mixed with 100ml water, applied to each litre of compost) and grown under long day (16/8h, 20°C/14°C) glasshouse conditions.

Four replicates of each accession per group were watered normally (twice-daily watering, average soil moisture content (SMC) = 36.6%), until plants in the drought group reached Zadoks' growth scale 13 (GS13; Zadoks, Chang and Konzak, 1974) whereby stress was applied by withholding water for a ten-day period. Normal watering then resumed for three-days to serve as a recovery period. Four replicates of each accession were grown at the same time, but not exposed to drought stress. All above-ground tissue from plants was harvested 13 days after GS13, before biomass was dried for two days at 70°C and weighed on a scale.

6cm of leaf tissue was collected from wheat seedlings upon reaching GS13 and at the end of the drought period. Tissue was collected individually for each sample, and immediately immersed in liquid nitrogen to prevent nucleic acid degradation. Tissue samples were stored at -80°C for later processing. At each sampling stage, as well as after drought recovery (13 days after GS13), SMC% was recorded using an ML3 Thetaprobe Soil Moisture Sensor with an HH2 Moisture Meter (Delta-T Devices, Cambridge, United Kingdom) to quantify the severity of the drought stress treatment. The probe was inserted into the soil to its full depth before moisture levels were recorded. Mean SMC% of conditions, at each time point, were compared via two-sample *t*-test.

6.2.2. RNA Isolation and Sequencing

Total RNA was extracted from ~100 mg of individual leaf tissue samples using the E.Z.N.A Plant RNA Kit (Omega Bio-Tek, GA, USA) including a DNase treatment, according to the manufacturer's protocol. RNA concentration was quantified using a Qubit 4 Fluorometer (Life Technologies, CA, USA), while RNA quality was assessed via both NanoDrop ND-1000 Spectrophotometer (Thermo-Fisher Scientific, MA, USA) and an Agilent Technology 2100 Bioanalyzer (Agilent Technologies, CA, USA). Samples with RNA Integrity Number (RIN) values greater than seven were deemed acceptable for use in subsequent analysis. Replicates were pooled into one sample per accession, per treatment, at equimolar proportions. Samples were stored at -80°C and shipped on dry ice to Novogene (Cambridge, United Kingdom) for sequencing, using the Illumina Novaseq 6000 platform (Illumina, CA, USA) with a 150bp paired-end strategy. The experimental design included both technical and biological replication. Prior to sequencing, RNA from 4 replicate plants per accession, per condition (pre- or post-drought) were pooled to help control the effect of the environment on the transcriptome, whilst the different accessions provided biological replication for each treatment.

6.2.3. Data Processing, Mapping, and Quality Control

After sequencing, quality control was carried out using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were then filtered by trimming low quality sequences (average Phred score < 15), trimming short length reads (< 36bp), and clipping Illumina adapters using Trimmomatic v0.39 (Bolger, Lohse and Usadel, 2014). Sequencing data is available at NCBI: GSE225797.

Salmon (Patro et al., 2017) was used to map reads to the IWGSC *Triticum aestivum* v1.0 reference assembly (GCA Accession: GCA_900519105.1) and the updated IWGSC *Triticum aestivum* v1.1 gene model annotation. Reference genome and gene model annotation files used can be found on the International Wheat Genome Sequencing Consortium (IWGSC) website (https://www.wheatgenome.org). Salmon's mapping-based mode was used to create an index from the reference genome, and then for quantification of the trimmed reads. Salmon output files were prepared for differential expression analysis using the R (version 4.1.2.; R Core Team, 2021) package TxImport (version 1.24; Soneson, Love and Robinson, 2015), generating a table containing transcript abundance (TPM), counts, and length from the Salmon quantification files.

6.2.4. Transcriptomic Overview and Differential Expression Analysis

Transcriptome data were initially explored using Principal Components Analysis (PCA) function of DESeq2 (version; 1.36.0; Love, Huber and Anders, 2014). Differential expression analysis was performed on the raw count data using the R package DESeq2. Genes with < 10 reads were filtered out before running DESeq2. An additive model was used to identify differentially expressed genes (DEGs) between pre- and post-drought samples. Expression fold changes were shrunk using the R package "Ashr" (version; 2.2-54; (Stephens, 2017) to account for variability in lowly expressed genes while preserving large fold changes.

Only genes with a log2FoldChange greater/less than 1.5/-1.5 and an FDR-adjusted (Benjamini and Hochberg, 1995) *p*-value < 0.05 were considered significantly differentially expressed and carried forward for GO enrichment analysis. Differential expression contrasts were visualised via volcano plots, made using the "ggplot2" package (version 3.4.0; Wickham, 2009) in R.

6.2.5. DEG Gene Ontology Term Enrichment Analysis

To identify gene ontology (GO) terms significantly enriched amongst upregulated and downregulated DEGs, identified via DESeq2, GO enrichment analysis was conducted. Because GO terms were only present for the IWGSC RefSeqv1.0 genome annotation, an

approach used previously (Borrill et al., 2019; Andleeb, Knight and Borrill, 2023) was adopted, whereby GO terms are transferred from the v1.0 annotation to the v1.1 annotation. This approach transfers the GO terms only from genes which were >99% identical across >90% of the sequence. The list of these genes can be found in (Andleeb, Knight and Borrill, 2023). IWGSC v1.0 GO terms were retrieved from: https://opendata.earlham.ac.uk/wheat/under_license/toronto/Ramirez-Gonzalez_etal_2018-06025-Transcriptome-Landscape/data/TablesForExploration/FunctionalAnnotation.rds. This RDS file was read in to R using the readRDS() function (in base R), prior to analysis.

GO terms associated with upregulated and downregulated DEGs were collated into two groups and submitted to the agriGO Singular Enrichment Analysis tool (Du et al., 2010; Tian et al., 2017). A Fisher's exact test was performed for each DEG group with the GO terms of all genes obtained after count filtering by DESeq2 serving as background; 0.05 as the *p*-value threshold; Hochberg (FDR) as the multi-test adjustment method (Benjamini and Hochberg, 1995), and 5 as the minimum number of mapping entries threshold. A GO term was considered enriched when its FDR-adjusted *p*-value was < 0.05. GO terms that were significantly enriched amongst upregulated and downregulated genes, compared to the background, were obtained for Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) categories, elucidating gene function and localisation within these DEG groups.

6.2.6. Network Construction and Module Detection

TPM data obtained from leaf tissue samples taken before and after drought stress exposure, described here, were used to construct a single co-expression network in R (version 3.6.3), using the WGCNA package (Langfelder and Horvath, 2008, 2012). 21,870 genes were removed due to too many zero values, leaving 84,888 genes, from 28 samples (14 accessions before and after drought stress) for network construction. Blockwise network construction and module detection was conducted using the blockwiseModules() function according to its default parameters, with several exceptions: network type = signed hybrid, maximum block size = 5000, soft threshold power = 16 (the first power to exceed a scale-free topology fit index of 0.9), minimum module size = 30, merge cut height = 0.25. The exportNetworkToCytoscape() function was used after module detection to create edge and node files for module visualization in Cytoscape. A threshold of 0.1 was used to filter out weak connections between genes.

6.2.7. Module GO Term Enrichment Analysis

The agriGO v2.0 Singular Enrichment Analysis tool (Du et al., 2010; Tian et al., 2017) was used to identify gene ontology (GO) terms significantly enriched in each module. To do this, GO terms of genes in each module were compared to GO terms of all genes in the co-expression network. The parameters used were the same as those described for the DEG GO term enrichment analysis above. GO terms used were also retrieved using the method described above.

6.2.8. DEG Enrichment Analysis

10,199 of the 84,888 genes included in the network were deemed to be DEGs – equating to 12% of all genes. If DEGs were distributed across modules according to module size, each module would be expected to contain this proportion of DEGs. To determine whether the observed proportion of DEGs in each module was significantly greater than this predicted proportion, a one-proportion Z test was employed. Modules were deemed to be significantly enriched in DEGs if $p < 0.05$.

6.2.9. Network Visualization and Hub Identification

To identify hub genes, degree (connection) scores were calculated for each gene within a module, either using the Cytoscape (version 3.9.1.; Shannon et al., 2003) network analyser tool (Assenov et al., 2008), or by counting the number of connections to and from each gene in the WGCNA edge file, using the table() function in R. The script used to calculate degree scores in R is available on GitHub (https://github.com/andreaharper/HarperLabScripts/). Cytoscape was used to visualize modules, and for hub gene identification in the majority of cases, however particularly large modules are often difficult to load, view and analyse in Cytoscape. In these cases (modules containing ~2000 genes or more), R was used to calculate degree score in the same way as in Cytoscape (i.e. counting the number of connections to and from each gene in the WGCNA edge file). Those genes in a module with the highest degree scores (most connections) were identified as the central hubs. In some cases, however, multiple genes within a module shared the highest degree score, whilst in other modules, the highest scoring genes were not found to be differentially expressed under drought conditions. In these cases, the highest-scoring DEG was identified as the module's hub gene, as these genes are both differentially expressed and well connected within the module, and so are more likely to regulate the transcriptional drought response, than a well-connected non-DEG. Those modules found to be significantly enriched in the "response to water" (GO:0009414) GO term (black and turquoise) were also amongst the largest in the co-expression network. These modules, therefore, likely contain genes involved in diverse processes – so, to focus on the response to water, subnetworks were created using genes annotated with the "response to water" (GO:0009414) GO term within the module as guide genes. It was thought that by only examining the connections to and from these genes, the subsequently identified hub gene would be a better candidate regulator of the drought response, than the hub gene of the entire, much larger, module. As with the other modules, the most well-connected DEG was identified as the hub gene in these subnetworks.

6.3. Results

6.3.1. Drought Stress Exposure

Drought stress was found to have a significant effect on plant growth, as both fresh and dry weight differed significantly (*t*-test: both $p < 2.2e\text{-}16$) between stressed and control plants (**Figure 6.1a**). Soil Moisture Content (SMC%) was measured over the course of the experiment (**Figure 6.1b**), with the drought stress treatment causing SMC% values of the control and drought groups to differ significantly ($p < 2.2e\text{-}16$) ten days after Zadoks' growth scale 13 (GS13; Zadoks, Chang and Konzak, 1974). No significant difference was identified between the two groups at GS13, before the start of the drought period ($p = 0.179$). A significant difference in SMC% was observed between the two groups at harvest ($p = 0.0006$), however. Although statistically significant, the difference in SMC% between the groups at harvest was slight, with average SMC% for both the control (45.9%) and drought (49.5%) groups being within the expected ranges for normal watering conditions. Data used to produce **Figure 6.1a** and **6.1b** are available in **Supplementary Data S6.1**.

6.3.2. Transcriptome Sequencing, Quantification, and Overview

921.6 Gb of raw data was generated as a result of sequencing with the Illumina paired-end Novaseq 6000 platform. From 28 samples (pooled RNA samples from 4 replicate plants, for each of the 14 accessions, before and after drought stress), 1.465×10^9 reads were generated; an average of 97.3% and 92.6% of bases had a q-value of ≥ 20 and ≥ 30, respectively, with an error probability of 0.03. GC content of the reads ranged from 53.4% to 57.2%. Data quality was assessed using FastQC, with data for each sample being deemed acceptable, before pre-processing and then quantification with Salmon. Average mapping rate across all samples

was 61%. Raw sequence read data were deposited in NCBI's Gene Expression Omnibus (GSE225797).

Counts of all 28 samples were variance-stabilised using DESeq2 and analysed using principal component analysis (PCA, **Figure 6.1c**). The clustering of the samples indicated that the variance within each group was smaller than the variance between groups, however there was more variance on PC2 after drought than before. PC1 and PC2 accounted for 67.9% of the total variance; PC1 (which explained 62.7% of the variation) was able to provide separation between the samples taken before and after drought stress, while PC2 provided separation potentially relating to a spread of tolerance phenotypes across the accessions, albeit explaining far less of the overall variance than PC1.

6.3.3. Identification and Functional Analysis of Drought-responsive Genes via Differential Expression and Gene Ontology Enrichment Analyses

To investigate the genes that responded to drought stress in wheat leaves during early growth stages, differential expression analysis between samples taken before and after drought stress was carried out. Genes were deemed to be differentially expressed (DEGs) when their FDR-adjusted *p*-value was < 0.05, and their log2FoldChange was greater/less than 1.5/-1.5.

A total of 10,199 DEGs were identified; 6051 and 4148 with significantly increased and decreased expression, respectively, in response to drought (**Figure 6.1d**). Wide dispersion of the genes in **Figure 6.1d** suggests a high level of difference in gene expression between the two groups. Normalised expression data from DESeq2 and differential expression analysis results can be found in **Supplementary Data S6.2** and **S6.3**, respectively.

To investigate gene function among DEGs, GO enrichment analysis was conducted on both the upregulated and downregulated genes. 231 GO terms were enriched amongst the upregulated genes, while 258 GO terms were enriched amongst from downregulated genes. Output from GO enrichment analyses can be found in **Supplementary Data S6.4** and **S6.5**.

GO terms related to the stress response were enriched amongst upregulated genes, such as; "response to water" (GO:0009415, FDR = 4.90e-27), "response to stress" (GO:0006950, FDR = 9.70e-11), "response to abiotic stimulus" (GO:0009628, FDR = 9.50e-16), and "response to oxidative stress" (GO:0006979, FDR = 0.0016). Other enriched terms were related to cell wall maintenance ("cell wall organization or biogenesis", GO:1903338, FDR = 5.10e-07; "cell wall biogenesis", GO:0042546, FDR = 2.10e-05), and the regulation of gene expression ("regulation of RNA transcription, DNA-templated", GO:0006355, FDR = 5.00e-19; "regulation of gene expression", GO:0010468, FDR = 4.60e-18). The most significant enriched GO term was "response water", followed by "response to acid chemical" (GO:0001101, FDR = 4.90e-27), and "oxidation-reduction process" (GO:0055114, FDR = 1.70e-23).

By contrast, GO terms enriched amongst downregulated genes were related to processes such as photosynthesis ("photosynthesis", GO:0015979, FDR = 3.10e-76; "thylakoid", GO:0009579, FDR = 1.20e-72; "chloroplast", GO:0009507, FDR = 3.70e-07), homeostasis

("cellular homeostasis", GO:0019725, FDR = 1.00e-11), and substance transport ("transport", GO:0006810, FDR = 0.0019).

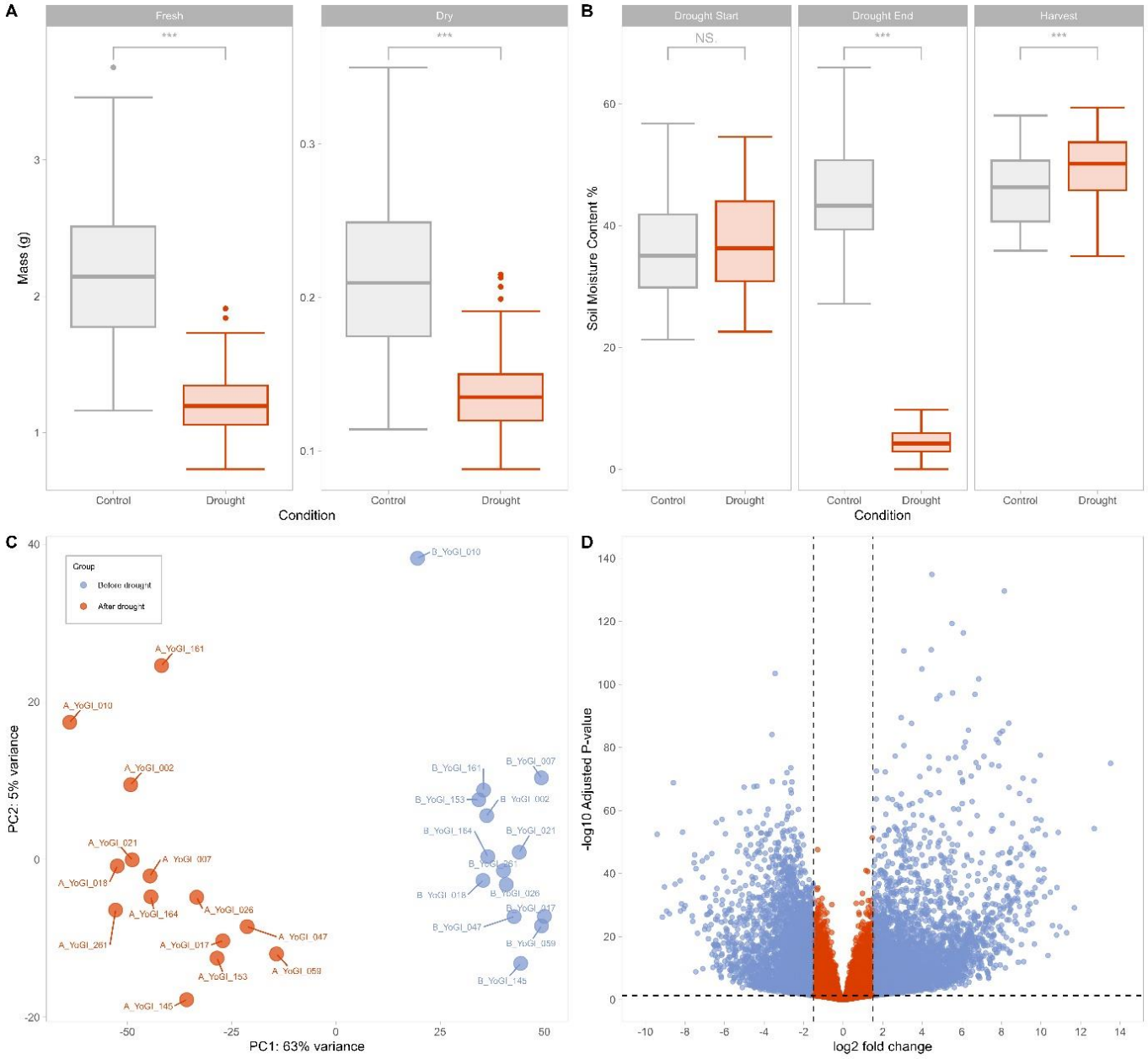

Figure 6.1: Drought treatment resulted in substantial differences across the panel in both phenotypic measurements and transcriptomic profiles. Ten days of drought stress was found to significantly reduce (*t*-test: both $p < 2.2e\text{-}16$) average fresh weight by 42.9% and average dry weight by 34.8% **(A)**, while soil moisture content (SMC%) was significantly different at the end of the drought treatment **(B)**. SMC% did not differ at the start of the drought period. 'Harvest' refers to the point where all above-ground biomass was harvested per individual, 13 days after GS13. An initial exploration of expression data suggested that early heat stress caused significant transcriptional changes **(C-D)**. Principal component analysis (PCA) of variance-stabilised transcript counts from all 28 samples **(C)** showed clear separation between the before and after groups on PC1, while differential expression analysis identified 10,199 DEGs, visualised via a volcano plot **(D)**. Dashed lines indicate DEG thresholds: vertical lines represent the log2FC thresholds of ±1.5, horizontal lines represent the *p*-value threshold of 0.05. DEGs that meet the criteria are beyond these threshold lines, coloured in light blue.

6.3.4. Identifying Stress-Associated Modules in Co-expression Network

The co-expression network contained 84,888 genes, housed within 81 modules (**Supplementary Data S6.6**). Mean module size was 1048, whilst median module size was 165. Module size ranged from 30 to 19,380 genes.

To identify modules associated with the drought response, GO enrichment analysis was conducted on each module, using all the genes included in network construction as background. Modules containing genes involved in regulating the drought response were expected to be enriched in stress-associated GO terms such as “response to water” (GO:0009414), “response to stress” (GO:0006950), or “response to abiotic stimulus” (GO:0009628). 10 of the 81 modules were significantly enriched in such GO terms (**Table 6.1**), with the black and turquoise modules being enriched in the GO term “response to water” (FDR = 4.8e-08 and 0.029, respectively).

To gain further insight into which modules may contain genes particularly associated with the drought response, DEG enrichment analysis was conducted. 10,199 genes (12% of the genes included in the co-expression network) were deemed to be DEGs. If the number of DEGs was distributed across modules according to size, it would be expected that 12% of the genes in each module would be DEGs. 17 modules contained a significantly higher proportion of DEGs than expected (**Table 6.2**), and so represent groups of co-expressed genes involved in the drought response – the hub genes of these modules, therefore, are promising candidates for master-regulators of the transcriptional drought response.

Combined, these analyses identified modules which were particularly stress-associated, either as a result of the enrichment of stress-associated GO terms, or the enrichment of DEGs. Only hub genes from those modules listed in **Tables 6.1** and **6.2**, therefore, were examined further to determine whether they may be promising candidate master-regulators of the transcriptional early drought response.

6.3.5. Hub Gene Identification

The hub genes within those modules deemed to stress-associated (**Tables 6.1** and **6.2**) may act as master-regulators of the transcriptional drought response, as they are significantly co-expressed with many stress-associated and/or drought-responsive genes. These hub genes (**Table 6.3**) seemingly play roles in diverse processes, such as stress hormone signalling (*TraesCS6A02G340100* and *TraesCS4D02G325200*) or the biotic stress response (*TraesCS5A02G052600* and *TraesCSU02G171500*). One hub gene, meanwhile, was found to be drought-responsive in the present work, but is likely a key actor in photosynthesis, and so is probably required to aid growth and development under normal conditions (*TraesCS6D02G247400*), whereas others were completely uncharacterized and do not share sequence identity with any well understood gene (*TraesCS3D02G361500, TraesCS4D02G251500, TraesCS4A02G212000, and TraesCS4A02G190700*), making their potential role as regulators of the drought response completely novel. Modules which were particularly large likely contained genes involved in diverse processes. Some of the largest modules were also significantly enriched in the “response to water” (GO:0009414) GO term, therefore to identify candidate master-regulators of processes of interest (namely, the drought response) subnetworks were created using genes annotated with this GO term as guide genes. This was done for the black and turquoise modules, with the subsequent subnetworks’ hub genes (*TraesCS5D02G379200* and *TraesCS6D02G234700*, respectively) being identified as dehydrins.

Hub genes in these stress-associated modules (**Table 6.3**) represent valuable targets for further inquiry into the regulation of the transcriptional drought response, and as targets for breeders in for the production of drought tolerant varieties. However, two of these hub genes,

TraesCS5D02G379200 (TaDHN4-D1) and *TraesCS3D02G361500* (uncharacterised gene), were deemed to be particularly promising candidate master-regulators of both the transcriptional and physiological drought responses, due to the likely functions of the genes they were connected to in the co-expression network. *TraesCS5D02G379200* may regulate the expression of a suite of fellow dehydrins, as well as stress-responsive transcription factors and genes which may affect stomatal dynamics – all of which show significant up-regulation of expression under drought stress. *TraesCS3D02G361500* may also regulate the expression of genes likely involved in controlling stomatal dynamics, as well as other potentially guard cell-localized genes involved in stomatal morphogenesis, and several aquaporins – however, unlike *TraesCS5D02G379200*, the hub, and the genes it is connected to, are downregulated significantly under drought stress.

Table 6.1: 10 modules were significantly enriched in GO terms related to the stress response, according to GO enrichment analysis by the AgriGO v2.0 Singular Enrichment Analysis tool (Du et al., 2010; Tian et al., 2017). The modules enriched in such GO terms are listed, as well as the most significantly-enriched GO term, and the stress-associated GO term they were also enriched in, respectively. In the instances where stress-associated GO terms were the most significantly enriched term in a module, only that term is given. The FDR-adjusted Fisher exact test *p*-values associated with each enriched GO term are given in brackets.

Module	Enriched GO Term
Black	Response to Water (4.8E-08)
Blue	Protein Phosphorylation (1.8E-110) Response to Stress (3.5E-06)
Cyan	Organonitrogen Compound Biosynthetic Process (5.3E-45) Response to Heat (6.2E-05)
Darkolivegreen	Protein Phosphorylation (6.3E-09) Response to Oxidative Stress (0.0023)
Magenta	Regulation of Multi-organism Process (8.7e-07) Regulation of Response to Stress (8.7e-07)
Midnightblue	Regulation of Primary Metabolic Process (0.046) Trehalose Biosynthetic Process (0.046)
Purple	Cellular Response to Stress (1.4e-06)
Salmon	Carbohydrate Metabolic Process (5.9E-10) Response to Oxidative Stress (0.031)
Tan	Phenylpropanoid Metabolic Process (1.8E-06) Response to Oxidative Stress (0.005)
Turquoise	Cellular Localization (1.5E-55) Response to Water (0.029)

Table 6.2: 17 modules were significantly enriched in DEGs, These modules contained a significantly higher proportion of DEGs than expected (12%) should the total number have been distributed across modules according to their size. These modules are listed, as well as the number of genes in each module, the proportion of these genes which were observed to be DEGs, the *p*-value result from the one-proportion Z-test, and the mean log2-fold change values of the DEGs within each module.

Module	**Number of Genes**	**Observed Percentage of DEGs**	***p*-value**	***Mean log2-Fold Change of DEGs***
Bisque4	111	23	3.23E-04	2.99
Black	2184	21	2.22E-38	3.41
Brown	3396	69	0	-2.58
Darkolivegreen	312	49	1.91E-90	-2.62
Greenyellow	1516	20	5.37E-22	3.7
Ivory	136	71	1.91E-98	-2.76
Lightsteelblue1	163	24	1.40E-06	3.66
Mediumpurple3	165	21	0.0001	2.34
Orangered4	174	40	1.06E-30	2.87
Plum2	105	22	0.0009	2.89
Skyblue	624	27	1.54E-29	-2.85
Steelblue	512	19	3.31E-07	-2.97
Turquoise	19380	15	2.18E-32	3.1
Yellow	2709	25	6.42E-96	2.55
Darkviolet	41	22	0.025	-2.39
Grey60	1024	14	0.027	-2.72
Salmon	1433	15	9.10E-05	3.23

Table 6.3: Hub genes identified in stress-associated modules may be strong candidates for master-regulators of the drought response, based on their high number of connections to other genes within stress-associated modules. Each hub gene's module membership and log2FC are given, as well as their identity and putative function.

Hub Gene	Module	Log2FC	BLAST Hit	Putative Function	Reference
TraesCS4D02G251500	Bisque4	1.99	*Aegilops tauschii subsp. strangulata B3 domain-containing protein Os03g0212300*	Uncharacterized	
TraesCS5D02G379200	Black	5.87	*TaDHN4-D1*	Drought tolerance and drought response	(Hao et al., 2022)
TraesCS5D02G194500	Blue	2.04	*Aegilops tauschii subsp. strangulata senescence-induced receptor-like serine/threonine-protein kinase*	Senescence	(Shin et al., 2019)
TraesCS6D02G247400	Brown	-2.26	*T. aestivum phosphoribulokinase, chloroplastic-like*	Calvin Cycle, Response to salt stress	(Xu et al., 2016; Yu et al., 2020a)
TraesCS5A02G087200	Cyan	-1.64	*Triticum aestivum psbP domain-containing protein 1, chloroplastic-like*	Photosystem I assembly factor	(Liu et al., 2012a)
TraesCS5A02G052600	Darkolivegreen	-3.28	*Triticum aestivum probable glucan 1,3-beta-glucosidase A*	Response to fungal pathogen	(Münch-Garthoff et al., 1997)
TraesCS2D02G127000	Darkviolet	-2.02	*Triticum aestivum quinone-oxidoreductase QR2-like*	Protection against oxidative stress	(Greenshields et al., 2005)
TraesCS4A02G212000	Greenyellow	5.23	*Triticum aestivum uncharacterized LOC123082151*	Uncharacterized	
TraesCS7A02G034500	Grey60	-3.76	*TaGSTU6*	Cold tolerance	(Lv et al., 2022)
TraesCS3D02G361500	Ivory	-3.75	*T. aestivum uncharacterized LOC123079795*	Uncharacterized	
TraesCSU02G171500	Lightsteelblue1	2.97	*Triticum aestivum esterase PIR7B-like*	Biotic stress response	(Wäspi et al., 1998)
TraesCS2A02G129200	Magenta	1.67	*Triticum aestivum cytochrome b561 and DOMON domain-containing protein At5g47530-like*	Electron transport	(Asard et al., 2013)
TraesCS5A02G477300	Mediumpurple3	2.01	*Triticum aestivum zinc finger protein ZAT8-like*	Regulation of programmed cell death	(Feng et al., 2023)
TraesCS3D02G144500	Midnightblue	3.3	*Triticum aestivum protein RICE FLOWERING LOCUS T 1-like*	Flowering activator	(Komiya et al., 2008; Komiya, Yokoi and Shimamoto, 2009; Ogiso-Tanaka et al., 2013)

TraesCS6A02G340100	Orangered4	2.23	*Triticum urartu ethylene-responsive transcription factor ERF018-like*	Regulation of ethylene and ABA signalling	(Chen et al., 2016)
TraesCS7D02G220700	Plum2	2.45	*Triticum aestivum probable serine/threonine-protein kinase PBL7*	Regulation of brassinosteroid signalling	(Nolan, Chen and Yin, 2017)
TraesCS4A02G462000	Purple	1.5	*Triticum aestivum noroxomaritidine synthase 2-like*	Noroxomaritidine synthesis	(Singh and Desgagné-Penix, 2017)
TraesCS2D02G224200	Salmon	10.36	*Triticum aestivum isocitrate lyase*	Glucnoegenesis, Salt tolerance	(Runquist and Kruger, 1999; Yuenyong et al., 2019)
TraesCS1A02G314800	Skyblue	-2.73	*Triticum aestivum high molecular mass early light-inducible protein HV58, chloroplastic-like*	Cold tolerance	(Lee et al., 2020)
TraesCS4A02G190700	Steelblue	-1.84	*Triticum aestivum uncharacterized LOC123082090*	Uncharacterized	
TraesCS2D02G518200	Tan	1.74	*Triticum aestivum tryptophan decarboxylase 1-like*	Serotonin biosynthesis	(Kang et al., 2009)
TraesCS6D02G234700	Turquoise	2.43	*Triticum aestivum dehydrin COR410-like (COR410)*	Cold tolerance	(Danyluk et al., 1994, 1998)
TraesCS4D02G325200	Yellow	1.65	*A. tauschii subsp. strangulata serine/threonine-protein kinase BSK1-2*	Regulation of brassinosteroid signalling	(Nolan, Chen and Yin, 2017)

6.4. Discussion

6.4.1. Utilizing Landraces to Future-proof Wheat Crops

It is widely believed that landraces are an important genetic resource available to breeders for the production of more climate-resilient wheat varieties, thanks to their extensive phenotypic and genetic diversity (Zeven, 1998; Reynolds, Dreccer and Trethowan, 2007; Corrado and Rao, 2017; Schmidt et al., 2019; Cseh et al., 2021; Tehseen et al., 2022). This diversity has been extensively exploited in grass crops such as rice and barley, with many landrace accessions either being screened for drought tolerance (Van Oosterom, Ceccarelli and Peacock, 1993; Tardy, Créach and Havaux, 1998; Munasinghe et al., 2017; Dbira et al., 2018; Kumar et al., 2019a; Mishra, Behera and Panda, 2019; Boudiar et al., 2020; Sabouri et al., 2022; Bakhshi and Shahmoradi, 2023), utilized to identify the genetic determinants of drought tolerance (Yu et al., 2012; Fan et al., 2015; Reinert et al., 2016; Hoang et al., 2019; Beena et al., 2021), or used to better understand the drought response (Cantalapiedra et al., 2017; Khodaeiaminjan et al., 2023). Wheat landraces, however, remain relatively underutilized in the study of drought tolerance and the drought response (Dodig et al., 2012; Lin et al., 2019; Naderi et al., 2020; Gómez-Espejo et al., 2022). After highlighting both its extensive genetic diversity, and its usefulness in the study of early thermotolerance (**Chapter 2**) and the transcriptional response to early heat stress (**Chapter 3**), this work shows that the YoGI landrace panel can also be used to effectively study the response to early drought stress, and aid the production of drought tolerant wheat varieties.

The effect of drought stress on yield is well studied (Zhang et al., 2018; Kim, Iizumi and Nishimori, 2019; Qaseem, Qureshi and Shaheen, 2019; Senapati et al., 2019; Abou-Elwafa and Shehzad, 2021; Lan et al., 2022; Wan et al., 2022), but as the climate continues to change, periods of water shortage coinciding with the early growth stages of spring wheat crops are likely to become more common around the world. There has already been evidence of this, with major spring wheat-producing countries such as the USA and the UK experiencing drier than average periods in the months after spring wheat sowing (NOAA National Centers for Environmental Information, 2022c, 2022d). The majority of the work examining the effect of drought stress on wheat seedling growth has not aimed to identify regulators of the drought response during this early stage of development, however (Guo et al., 2017; Sallam et al., 2018; Ahmed et al., 2020, 2022; Mahpara et al., 2022; Nardino et al., 2022; Sharma et al., 2022) – something that remains relatively understudied (Ajigboye et al., 2017; Vuković et al., 2022; Mao et al., 2020). The present work, therefore, takes a novel approach to elucidate how the early drought response is transcriptionally controlled in wheat landraces, and represents a promising step towards the production of more drought tolerant varieties

6.4.2. Drought Stress Causes Substantial Changes in the Wheat Transcriptome

This analysis demonstrates that the expression profiles of spring wheat are vastly different before and after drought; over 10,000 genes were differentially expressed between the two groups. GO term enrichment analysis of DEGs indicated that growth and development was deprioritised; DEGs annotated with photosynthesis-, and chlorophyll-related GO terms were largely downregulated. Similarly, there was widespread downregulation of genes annotated with enriched Cellular Component GO terms such as “thylakoid”, suggesting a reduction in light-dependent reactions. Photosynthetic regulation is associated with both oxidative and drought stress responses; stress-related changes in photosynthetic activity under various environmental stress conditions have been identified in other cereals such as rice (Yu et al., 2020b; Gan et al., 2019), as plants seek to limit damage to critical components. Downregulation of genes involved in photosynthesis under drought is common among grasses, with studies in *Miscanthus* (De Vega et al., 2021), *Brachypodium distachyon* (Priest et al., 2014), and rice (Liang et al., 2021) demonstrating similar trends.

Reduced photosynthetic activity can result in an excess of absorbed light energy, inducing the generation of toxic reactive oxygen species (ROS; Pospíšil, 2016). GO enrichment analyses conducted on both up- and downregulated DEGs identified a number of enriched GO terms (such as “response to oxidative stress”) involved in both the production and mitigation of ROS and other oxidative agents. Both up- and down-regulation of genes involved in cellular oxidation and reduction has previously been observed in other grasses, like rice (Sirohi et al., 2020). ROS accumulation, while promoting immune responses and stomatal guard cell closure (Song, Miao and Song, 2014), can also cause oxidative damage to DNA and photosynthetic machinery, potentially leading to cell death (Huang et al., 2019; Ye et al., 2021). DEGs annotated with such terms were primarily identified as peroxidases and oxidases; their presence among both up- and downregulated DEGs is likely due to their cellular localisation, mediating ROS accumulation in some tissues over others (Csiszár et al., 2012).

GO enrichment of the upregulated genes identified a number of DEGs annotated with drought- and osmotic-stress enriched GO terms. These genes included a variety of dehydrins and other late embryogenesis abundant (LEA) genes, known key actors in various abiotic stress responses in wheat (Kosová, Vítámvás and Prášil, 2014; Hassan et al., 2015; Liu et al., 2019). Studies in species such as *B. distachyon* and *O. sativa* were similarly able to identify an upregulation of dehydrins (Sancho et al., 2022; Smita et al., 2013), suggesting that this is a common response among grasses.

Downregulation of genes under the term “transport”, which included genes involved in water transport processes, likely facilitated the conservation of water for critical organelles and guard cells, as well as mediating water loss by decreasing membrane permeability (Maurel et al., 2008; Patel and Mishra, 2021).

These trends in the expression of stress and growth-associated genes indicate a shift towards stress-mitigation, often seen with abiotic stresses such as harsh drought (Zhang, Zhao and Zhu, 2020).

6.4.3. *TaDHN4* May Regulate the Expression of Dehydrins and Drought Tolerance Genes under Drought Stress

The black module was significantly enriched in DEGs (**Table 6.2**), as well as the GO term “response to water” (FDR-adjusted *p*-value = 4.8E-08, **Table 6.1**), suggesting the module houses genes which play key roles in the drought response. Due to the size of the module (2184 genes), it is likely to contain genes involved in various processes besides the drought response. To focus on those genes most likely to play a role in the drought response, a subnetwork was created using the genes within the module which possessed the significantly enriched GO term “response to water” as guide genes. The resulting subnetwork contained 1544 genes, and 6562 connections between genes (**Figure 6.2a**).

The central hub gene was *TraesCS5D02G379200*, which possessed the enriched GO term “response to water” and was connected to 1222 other genes in both the full module (where it had the sixth highest degree score) and the subnetwork. The gene shares 100% sequence identity with *Aegilops tauschii subsp. strangulata dehydrin DHN2*, but has been classed as *TaDHN4-D1* in recent work (Hao et al., 2022). Expression of the gene was upregulated significantly (log2FC = 5.87) after drought stress (**Figure 6.2b**); consistent with the commonly observed expression responses of dehydrins in response to drought stress (Tiwari and Chakrabarty, 2021; Sun et al., 2021). *TaDHN4* belongs to the YSK_2 sub-family of dehydrins (Wang et al., 2014), a sub-family shown to increase stress tolerance when overexpressed in *Arabidopsis* (Brini et al., 2007) and whose expression, consistent with the present work, was most strongly upregulated in dehydrated leaves of wheat seedlings (Wang et al., 2014). Four of the five most well-connected genes in the subnetwork were homeologues, or duplicates, of *TaDHN4*; *TraesCS5D02G379200* (hub, *TaDHN4-D1), TraesCS5B02G372100* (*TaDHN4-B1*), *TraesCS5B02G372200* (*TaDHN4-B2*) and *TraesCS5A02G369900* (*TaDHN4-A2*) – suggesting both that all homeologues share similar expression responses, and that there is likely functional redundancy amongst the homeologues, meaning they may all play roles in regulating the drought response.

Further support for the hypothesis that the hub gene may act as a master-regulator of the drought response comes from the genes it is connected to in the subnetwork. The hub was connected to 220 DEGs in the subnetwork, 62.3% of all DEGs within it. Amongst these DEGs were several other members of the dehydrin family, besides the hub’s homeologues and duplicates to which it was also connected: *TraesCS5B02G426800* (log2FC = 9.89) encodes *T. aestivum dehydrin Rab15-like*, whilst *TraesCS6A02G350600* (log2FC = 8.39) is *T. aestivum dehydrin DHN3-like*. The hub gene is also connected to other genes with different functions related to the drought response: *TraesCS2D02G364500* (log2FC = 8.83), and its homeologue *TraesCS2A02G367700*, are *T. aestivum chromosome D caleosin (Clo10)* – a member of another drought-responsive gene family thought to be involved in the drought response, via action on stomatal aperture and transpiration (Aubert et al., 2010; Kim et al., 2011). The hub may also have far-reaching effects on global gene expression, due to its connection to drought-responsive transcription factors such as *TaNAC29*, *TraesCS2A02G367700* (log2FC = 7.32), which has been shown to increase drought and salinity tolerance when expressed in

Arabidopsis (Huang et al., 2015). The hub's connection to drought-responsive genes with these kinds of functions further suggests that it may act as a master-regulator of the drought response.

Dehydrins act as molecular chaperones to maintain protein structure and functional folding under stressful cellular conditions, so the hub gene's ability to regulate gene expression may not be immediately apparent. Recent evidence, however, suggests that there are multiple potential mechanisms by which dehydrins can control the expression of other genes. This can occur as a result of their chaperone activity, protecting transcription factors and other transcriptional regulators from damage by cellular stress, ensuring their function and subsequent effect on gene expression is maintained (Tiwari and Chakrabarty, 2021). There is also emerging evidence that dehydrins themselves may act as transcription factors, with rice lines overexpressing *OsDhn-Rab16D* showing increased expression of ABA signalling and stress-responsive genes (Tiwari et al., 2019). Dehydrins may also effect gene expression by binding directly to DNA and protecting it from damage by ROS during stress events; this is not a commonly-observed role played by dehydrins, however, only being reported in grape and citrus (Hara et al., 2009; Boddington and Graether, 2019). Each of these roles would rely on the hub gene protein being localized in the nucleus, but, according to recent work, the hub gene appears to be localized to the cytoplasm (Hao et al., 2022). This chapter has seen evidence that the hub gene dehydrin may act to control the expression of other drought-responsive dehydrins, as well as several other stress-responsive genes which seemingly play roles in the drought response, suggesting either the hub gene may in fact be localized to the nucleus under drought stress, or that the protection it provides transcriptional regulators in the cytoplasm is sufficient to allow them to act functionally once translocated to the nucleus.

6.4.4. Uncharacterized Hub Gene Potentially Controls Stomatal Dynamics, Water Movement and Stress Hormone Signalling under Drought Stress

The ivory module (**Figure 6.2c**) was identified as drought-associated, as it was significantly enriched in DEGs (**Table 6.2**). The most well-connected gene in the module was *TraesCS3D02G361500*, with its homeologues (*TraesCS3A02G368600* and *TraesCS3B02G400100*) also amongst the top five most well-connected genes in the module. Expression of the hub gene, *T. aestivum uncharacterized LOC123079795*, was found to be downregulated under drought stress (log2FC = -3.75, **Figure 6.2d**), suggesting the gene may play a repressive role during the transcriptional and physiological drought responses.

35 of the 41 genes the hub was connected to were also DEGs, all of which were downregulated under drought stress, with several having functions related to the drought response. *TraesCS1A02G070200* (log2FC = -4.79) is *T. aestivum jasmonate-induced oxygenase 1-like*, and also shared some sequence identity (69%) to a large region of its *Arabidopsis* namesake, and orthologue (identified using Ensembl Plants; Yates et al., 2022), *AtJOX1*. The gene is a negative regulator of jasmonic acid (JA) signalling, conducting hydroxylation of JA, inactivating it in the signalling pathway (Caarls et al., 2017). JA is known to accumulate in plant cells during drought stress and increase tolerance to drought stress in wheat (Wasternack, 2014; Ali and Baek, 2020; Wang et al., 2021c). JA has also been shown to act in unison with ABA to control stomatal closure in *Arabidopsis* (Hossain et al., 2011), suggesting the hub gene may be able to determine stomatal aperture via control over *TraesCS1A02G070200* expression, and subsequently, JA signalling.

The hub gene is also connected to several other DEGs potentially involved in regulating stomatal opening. *AtAO1* plays a role in programmed cell death via its production of reactive oxygen species, as well as a role in protoxylem differentiation in root tissue (Møller and McPherson, 1998; Ghuge et al., 2015a, 2015b), and is the *Arabidopsis* orthologue of

TraesCS4B02G282700 (log2FC = -4.61) which encodes *T. aestivum primary amine oxidase 1-like*. As well as this, *AtAO1* expression was found to be both induced by methyl-jasmonate, and localized in guard cells, and other tissues involved in regulating water homeostasis – leading the authors to suggest that the gene may play a key role in regulating stomatal closure (Ghuge et al., 2015b). Previous work suggests *AtAO1* promotes stomatal closure, however here *TraesCS4B02G282700* expression is downregulated under drought stress, suggesting it may act to repress stomatal closure in wheat. *TraesCS4A02G398700* (log2FC = -4.2) was also connected to the hub gene, and similarly may play role in stomatal dynamics. The gene is *T. aestivum GDSL esterase/lipase APG-like*, whilst also sharing sequence identity (66%) with large regions of *AtGGL19*, a gene found to be expressed in *Arabidopsis* guard, pavement and mesophyll cells, whose expression was also downregulated under drought stress, suggesting the gene may play a role in stomatal closure (Xiao et al., 2021). These observations, paired with the downregulation of *TraesCS4A02G398700* under drought stress, suggest the gene may act to repress stomatal closure. *TraesCS1B02G176000* was another downregulated DEG (log2FC = -3.96) connected to the hub gene, and encodes *T. aestivum cytokinin dehydrogenase 3-like*. The gene appears to also be involved in stomatal biology, as a result of its inactivation of cytokinins. However, overexpression of *TraesCS1B02G176000*'s *Arabidopsis* namesake, *AtCKX3*, improved drought tolerance in tomato and *Arabidopsis* thanks to reduced transpiration, likely from reduced leaf area and stomatal density (Werner et al., 2010; Farber, Attia and Weiss, 2016). The downregulation of *TraesCS1B02G176000* in the present work, however, suggests it may act to increase water loss, unlike its *Arabidopsis* namesake. Despite the gene's name, *TraesCS1B02G176000* showed the highest level of sequence identity to *AtCKX6* – a guard cell-localized gene with a potential role in stomatal morphogenesis (Werner et al., 2003). Because of this, and its downregulation under drought stress in the present work, *TraesCS1B02G176000* may play a positive role in stomatal morphogenesis, as reducing the production of stomata under drought stress is likely to limit the amount of water loss via transpiration (Bertolino, Caine and Gray, 2019).

Two genes involved in water transport were also connected to the hub. *TraesCS4D02G024400*, *T. aestivum protein NRT1/ PTR FAMILY 8.3-like*, was downregulated under drought stress (log2FC = -3.34) and shares sequence identity (63%) with a large region of its namesake, *AtNPF8.3*. The gene appears to play a role in water uptake in germinating *Arabidopsis* seeds, as knockout mutant seeds showed a 17% lower water content compared to WT (Choi et al., 2020). *TraesCS4B02G310900* (log2FC = -1.74) also appears to be involved in water transport, as it is *T. aestivum aquaporin TIP1-1-like*, but shares marginally more sequence identity with *AtTIP2* (73%) than *AtTIP1* (72%). The downregulation of these genes under drought stress in the present work, paired with their membership of a module containing so many potential guard cell-localized genes, suggests that these genes may act to control guard cell turgidity, via their control of water movement in and out of the cells. When guard cells are turgid, stomata are open, whilst flaccid guard cells cause stomata to close – suggesting that the downregulation of these water uptake genes in response to drought stress may be a mechanism to cause stomatal closure, and prevent excess moisture loss under water shortage. Recent work has shed light on the relationship between water uptake proteins, such as aquaporins, and stomatal dynamics (Grondin et al., 2015; Ding and Chaumont, 2020b, 2020a; Cui et al., 2021), suggesting the hub may act to reduce water loss via its downregulation of these water uptake genes under drought stress.

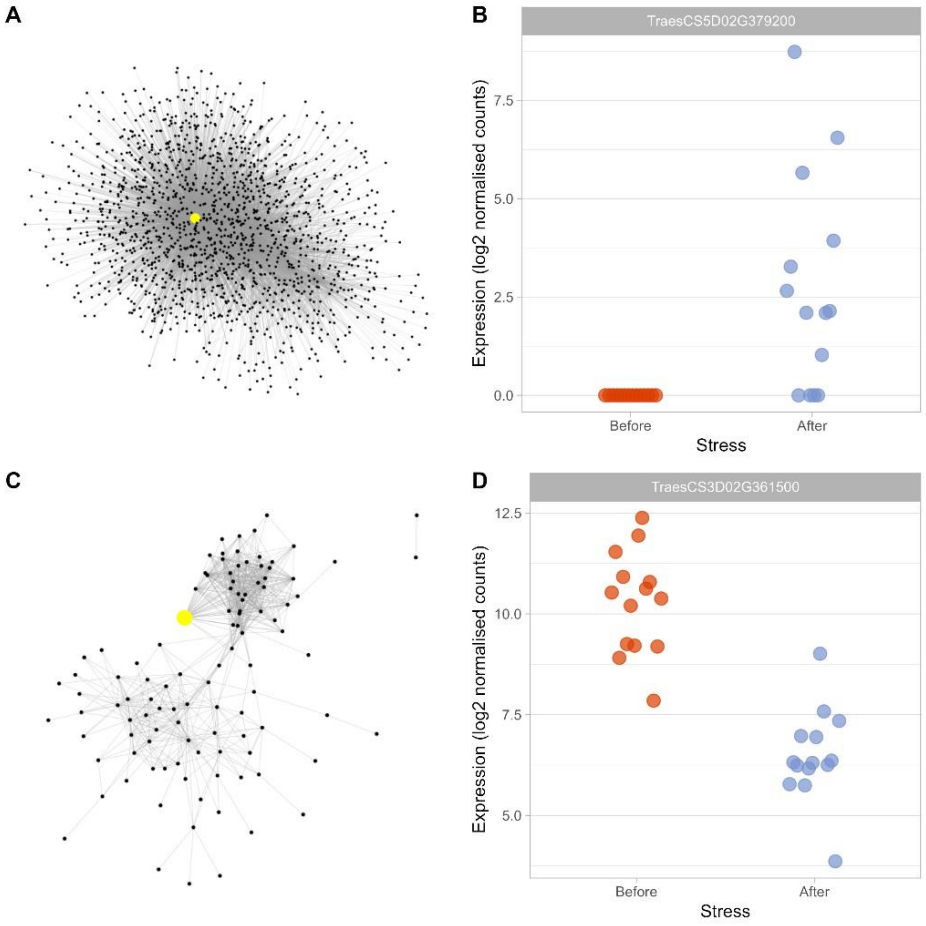

Figure 6.2: Drought-associated modules house candidate master-regulators of the early drought response. To focus on genes likely involved in the drought response within the large black module, a subnetwork was created (**A**), whereas the ivory module (**C**) was small enough to be analysed in its entirety. The hub genes within the black subnetwork (*TraesCS5D02G379200*) and ivory module (*TraesCS3D02G361500*) are highlighted in yellow and enlarged. Expression of *TraesCS5D02G379200* **(B)** was found to be significantly upregulated (log2FC = 5.87) in response to drought stress, whereas *TraesCS3D02G361500* **(D)** expression was significantly downregulated (log2FC = -3.75).

6.5. Conclusions

This chapter presents the YoGI landrace panel as a valuable resource for the study of the transcriptional control of the drought response, and useful tool for breeders in the development of climate-resilient wheat varieties. Thousands of genes, differentially expressed before and after exposure to drought stress during early development, were identified. The use of co-expression network analysis permitted the identification of several hub genes which may act as master-regulators of the transcriptional response to early drought stress. Two very promising candidate hub genes, however, may act to coordinate both the transcriptional and physiological early drought responses, as they potentially control the drought-responsive expression of stress-associated genes such as dehydrins, aquaporins and genes involved in stomatal dynamics. Further work is required, however, to make the link between the potential action of these hub genes on drought-responsive gene expression, and the physiological drought response.

7. Discussion

7.1. Summary

The work presented in this thesis is fundamentally an exploratory look into the physiological and transcriptomic responses of hexaploid wheat to heat and drought stresses, utilizing the vast genetic and phenotypic diversity of a new wheat landrace panel, and open-source gene expression data to do so. The present work introduces the YoGI landrace panel, and its accompanying transcriptomic data under control conditions, as well as transcriptome data from a select number of landrace accessions exposed to heat and drought stresses during early development – key community resources for both wheat breeders and researchers. The often underutilized network approach was applied to these data to identify novel candidate master-regulators of the responses to these stresses, and of basal thermotolerance, whilst this approach was also employed to make use of open-source gene expression data and identify candidate master-regulators of the response to drought stress during the latter stages of development, which, when mutated, may lead to alterations in growth/survivability under drought stress. These works not only contribute towards the community's understanding of how these responses and traits may be regulated transcriptionally, but this thesis also provides an abundance of targets for wheat breeders to use during marker-assisted breeding for the production of more stress-tolerant HYVs. Similarly, characterization of the phenotypes and gene expression responses of a selection of the landrace accessions in the YoGI panel also identifies favourable accessions which these breeders could include in such breeding programmes – capturing the, as yet, relatively untapped genetic and phenotypic diversity of wheat landraces. Not only has this thesis characterized the stress tolerance and stress-responsive gene expression of some of these landrace accessions, but it has also identified a novel flowering response to early heat stress exposure in a selection of these landrace accessions – a delay of flower emergence after exposure to early heat stress, never before described in wheat. This thesis also proposes that this novel phenotypic response may be a consequence of the downregulation of several genes, putatively involved in promoting flowering and phytohormone signalling, after heat stress exposure during early vegetative development.

7.2. General Reflections

This thesis has repeatedly made reference to stress tolerance, and basal thermotolerance in particular. The measure used to define stress tolerance in the present work was normalized difference in biomass production (dry weight) between plants under stressed conditions, and plants grown under control conditions. It can be argued, therefore, that the work in this thesis, instead of measuring stress tolerance, measured growth and survivability under stress – important, albeit different, traits to stress tolerance. In the extant literature, stress tolerance is often defined differently between publications, with Tiwari, Khungar and Grover (2020) defining thermotolerance via a binary survival measure (dead or alive), whilst Zang et al., (2017) defined thermotolerance using membrane stability and PSII efficiency measures. Such variation in the definitions of stress tolerance used throughout the literature reflects how multi-factored this "trait" is, with multiple tolerance mechanisms (often those traits used as measures of stress tolerance, such as those described above) all contributing to an accession's ability to tolerate environmental stress. Although the present work was explicit about its definition of stress tolerance, perhaps it would be more accurate to say that those hub genes identified as candidate master-regulators of basal stress tolerance (such as those identified in **Chapter 2**) are actually candidate master-regulators of growth and survivability under stress, whose basal expression level pre-disposes accessions to either grow well, or poorly under periods of environmental stress.

Similarly, although this thesis identified a novel response related to flower emergence in **Chapter 4**, this is different to the identification of a novel flowering time response. Flowering itself can be argued to have begun long before the visible emergence of the flower on wheat heads, namely at the point of floral induction, whereby meristem identity changes from vegetative to floral – marking the point where reproductive cells and tissues begin to be formed. Therefore, the effect of early heat stress exposure on flowering time is not truly determined in **Chapter 4**. To gain an understanding of how this stress treatment affects this transition, and, therefore, how it truly affects flowering time, meristem dissections are required at regular intervals to determine when this transition takes place under the control and stressed treatments used in **Chapter 4**. These insights would allow conclusions to be made about whether this stress treatment does delay flowering, and whether this delay comes as a result of a prolongation of vegetative development, as expected. The insights from the proposed experiments would also provide a more convincing account of the effect of exposure to, and then removal of, early heat stress than those provided in the present work – perhaps leading to more engagement with academic colleagues, and increased cut-through with wheat breeders and farmers. It was hoped that this experiment could be completed as part of the present work, however time constraints meant this was not possible.

The present work has generated extensive amounts of new data, particularly gene expression data, from which certain insights have been drawn – however, these data have not been exhaustively utilized by any means, largely due to time restrictions. Due to the amount of data generated in the present work, this thesis represents a key resource for other researchers, so they can further utilize these data and use them to answer their own questions, draw their own insights, and form their own conclusions. The data generated in the present work are publicly available, and thus are ready to be mined further by colleagues. The gene expression data of the whole YoGI landrace panel, and the subsequently formed co-expression network (**Chapter 2),** are perhaps the most widely useful for researchers, as these gene expression data are gathered from plants grown under control conditions, meaning a variety of traits can be studied – ranging from basal stress survivability, to plant height, or photosynthetic efficiency. These data could have been utilized more extensively in the present work, as although **Chapter 2** focussed solely on basal survivability to early heat stress, modules in the co-expression network associated with basal survivability under drought stress could have been identified, before candidate hub genes within these modules were tested as predictive markers in the same way as they were in **Chapter 2**. This would have allowed a more comprehensive understanding of wheat's response to drought stress, and survivability under drought stress, to be gained and discussed in this thesis – perhaps, therefore, this should be the next step for colleagues looking to build on the findings of this thesis.

Despite generating such a vast wealth of gene expression data, the present work was not able to provide the same level of information on the phenotypes of the entire YoGI landrace panel. A full-panel screen of growth and survivability under drought stress was conducted, however the results are not discussed or presented in the present work. Due to the size of the panel, completion of such a screen was incredibly time-consuming (taking over 6 months) and laborious, especially when the work had to be completed alone due to a lack of available support, and COVID-19 restrictions. Repeating such screens would mean, however, that trait information could be used to gain a deeper understanding of the YoGI landrace panel, and perhaps provide a "profile" of each accession – characterizing things such as inherent survivability under stresses, developmental speed, and biomass production. Collection of such trait data could also further maximise the value of the gene expression data and co-expression network in **Chapter 2**, as phenotype data for any trait of interest (or multiple traits of interest) can be fed into the network construction, before modules particularly associated with the observed trait variation are identified – perhaps allowing more accurate and direct

identification of candidate hub genes which may act to transcriptionally coordinate certain traits. The lack of such screens is a major failing of the present work, however were not able to be conducted due to the reasons described above – perhaps, therefore, colleagues with more time and resources will be able to conduct such screens, building knowledge of the YoGI landrace accessions, and further utilizing the data generated in **Chapter 2**.

It is also worth noting that the failed attempt to backcross TILLING mutant lines in **Chapter 5** limited the usefulness of the experiments conducted to validate the identified candidate hub genes, and led to less robust conclusions about the putative functions of these genes in wheat. The backcrossing protocol used here likely failed due to limited expertise and practical experience within the research group, and a lack of skills required to generate a sufficient number of backcrossed offspring containing the mutant allele necessary to ensure that, after selfing, some offspring would still possess it. Not only this, but despite the use of "speed-breeding" growth conditions (as detailed in **Chapter 5**), the backcrossing programme still took several months to complete, meaning time constraints were an ever-present issue. This was compounded by the fact that TILLING seeds took almost one year to arrive after being ordered – hindering any chance that this work had of being completed on time. Although the work done in **Chapter 5** was not as complete as intended, the hub genes identified here are the closest of all those identified in this thesis to having their function, at least partially, validated, due to the research group's possession of TILLING mutant seed, and results of the non-backcrossed drought tolerance/survivability screen which can guide future work (detailed in **Chapter 5**). Non-backcrossed TILLING mutant seeds for the lines used in **Chapter 5** are available for use, should colleagues desire to repeat this backcrossing programme, with the most promising target (perhaps of all those identified in this thesis) being *TraesCS4D01G050400* as, as well as the reasons listed above, the hub gene is an orthologue of a repressor of the drought response in Arabidopsis that is yet to be fully characterized in wheat.

7.3. Hexaploid Wheat Yields must Increase to Support Population Growth, however Climate Change will likely threaten these Increases

Formation of hexaploid bread wheat (AABBDD) via hybridization of the tetraploid *Triticum turgidum* (AABB) and the diploid *Aegilops tauschii* (DD) is arguably one of the most important events in human history, as the resulting species went on to support the Neolithic Revolution in the 1st century CE, and now accounts for 20% of the protein and calories consumed by over 8 billion people (Petersen et al., 2006; International Wheat Genome Sequencing Consortium (IWGSC), 2014, 2018; Bowles and Choi, 2019; Ritchie and Roser, 2023). This level of reliance on a single crop species not only means it is cultivated all around the world, from 67° North to 45° South, but also that the lives and livelihoods of billions of people depend on its success in the field (United States Department of Agriculture - Foreign Agricultural Service, 2023; Levy and Feldman, 2022). However, as the global population continues to grow, being expected to reach 9 billion people by 2036 (Ritchie and Roser, 2023), the production of key crops, such as wheat, will also need to increase to meet the growing demand for food. Wheat is currently showing a steady rate of yield increase, ~0.9% per year, which will see yields rise by only 38% by the year 2050 – below the level expected to be required to support the projected increase in population growth (Hawkesford et al., 2013; Ray et al., 2013).

Achieving these necessary yield increases is likely to be complicated by climate change, however, as global warming is occurring at faster rate than ever before, resulting in average global temperatures reaching heights never before seen in the last 100,000 years (Calvin et al., 2023). Such drastic temperature rises are likely the result of excessive anthropogenic greenhouse gas emissions, with atmospheric CH_4 and N_2O reaching levels unprecedented in 800,000 years, whilst CO_2 levels are predicted to be higher than at any point in the last 2 million years (Calvin et al., 2023). This accumulation of greenhouse gases in the Earth's

atmosphere accentuates the greenhouse effect, absorbing thermal radiation from the sun and preventing its escape into space – thus, explaining the rapid global warming seen in recent decades. Periods of extreme weather are also likely to occur more frequently around the world in this changing climate, with heatwaves becoming more common in almost every global region since 1950, and periods of ecological and agricultural drought occurring more frequently in over 25% of these regions (Calvin et al., 2023). Not only are periods of extreme heat and drought occurring more frequently, but the continued rate of climate change may also lead to shifts in the conventionally-defined seasons, with rising global temperatures effectively causing spring to occur much earlier, relative to 1952 (Wang et al., 2021a). Periods of extreme heat and drought, therefore, are also likely to occur earlier in the year, with evidence of this being observed in recent times, as temperatures approaching record levels (in excess of 40°C in some cases) were observed in some states of the USA in May 2022, whilst April 2022 saw almost 50% of the United States experiencing moderate to exceptional drought (NOAA National Centers for Environmental Information, 2022d, 2022b).

Although the plasticity of the hexaploid wheat genome aided its colonization of, and cultivation in, many different environments shortly after its formation, such periods of extreme temperature and water limitation will likely cause damage to wheat yields around the world thanks to the genetic uniformity of modern hexaploid wheat varieties. Produced during, or descended from varieties produced during, the Green Revolution, modern high-yielding varieties (HYVs) subsequently show little genetic diversity, as they share a significant amount of recent common ancestry – meaning these varieties are largely adapted to grow under similar environmental conditions, and thus are susceptible to damage by similar environmental perturbations. Exposure to these stresses will therefore lead to yield losses, particularly if they occur during the susceptible reproductive stages of development; however, the physiological and molecular damage caused by heat and drought stress will also disturb growth at any point during development, likely also causing ramifications for yield.

7.4. Wheat Landraces are an Untapped Source of Novel Genetic Diversity

Landraces formed as wheat spread beyond the Middle East, accumulating mutations that conferred improved growth in its new environment when selected for naturally, or artificially by early farmers. These selection events occurred independently, many times over, in different regions of the world, resulting in distinct landrace varieties adapted to grow in a wide range of environmental conditions, and showing phenotypes which aid this adaptation (Charmet, 2011; Peng, Sun and Nevo, 2011; Lopes et al., 2015). Subsequently, landrace varieties show extensive levels of genetic diversity, and improved growth under sub-optimal growth conditions, compared to HYVs. It is thought, therefore, that landrace varieties may be a good source of novel genetic diversity that could be introduced into HYVs, via conventional breeding, in an attempt to improve their tolerance to heat and drought stresses. Similarly, understanding how basal tolerance, and the response, to these stresses is regulated in these landraces may allow for the production of HYVs better able to tolerate heat and drought stresses, as key players in the regulation of these processes and traits are identified.

One of the most widely-used approaches to identify such master-regulators is co-expression network analysis, however, despite its power, this approach has only been used a handful of times to identify candidate master-regulators of basal tolerance, and the response, to heat and drought stress in hexaploid wheat (Girousse et al., 2018; Lv et al., 2020; Mishra et al., 2021; Du et al., 2022; Tian et al., 2022). Further, despite their extensive genetic and phenotypic diversity, wheat gene expression data from wheat landraces have never been used to identify candidate master-regulators of these processes via co-expression network analysis. The present work, therefore, employed these underutilized resources in combination to identify candidate master-regulators of thermotolerance, and of the transcriptional response to heat

and drought stresses – providing a better understanding of how these processes are regulated, and identifying targets for wheat breeders to aid them in the production of heat and drought tolerant HYVs.

7.5. YoGI Landrace Panel can be used to Identify Novel Candidate Master-regulator of Basal Thermotolerance

This thesis firstly introduced the YoGI landrace diversity panel, containing varieties from different landrace collections, such as the A. E. Watkins and CIMMYT collections, that have adapted to grow in range of different environments, as varieties were selected to represent different countries across all global wheat mega-environments (Sonder, 2016). This thesis then described how gene expression data, under control conditions, were gathered for all 342 accessions within the YoGI panel, before identifying regions of genome where large structural rearrangements or deletions have occurred, and determining how the extent of such rearrangements varied across the panel. This thesis then outlined how these gene expression data were utilized to construct a weighted gene co-expression network via WGCNA. Within the co-expression network, several modules were deemed to contain genes which may determine an accession's degree of basal stress tolerance, with the hub genes within these modules therefore being good candidates for master-regulators of basal thermotolerance. One of these modules contained three hub genes (*TraesCS4D01G207500.1*, *TraesCS7B01G149200.1*, and *TraesCS7D01G241100.1*) which were deemed to be particularly promising candidates, due to their characterizations as HSPs. The expression of these three hub genes under control conditions was then determined to be significantly associated with an accession's degree of thermotolerance during early vegetative development, as tested by linear regression analysis. This was perhaps unsurprising for *TraesCS7B01G149200.1* and *TraesCS7D01G241100.1* (also known as *TaHSP90.2-B1* and *TaHSP90.2-D1*, respectively) as they shared remarkable sequence identity (99.9% and 97.4%, respectively) with *TaHSP90* – a gene previously identified as being highly expressed in the thermotolerant hexaploid wheat cultivar C306, as well as being shown to improve thermotolerance of *E. coli* when transgenically overexpressed (Vishwakarma et al., 2018). The fact that increased expression of *TraesCS4D01G207500.1* was associated with improved thermotolerance in wheat was more surprising, however, given that its Arabidopsis orthologue, *AtHSC70-1*, is a repressor of basal thermotolerance (Tiwari, Khungar and Grover, 2020). However, the co-expression network revealed that *TraesCS4D01G207500.1* may be able to confer improved thermotolerance thanks to seeming regulation over the expression of a suite of other HSPs (including *TraesCS7B01G149200.1* and *TraesCS7D01G241100.1*), as well as five heat-shock transcription factors – the master-regulators of the transcriptional response to heat stress.

This work, therefore, not only introduced a new landrace diversity panel, and accompanying transcriptome data (SRA: PRJNA912645), for use by the wheat research community as a key resource, but it also exemplified, for the first time, that gene expression data from hexaploid wheat landraces could be used to construct a co-expression network for the identification of candidate master-regulators of basal thermotolerance. These hub genes therefore serve as valuable targets for wheat breeders for the production of thermotolerant HYVs, whilst the gene expression data can be used to identify landrace accessions from the YoGI panel which could be included in breeding programs to introduce novel genetic diversity, conferring increased expression of these hub genes, into HYVs.

Further work employing the use of mutant lines with disrupted expression, or function, of these hub genes would allow the subsequent effect on phenotype to be seen – further validating these hub genes as determinants of basal thermotolerance – currently, however, no such mutants exist within the JIC TILLING mutant population for the hub genes identified in this

chapter. Similarly, RNA-sequencing, or targeted RT-qPCR of the genes connected to *TraesCS4D01G207500.1* in the co-expression network, in *TraesCS4D01G207500.1* mutants and wild-type plants could be conducted to confirm whether *TraesCS4D01G207500.1* does indeed act as a master-regulator of HSP and Hsf gene expression, as suggested by the co-expression network. It would be hypothesized that, if so, the expression of these genes would be reduced in *TraesCS4D01G207500.1* mutants, given the positive correlations seen in the present work between hub gene expression and the expression of the genes it is connected to in the co-expression network.

After determining whether the hub gene does appear to regulate the expression of the genes it is connected to in the co-expression network, further work will then be required to determine the molecular mechanism underlying this regulation. HSPs are unlikely to directly affect gene expression in the same way as a transcription factor, therefore the methods used to probe a potential regulatory mechanism will differ than those used conventionally. Because HSPs bind proteins in the cytoplasm, it may be possible that *TraesCS4D01G207500.1* binds to negative regulators of the genes it is connected to in the co-expression network, retaining them in the cytoplasm and inhibiting their translocation to the nucleus and subsequent repressive action. Alternatively, it may be possible that higher levels of *TraesCS4D01G207500.1* pre-dispose accessions to show increased survival under heat stress, because the protein binds to transcriptional activators in the cytoplasm – protecting them from the effects of heat stress, and enabling their translocation to the nucleus where they remain functional to act on the genes connected to the hub in the co-expression network. To test either of these hypotheses, co-immunoprecipitation experiments will be required to determine which proteins are bound by *TraesCS4D01G207500.1*, and subsequently how this may affect the expression of other genes. Determining whether *TraesCS4D01G207500.1* binds to transcriptional activators or repressors will likely hold the key to testing these hypotheses. Similarly, it may also be beneficial to confirm the cellular localization of the hub gene via Western blot, or proteomic sequencing of nuclear and cytoplasmic proteins, to determine whether the hub gene protein is itself unexpectedly translocated to the nucleus – the results of which will help form a hypothesized mechanism of regulation.

7.6. Transcriptomic and Co-expression Network Analyses Revealed Large Shifts in the Wheat Landrace Seedling Transcriptome in Response to Heat Stress, and Identified Candidate Master-regulators of the Response

A similar approach was then taken to build on the findings of the previous chapter, as this thesis then described how transcriptome data from 13 accessions (from the 15 used previously to validate the identified thermotolerance hub genes in **Chapter 2**), before and after seedlings were exposed to the same heat stress treatment as used previously, were gathered. Gathering data from two time points allowed differential expression analysis to be conducted, subsequently identifying 7827 genes whose expression was significantly changed in response to early heat stress exposure. Genes whose expression were upregulated by heat stress tended to be involved in cell wall modification or the protection against DNA damage, whereas downregulated genes were largely involved in photosynthesis or the response to abiotic stresses other than heat. Such downregulation of genes putatively involved in the response to other abiotic stresses was not reported in similar work in hexaploid wheat (Qin et al., 2008; Rangan, Furtado and Henry, 2020; Azameti et al., 2022; Lee et al., 2022), nor was a comparable shift seen in **Chapter 6** during the examination of the effect of early drought stress on the wheat transcriptome; for example, only 161 (2.99%) of the 5384 upregulated heat-responsive DEGs identified in **Chapter 3** were also downregulated under early drought stress in **Chapter 6**, whilst almost double this number of upregulated drought DEGs (321), identified in **Chapter 6**, were downregulated in **Chapter 3** after heat stress exposure. This, therefore, may suggest that these genes alleviate the symptoms of specific cellular environments caused

by these other stresses, such as desiccation, ion imbalance or ice crystal formation, and thus are superfluous under heat stress as such symptoms are absent – leading to their downregulation. On the other hand, those genes involved in responding to heat stress are largely involved in mitigating the effects common amongst most abiotic stresses, particularly damage to proteins and membranes; this potential functional cross-over of such genes is evidenced by the fact that 1184 of the upregulated DEGs identified in **Chapter 3** were also upregulated after exposure to early drought stress in **Chapter 6**.

Gene expression data were then used to construct a co-expression network via WGCNA, before stress-associated modules were identified via GO term enrichment analysis (as before), and differentially expressed gene (DEG) enrichment analysis, by the way of a one-proportion Z-test. The hub genes within these modules were therefore candidate master-regulators of the transcriptional response to early heat stress, particularly those hub genes within modules significantly enriched in DEGs. However, three hub genes from this list were identified as particularly promising candidate master-regulators of this response, based on their own putative function, or the putative functions of the genes they were connected to within the co-expression network. Two hub genes, *TaMAPKKK18-like* and *TaERD15-like*, were downregulated after heat stress exposure, and seemingly act to downregulate the expression of genes involved in responding to other abiotic stresses, particularly drought and cold. A small HSP, *HSP26*, was also identified as a hub gene within a stress-associated module, however it was upregulated after early heat stress exposure, and likely acts as a positive regulator of the heat stress response thanks to connections to a large number of fellow HSPs, as well as a group of stress-responsive transcription factors, including Hsfs as well as members of the WRKY, ERF, and NAC families – all of which were also upregulated.

The approach taken in this chapter of the thesis was completely novel, as although previous work has described the transcriptional response of hexaploid wheat seedlings to heat stress (Qin et al., 2008; Jin et al., 2020; Liu et al., 2015), such exploratory comparative transcriptomic analysis, paired with co-expression network analysis has never been done in non-mutant wheat seedlings. This work builds on the findings of those described in the previous chapter, as, when viewed together, these results provide insights into how both basal thermotolerance, and the response to early heat stress may be regulated in wheat seedlings. Again, the hub genes identified in this chapter could be good targets for genetic manipulation by wheat breeders, as they may act to coordinate the response to early heat stress.

Further work could screen the expression responses of the three promising hub genes to early heat stress in other accessions within the YoGI panel, before predictions about their growth and survivability under heat stress could be made based on these responses. It would be hypothesized that those showing greater upregulation of the sHSP hub gene and greater downregulation of *TaMAPKKK18-like* and *TaERD15-like* may show improved growth and survival under early heat stress, due to the putative effects these hub genes have on global gene expression. Given that *TaMAPKKK18-like* and *TaERD15-like* may regulate the expression of genes involved in responses to drought, salinity and cold, it would also be interesting to determine the expression responses of these hub genes, and the genes they are connected to in the co-expression network, to such stresses via global RNA-sequencing. It would be hypothesized that these genes would now be upregulated, under stress, whilst the same may also be true of the sHSP hub gene, given its likely crossover potential as a chaperone protein. Further work is required to determine whether these hub genes do indeed regulate the expression of the genes they are connected to in the co-expression network under early heat stress, with a similar protocol as described above also likely proving to be useful in providing insight on this for the sHSP hub gene. The remaining hub genes, however, would require a different approach to elucidate their regulatory mechanism – starting with

determining whether their expression effects ABA signalling. ABA concentration in leaf tissue of TILLING lines carrying mutations in these hub genes could be determined before, during and after early heat stress exposure by gas chromatography-mass spectrometry, and compared to those seen in wild-type plants. However, these hub genes may not influence ABA concentration, but instead influence ABA signalling; to test this, the expression responses of known ABA-responsive genes to ABA treatment should be compared in TILLING mutant and wild-type lines. Assessing the expression responses of these hub genes, and the putatively superfluous genes they are connected to in the co-expression network, to ABA treatment would suggest that any effect these hub genes have on ABA signalling has downstream effects on their expression.

7.7. Exposure to Early Heat Stress Provokes a Novel Flower Emergence Response, but has no Significant Effect on Yield

This thesis then aimed to build on the work described in **Chapters 2** and **3**; firstly, aiming to determine whether seedling thermotolerance could be used to predict the effect of early heat stress on yield, before also aiming to determine whether exposure to early heat stress provokes a delay in flower emergence time. Much of the work examining the effect of heat stress on wheat yields exposes plants to elevated temperatures during the susceptible reproductive stages of development (Dias and Lidon, 2009; Pradhan and Prasad, 2015; Vignjevic et al., 2015; Balla et al., 2019; Qaseem, Qureshi and Shaheen, 2019; Djanaguiraman et al., 2020; Schittenhelm et al., 2020; Shenoda et al., 2021), whilst there is only one example of wheat plants being exposed to early heat stress, before the stress is removed and yield traits are examined. Therefore, there is little understanding, especially in hexaploid wheat landraces, about whether exposure to early heat stress leads to yield losses, and, if so, whether those accessions better able to grow under these conditions as seedlings also show lesser yield losses. Across all the accessions screened, exposure to early heat stress had no significant effect on any of the yield traits measured – however, individual accessions did show varying differences in these traits, with some showing widespread significant reductions, whilst others were unaffected by the stress. There was also no relationship between seedling thermotolerance (as defined in **Chapter 2**) and the normalized difference in any of these yield traits, suggesting early thermotolerance cannot be used to predict how yield will be affected after early heat stress exposure. This was the case for the definition of seedling thermotolerance used in **Chapter 2**, however, perhaps a significant relationship between seedling thermotolerance and yield traits would have been observed if thermotolerance was defined differently; for example, by membrane thermostability, or photosynthetic rate under heat stress. Further work is required to screen these traits in the landrace panel and determine whether these traits influence yields after early heat stress exposure.

This chapter then built on the findings of the previous one, as the differential expression analysis experiment done in **Chapter 3** identified that a large number of genes, putatively involved in promoting flowering, were significantly downregulated after early heat stress exposure. This led to the hypothesis that exposure to, and then removal of, early heat stress may lead to delayed flower emergence, due to the downregulation of these genes. Subsequently, this heat stress treatment led to delayed flower emergence in all the accessions screened, with this delay being significant ($p < 0.05$) for 13 out of 16. This finding is at odds with the widely observed response of wheat to elevated temperatures, as plants usually show accelerated development, and faster flowering, after exposure to stress (Rahman et al., 2009; Nahar, Ahamed and Fujita, 2010; Hakim et al., 2012; Hemming et al., 2012; Hossain and da Silva, 2012; Hossain et al., 2012, 2013). To identify potential transcriptional regulators of this novel response, the relationship between the expression response of every DEG identified in **Chapter 3**, and the flower emergence response shown by each accession were tested via

linear regression analysis. There was deemed to be a significant relationship between these factors for 38 of the 7827 DEGs; seven of which are particularly promising markers due to their putative roles in promoting flowering, or in phytohormone signalling.

The work discussed in this chapter, therefore, aimed to tie together the work done in the previous two research chapters, and determine whether the observed effects of heat stress exposure on the physiology and transcriptomes of wheat seedlings would subsequently lead to agriculturally-relevant phenotypic effects later in development. Although this heat stress treatment had no significant effect on yield traits, the present work is the first example of this screen being done in wheat landraces, as although Matsunaga et al. (2021) conducted a similar stress treatment, they only examined yield traits in one HYV (Matsunaga et al., 2021). Similarly, the work described in this chapter was novel, as it identified a flowering response never before observed in hexaploid bread wheat, and offered an explanation as to how this novel response may be coordinated transcriptionally, via the differential expression of seven particularly promising genes. As well as these seven, the expression responses of 31 other genes were identified as significant markers of the observed flower emergence delay – providing wheat breeders with a large number of targets for genetic manipulation to ensure HYVs do not show a similar delay, and likely development stunting, after exposure to early heat stress.

Future work is required to determine whether this novel flower emergence response is also seen in modern HYVs, however, because, as the Spring months become warmer and more variable over the coming years, spring habit wheat crops in many regions are likely to experience this early heat stress, and subsequently may exhibit significantly delayed flower emergence. This would not only result in changes to the crop cycle, but could also lead to reduced yields by limiting the amount of time spent in reproductive development before harvest (reducing the amount of time for grain formation and filling, for example), whilst also causing these developmental stages, which are susceptible to damage by periods of stress (as discussed in **Chapter 1**), to be more closely aligned with the warmest summer months (for example, crops in the UK may flower during August, when crops are usually harvested), increasing the risk of yield losses by damage to reproductive tissues and processes. If this response is seen in modern HYVs, the present work also identified landrace accessions which did not show a significant delay after early heat stress exposure, and thus may be beneficial to include in breeding programmes to introduce this trait into HYVs, if necessary.

As discussed above, future work examining meristem identity in plants under control and stressed treatment regimes is required to determine whether exposure to early heat stress truly causes delayed flowering, and whether this delay comes as a result of a developmental shift, prolonging time in vegetative development and delaying the floral transition at the meristem. The expression responses of the putative flowering-promoting genes identified and described in **Chapters 3** and **4** suggest such a shift, and subsequent delay, occurs, however this conclusion could perhaps be more robust. Collecting gene expression data from the shoot apex/meristem at the same time as these regular meristem dissections would allow direct correlations to be made between the expression of these (and other) genes, and the identity of the meristem. The use of such expression data, as opposed to the expression data gathered from leaf tissue in the present work, would provide accurate insights into the gene expression changes occurring in the meristem which likely determine any meristematic response seen under early heat stress – unlike the insights currently provided by gene expression data from leaf tissue, which is not undergoing such a transition. Gene expression data could be gathered after the floral transition has occurred at the meristem, to determine whether expression of these genes remain low until the point of flower emergence – insight currently absent from the

present work, which collected gene expression data at two time points long before flower emergence.

As with the work in previous research chapters, further work is required to confirm whether the identified markers do indeed control this phenotypic response; screening the flower emergence times of lines carrying mutations in these genes which either eliminates their expression, or prevents the heat-induction of their expression, would allow these markers to be validated. Finally, although no similar widespread downregulation of putative flowering promoting genes was observed after exposure to early drought stress, it may also be interesting to determine whether accessions show the same flower emergence response after such treatment – this work is currently ongoing.

7.8. Identifying Candidate Master-regulators of the Transcriptional Drought Response using Open-Source Data

This thesis then aimed to apply the approaches used in **Chapters 2** and **3** to study drought tolerance and the drought response in hexaploid wheat. However, due to COVID-19-enforced restrictions, transcriptome data from landrace accessions before and after plants were exposed to drought stress could not be gathered. Despite this, the vast wealth of open-source gene expression data meant that the effects of drought stress on the wheat transcriptome could still be studied in the present work, with new insights being able to be drawn from these data via the application of novel analyses.

Across the microarray meta-dataset that was gathered, 2916 probes were deemed to be significantly differentially expressed in at least one instance. Interestingly, similar to the trend seen in **Chapter 3**, genes (corresponding to probes) involved in the response to other stresses were largely downregulated – demonstrating that, although not widely reported in similar work, the downregulation of genes involved in responding to absent environmental stimuli seems to be an important part of the tailored response to drought stress, as well as heat stress (as identified in **Chapter 3**). However, these trends may not be directly comparable, given that **Chapter 3** exposed wheat plants to heat stress during early vegetative development, whereas many of the studies whose data are included in the meta-dataset exposed plants to drought stress much later in development.

This chapter then identified several hub genes within stress-associated modules which may contain genes that act during the response to drought stress. To validate the function of these candidate hub genes, TILLING mutant lines, containing nonsense or missense mutations in their gene sequence, were sourced. These TILLING lines showed a range of drought tolerance levels, with most exhibiting improved drought tolerance compared to the wild-type Cadenza variety, possibly as a result of the mutation nullifying the action of these hub genes in the drought response. Although an attempt to backcross these mutant varieties into the wild-type Cadenza failed, confirming the genotypes of some TILLING mutants in this screen went some way to validating the roles of *TraesCS4D01G050400* and *TraesCS7D01G347300* as part of the drought response, and/or as determinants of drought tolerance. The work described in this chapter also identified one promising hub gene which may act to coordinate the ABA-mediated response to environmental stimuli other than drought, as the hub gene was connected to several genes encoding proteins which are putatively integral in the response to cold and salinity stresses, but were only differentially expressed in response to ABA treatment, and not drought stress, in the meta-dataset.

Although there were no similar examples of a network approach being applied to open-source gene expression data, and used to identify candidate regulators of drought tolerance or the drought response during the completion of the work described in this chapter, one example has since been published (Lv et al., 2020). However, it was clear that the network approach

used by Lv et al. (2020) could be further optimized, whilst validation of the identified hub genes using mutant lines would take the approach further than this work had done. Although only partial validation of some of the identified hub genes was able to be executed in the present work, further work repeating the backcrossing of TILLING mutant lines into Cadenza, to remove the level of background mutations present, is current ongoing. The progeny of these crosses, after selfing and further genotyping, will then be used to repeat the drought tolerance screen – definitively validating whether mutation to these genes significantly impedes the drought response and has consequences for drought tolerance.

Further work is also required to determine whether the hub genes identified in this work indeed act to coordinate the drought response; doing so for *TraesCS4D01G050400* initially, for example, would perhaps be most logical, as its Arabidopsis orthologue is a known repressor of the drought response. To do this, the failed backcrossing protocol should be repeated for the TILLING lines containing mutations in this gene, which showed improved growth under drought stress in the initial screen of non-backcrossed TILLING lines. Once backcrossed mutant seed have been generated and selfed, and the presence of the mutant allele confirmed in selfed offspring, then the drought tolerance/survivability screen should be repeated. The results of this screen will confirm or deny whether *TraesCS4D01G050400* likely plays a similar role to its Arabidopsis orthologue, in wheat. Unlike the other hub genes mentioned above, previous work on the hub gene's Arabidopsis orthologue, *AtAHK4*, can help form hypotheses on how the hub gene may act to regulate the expression of the genes it is connected to in the co-expression network, under drought stress. The above-mentioned backcrossed TILLING mutants can again be utilized here to determine whether the presence of non-functional *TraesCS4D01G050400* leads to altered ABA and cytokinin responses, as seen in Arabidopsis when *AtAHK4* is mutated (Tran et al., 2007; Jeon et al., 2010; Kang et al., 2012) – levels of these phytohormones can be measured via gas chromatography-mass spectrometry, whilst the expression responses of known ABA- and cytokinin-responsive genes can be determined via qRT-PCR. Further transcriptomic work, such as qRT-PCR or whole transcriptome sequencing, would allow the identification of any transcription factors which show differential responses to drought stress in wild-type and TILLING mutant plants to be identified. To then identify which of these transcription factors may be affecting the regulation of the genes connected to the hub in the co-expression network, the results of the recently-developed promoter pull-down assay (Chaparian and van Kessel, 2021) could be compared to the results of the transcriptomic work – identifying proteins which bind the regulatory regions of the genes, and also show differential responses to drought stress in wild-type and TILLING mutant plants.

Despite the need to complete such further work to truly validate the functions of the identified hub genes, the present work identifies a large number of candidate master-regulators of the drought response, whose putative action may also determine drought tolerance. This work, therefore, not only acts as a valuable resource for wheat breeders to aid their production of drought tolerant HYVs, but also provides new insights into how the response to drought stress may be regulated transcriptionally.

7.9. Assessing the Transcriptional Drought Response in YoGI Landrace Accessions, and Identifying Candidate Master-regulators of this Response

Although work in the previous chapter identified candidate regulators of the transcriptional response to drought stress, this thesis had not yet assessed the transcriptional response to drought stress in wheat landrace seedlings, nor had it sought to understand how this response may be regulated. Therefore, this chapter aimed to answer these questions; gathering transcriptome data from landrace seedlings before and after drought stress exposure, before using these gene expression data to construct a co-expression network. Exposure to drought stress during early vegetative development, much like exposure to early heat stress as

described in **Chapters 2** and **3**, was found to both significantly impede growth, and elicit large changes in the hexaploid wheat transcriptome. 10,199 genes were deemed to be significantly differentially expressed after drought stress exposure, with upregulated genes putatively involved in the response to water deprivation and cell wall maintenance, and genes likely involved in photosynthesis being enriched amongst downregulated genes. Therefore, unlike previous transcriptional responses, described in **Chapters 3** and **5**, the present work did not identify widespread downregulation of genes putatively involved in responding to other stresses. Co-expression network construction resulted in the formation of 23 modules containing genes likely associated with the drought response, with two hub genes within these modules being identified as particularly promising candidate master-regulators of the physiological and transcriptional responses to water limitation. One, a dehydrin, may positively regulate the expression of fellow dehydrins as well as other genes putatively involved in the drought response, whereas another, an uncharacterized gene, may act to downregulate the expression of genes involved in water movement, stomatal opening, and stomatal morphogenesis under drought stress.

Identification of these hub genes therefore, again, aids our understanding of how the transcriptional response to drought stress may be coordinated in wheat; this time in wheat seedlings, unlike **Chapter 5** which identified candidate master-regulators of the response to drought stress later in development. The work described in this chapter may also provide a more comprehensive insight into the transcriptional response to drought stress than given in **Chapter 5**, due to differences in the technologies used between these works; **Chapter 5** utilized microarray data and thus provided an insight into the drought-responsiveness of 55,052 transcripts, whereas the work in this chapter, as in previous chapters, utilized transcriptome-wide gene expression data generated by RNA sequencing, thus providing information on the drought responsiveness of over 100,000 genes. Similarly, direct comparisons with the work described in **Chapter 3** must be made with caution, as the landrace accessions used in these works were different, and some of these transcriptional responses are likely to be accession-dependent, as a result of the extensive genetic diversity between accessions in the YoGI panel. Further work examining the effects of both heat and drought stress on the transcriptomes of all these accessions (via whole transcriptome RNA sequencing, as used throughout this thesis) would allow direct comparisons between the transcriptional shifts and trends seen under these stresses to be made with more confidence.

The opportunity to conduct further work is plentiful given the unknown function of one of the candidate hub genes identified in this work. Proteomic work, such as the employment of AlphaFold, to predict protein structure and putative function would allow a potential mechanism of how the uncharacterized hub gene may regulate gene expression under drought stress to be hypothesized. Despite being more well characterized, the exact mechanism of how the dehydrin hub gene may regulate the expression of genes under drought stress is also unclear, and requires further work to determine. Previous work led to several hypothesized mechanisms (detailed in **Chapter 6**), which could be tested. Co-immunoprecipitation can be employed to identify what proteins are bound by TaDHN4 under control and stressed conditions, with the identification of any transcription factors being bound under stressed conditions suggesting TaDHN4 may be able to mediate gene expression through this activity. Determining whether functional copies of such transcription factors are able to translocate to the nucleus would shed further light on the exact mechanism; if not, then perhaps binding by TaDHN4 holds the regulators in the cytoplasm and prevents them from acting to regulate gene expression, whereas if so, then perhaps TaDHN4 binding allows a functional protein to translocate to the nucleus and act to regulate gene expression. Chromatin-immunoprecipitation could be used to determine if TaDHN4 itself acts as a transcriptional regulator by binding to regulatory regions of DNA. This method would also allow

the DNA protection hypothesis to be tested, as perhaps TaDHN4 is able to bind DNA in regions surrounding the genes it is connected to in the co-expression network, but not in the regulatory regions of these genes – suggesting the protein does, in fact, bind DNA and protect it from oxidative damage, hence influencing expression at these loci.

7.10. Future Perspectives

Although the work presented in this thesis represents an advancement in our understanding of both the characteristics of the YoGI landrace panel, and how basal thermotolerance and the response to heat and drought stresses are regulated transcriptionally, further work could be done to gain an even more comprehensive understanding of these. For instance, further value could be added to the YoGI landrace panel if growth and survival of the whole panel under heat and drought stresses, at multiple different growth stages, were assessed, whilst the examination of certain traits associated with these environmental perturbations (such as stomatal conductance, membrane stability, Rubisco activity, photosynthetic capacity, and ROS detoxification enzyme abundance) would shed light on how accessions respond, physiologically and biochemically, to these stresses, and allow for comparisons to be made across the panel. Increasing knowledge about such traits across the panel would effectively allow each accession to be profiled, meaning breeders could easily select accessions for use in breeding programmes based on the traits they exhibit (under control conditions, and under stress), and the transcriptional markers they possess.

In addition, value could be added to the landrace panel, especially for breeders, via the identification of sequence variants, arising from genetic divergence, associated with the gene expression markers discussed at length throughout this thesis, via eQTL analysis. The presence or absence of such markers will be easier to track throughout the breeding process, and thus could lead to more engagement with breeders, and subsequently more real-world impact as a result. Similarly, the identification, via GWAS, of sequence variants significantly associated with the traits described in this thesis, or those yet to be measured across the entire panel, would be valuable for breeders to predict whether accessions, and offspring of crosses using these accessions, are likely to exhibit certain traits. Due to the extensive amount of RNA-sequencing data conducted as part of the present work, the data required to identify these sequence variants are readily available, and waiting for other researchers to identify those significantly associated with different traits.

Further value can be extracted from the work described in this thesis, especially with respect to the amount of data generated, which can be utilized by other researchers to aid their own work. This is particularly true of the gene expression data described in **Chapter 2**, and the co-expression network created from it. Although these data, and this network, were used in the present work to gain a better understanding of the putative transcriptional regulation of basal thermotolerance, inferences about the transcriptional regulation of many other traits and processes could also be made using this co-expression network, as it was constructed using gene expression data gathered from plants grown under control conditions. Unlike the other co-expression networks described in this thesis, this means that there is no environmental change driving variation in gene expression between samples, and subsequently affecting how genes cluster in the network – instead, the driving factor behind gene clustering in the network is inherent differences in basal gene expression, likely due to sequence variation in regulatory regions, or, as observed in **Chapter 2**, large-scale chromosomal rearrangements. Such variation will likely cause large phenotypic variation for many traits, therefore fellow researchers should be able to identify key modules containing genes putatively involved in controlling their trait of interest (using methods similar to those used throughout this thesis), and subsequently make hypotheses about how these traits may be transcriptionally controlled. Therefore, although beyond the scope of the present work, the data generated in this thesis,

particularly those described in **Chapter 2**, are a valuable resource for other researchers, and can help improve the community's understanding of the transcriptional regulation underpinning many key traits – especially control-condition traits such as biomass production, plant height, yield characteristics, nutrient content, chlorophyll content, and root length, to name but a few.

The work described in this thesis only examines stress events occurring once during early development, with no examination of the effect of repeat exposure to heat or drought stress on wheat growth, or gene expression. Obviously, such independent stress events are unlikely to occur in the natural world, with crops in the field likely being exposed to various periods of challenging growth conditions throughout their life. In turn, there is scope for future work to examine the effect that repeat exposure to heat or drought stress has on wheat growth and gene expression, and whether exposure to such stresses early in development improves the ability of plants to tolerate a second period of stress much later in development. This process, known as priming, has been studied in wheat, with evidence indicating that exposure to early stress leads to increased tolerance later in development, often as a result of modified phytohormone levels (Abid et al., 2017; Wang et al., 2021c; Li et al., 2023). Once the existence of a priming effect has been determined among these landraces, tracking the expression of the identified hub genes throughout development, after an initial stress exposure, would provide insight into whether gene expression changes made in response to an early stress event are maintained until the second stress event, and aid accessions' ability to tolerate the second stress. As discussed for other traits, the variation in any priming effect could be assessed across the panel, before common epigenetic and transcriptomic changes made uniquely in accessions which show a priming effect are identified – providing an understanding of how this process may be coordinated, and markers for breeders who want to safeguard varieties against the effects of multiple stress events.

Similarly, given that this thesis identified instances of genes putatively involved in responding to other stresses being downregulated in response to heat and drought stresses, future work could examine whether such transcriptomic changes are maintained throughout development, and whether they effect an accession's ability to tolerate exposure to a different stress later in development. For instance, **Chapter 3** identified the downregulation of genes putatively involved in the response to drought and salinity stresses after exposure to early heat stress. Future work could examine whether exposure to early heat stress subsequently causes a reduction in the ability of plants to grow well and survive when exposed to drought or salinity stress later in development, compared to plants who have not been exposed to early heat stress, whilst the extent of this effect in different accessions can be correlated with their observed expression changes of these genes under the first stress event. Again, the transcriptomic changes of these genes could be tracked throughout development to determine whether the expression of these genes remain low until the point of the second stress, or whether their expression response to the second stress is diminished compared to their counterparts who have not been exposed to the initial stress – perhaps identifying how any differential response to the second stress is coordinated. Such work would need to be conducted to reassure breeders that exposure to early heat stress does not render plants vulnerable to damage from a different stress later in development, as a result of the action of the hub genes identified in the present work, which appear to coordinate this downregulation of such genes, and may affect growth under the initial stress – especially if the expression of these hubs genes are to be manipulated to improve growth under the initial stress.

To truly determine whether these genes are downregulated to aid the response to early heat stress, various changes likely need to be made to their regulatory regions. Modification of the promoter regions of several of these genes, to promote the induction of expression under early

heat stress, would allow for comparisons to be made between the modified line and wild-type. If, as hypothesized, these genes are downregulated under early heat stress because they are not needed, and thus the production of their encoded protein detrimentally effects the production of other more vital proteins, it would be expected that the modified lines are less able to tolerate exposure to early heat stress, and show poorer growth as a result. Alternatively, eliminating the expression of these genes entirely, or reducing via RNAi (this could be done on a gradient to observe any effect of dosage), may improve the growth of modified plants, if the proposed hypothesis is correct – further suggesting that their expression under early heat stress utilizes resources which would be better used producing proteins tailored to the response to elevated temperatures. Determining why these genes, specifically, are downregulated under early heat stress, however, would require much closer exploration of their function within the cell, under stressed and control conditions – given the number of such downregulated genes, this would likely be laborious and time-consuming. However, one approach may be to overexpress these genes and examine growth of these plants under early heat stress, compared to WT plants. If, as hypothesized, the function of the proteins encoded by these genes is redundant under heat stress, then it would be expected that lines with heat-inducible expression of these genes show either poorer growth under heat stress, or growth similar to that of the WT. Alternatively, complementation experiments could be conducted in yeast to determine whether expression of these genes restore the ability of strains containing mutations in known thermotolerance genes, and subsequently showing reduced thermotolerance, to grow under elevated temperatures – if not, then it could be concluded that these genes play other cellular roles. Together, these experiments could provide evidence that the genes putatively involved in responding to drought, salinity and cold stresses, which were downregulated under early heat stress in **Chapter 3**, are downregulated because the role of their encoded protein does not aid growth under early heat stress, and thus their production would be a drain on valuable resources.

7.11. Concluding Remarks

The work outlined in this thesis represents significant steps taken towards a better understanding of the transcriptional and physiological responses to heat and drought stress in hexaploid wheat. Examples of further work are also provided, identifying the most promising candidate genes to initially pursue, and experiments to conduct in order to answer the most pressing questions, and enable fellow researchers to take further steps, building on the findings of the present work, to provide an even more comprehensive understanding of these responses and processes. Combined, the present work and the results of the proposed future work will hopefully play a small, yet significant, role in the production of stress-tolerant wheat varieties. Varieties which are able to produce high yields, and support the current rate of population growth, despite the challenges presented by the ever-changing climate – varieties needed now, more than ever.

8. References

Aalto, M. K. et al. (2012). ERD15--an attenuator of plant ABA responses and stomatal aperture. *Plant science: an international journal of experimental plant biology*, 182, pp.19–28. [Online]. Available at: doi:10.1016/j.plantsci.2011.08.009.

Abd El-Daim, I. A., Bejai, S. and Meijer, J. (2014). Improved heat stress tolerance of wheat seedlings by bacterial seed treatment. *Plant and soil*, 379 (1), pp.337–350. [Online]. Available at: doi:10.1007/s11104-014-2063-3.

Abebe, T. et al. (2003). Tolerance of mannitol-accumulating transgenic wheat to water stress and salinity. *Plant physiology*, 131 (4), pp.1748–1755. [Online]. Available at: doi:10.1104/pp.102.003616.

Abeysingha, D. N. et al. (2021). The effect of auxins on amelioration of heat stress-induced wheat (Triticum aestivum L.) grain loss. *Journal of agronomy and crop science*, 207 (6), pp.970–983. [Online]. Available at: doi:10.1111/jac.12555.

Abhinandan, K. et al. (2018). Abiotic Stress Signaling in Wheat - An Inclusive Overview of Hormonal Interactions During Abiotic Stress Responses in Wheat. *Frontiers in plant science*, 9, p.734. [Online]. Available at: doi:10.3389/fpls.2018.00734.

Abid, M. et al. (2017). Pre-drought priming sustains grain development under post-anthesis drought stress by regulating the growth hormones in winter wheat (Triticum aestivum L.). *Planta*, 246 (3), pp.509–524. [Online]. Available at: doi:10.1007/s00425-017-2698-4.

Abou-Elwafa, S. F. and Shehzad, T. (2021). Genetic diversity, GWAS and prediction for drought and terminal heat stress tolerance in bread wheat (Triticum aestivum L.). *Genetic resources and crop evolution*, 68 (2), pp.711–728. [Online]. Available at: doi:10.1007/s10722-020-01018-y.

Agurla, S. et al. (2018). Mechanism of Stomatal Closure in Plants Exposed to Drought and Cold Stress. *Advances in experimental medicine and biology*, 1081, pp.215–232. [Online]. Available at: doi:10.1007/978-981-13-1244-1_12.

Ahmed, H. G. M.-D. et al. (2020). Conferring drought-tolerant wheat genotypes through morpho-physiological and chlorophyll indices at seedling stage. *Saudi journal of biological sciences*, 27 (8), pp.2116–2123. [Online]. Available at: doi:10.1016/j.sjbs.2020.06.019.

Ahmed, H. G. M.-D. et al. (2022). Conferring of drought tolerance in wheat (Triticum aestivum L.) genotypes using seedling indices. *Frontiers in plant science*, 13, p.961049. [Online]. Available at: doi:10.3389/fpls.2022.961049.

Ahn, H.-K. et al. (2015). Physiological Functions of the COPI Complex in Higher Plants. *Molecules and cells*, 38 (10), pp.866–875. [Online]. Available at: doi:10.14348/molcells.2015.0115.

Ajigboye, O. O. et al. (2017). Altered gene expression by sedaxane increases PSII efficiency, photosynthesis and growth and improves tolerance to drought in wheat seedlings. *Pesticide biochemistry and physiology*, 137, pp.49–61. [Online]. Available at: doi:10.1016/j.pestbp.2016.09.008.

Akerfelt, M. et al. (2007). Heat shock factors at a crossroad between stress and development. *Annals of the New York Academy of Sciences*, 1113, pp.15–27. [Online]. Available at: doi:10.1196/annals.1391.005.

Alabdullah, A. K. et al. (2019). A Co-Expression Network in Hexaploid Wheat Reveals Mostly Balanced Expression and Lack of Significant Gene Loss of Homeologous Meiotic Genes Upon Polyploidization. *Frontiers in plant science*, 10, p.1325. [Online]. Available at: doi:10.3389/fpls.2019.01325.

Alam, M. M. et al. (2013). Exogenous salicylic acid ameliorates short-term drought stress in mustard (Brassica juncea L.) seedlings by up-regulating the antioxidant defense and glyoxalase system. *Australian journal of crop science*, 7 (7), pp.1053–1063. [Online]. Available at: http://www.cropj.com/mirza_7_7_2013_1053_1063.pdf [Accessed 21 August 2023].

Ali, M. S. and Baek, K.-H. (2020). Jasmonic Acid Signaling Pathway in Response to Abiotic Stresses in Plants. *International journal of molecular sciences*, 21 (2). [Online]. Available at: doi:10.3390/ijms21020621.

Ali, Z. et al. (2022). Wetting mechanism and morphological adaptation; leaf rolling enhancing atmospheric water acquisition in wheat crop-a review. *Environmental science and pollution research international*, 29 (21), pp.30967–30985. [Online]. Available at: doi:10.1007/s11356-022-18846-3.

Allan, R. E., Vogel, O. A. and Craddock, J. C., Jr. (1959). Comparative response to gibberellic acid of dwarf, semidwarf, and standard short and tall winter wheat varieties. *Agronomy journal*, 51 (12), pp.737–740. [Online]. Available at: doi:10.2134/agronj1959.00021962005100120013x.

Altenbach, S. B. et al. (2003). Temperature, Water and Fertilizer Influence the Timing of Key Events During Grain Development in a US Spring Wheat. *Journal of cereal science*, 37 (1), pp.9–20. [Online]. Available at: doi:10.1006/jcrs.2002.0483.

Al-Whaibi, M. H. (2011). Plant heat-shock proteins: A mini review. *Journal of King Saud University - Science*, 23 (2), pp.139–150. [Online]. Available at: doi:10.1016/j.jksus.2010.06.022.

Amrine, K. C. H., Blanco-Ulate, B. and Cantu, D. (2015). Discovery of core biotic stress responsive genes in Arabidopsis by weighted gene co-expression network analysis. *PloS one*, 10 (3), p.e0118731. [Online]. Available at: doi:10.1371/journal.pone.0118731.

Andleeb, T., Knight, E. and Borrill, P. (2023). Wheat NAM genes regulate the majority of early monocarpic senescence transcriptional changes including nitrogen remobilization genes. *G3* , 13 (2). [Online]. Available at: doi:10.1093/g3journal/jkac275.

Aprile, A. et al. (2009). Transcriptional profiling in response to terminal drought stress reveals differential responses along the wheat genome. *BMC genomics*, 10, p.279. [Online]. Available at: doi:10.1186/1471-2164-10-279.

Arjona, J. M. et al. (2020). The Effect of Photoperiod Genes and Flowering Time on Yield and Yield Stability in Durum Wheat. *Plants*, 9 (12). [Online]. Available at: doi:10.3390/plants9121723.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant physiology*, 141 (2), pp.391–396. [Online]. Available at: doi:10.1104/pp.106.082040.

Asard, H. et al. (2013). Cytochromes b561: ascorbate-mediated trans-membrane electron transport. *Antioxidants & redox signaling*, 19 (9), pp.1026–1035. [Online]. Available at: doi:10.1089/ars.2012.5065.

Assenov, Y. et al. (2008). Computing topological parameters of biological networks. *Bioinformatics* , 24 (2), pp.282–284. [Online]. Available at: doi:10.1093/bioinformatics/btm554.

Aston, M. J. and Lawlor, D. W. (1979). The Relationship between Transpiration, Root Water Uptake, and Leaf Water Potential. *Journal of experimental botany*, 30 (1), pp.169–181. [Online]. Available at: doi:10.1093/jxb/30.1.169 [Accessed 21 August 2023].

Aubert, Y. et al. (2010). RD20, a stress-inducible caleosin, participates in stomatal control, transpiration and drought tolerance in Arabidopsis thaliana. *Plant & cell physiology*, 51 (12), pp.1975–1987. [Online]. Available at: doi:10.1093/pcp/pcq155.

Avni, R. et al. (2014). Functional characterization of GPC-1 genes in hexaploid wheat. *Planta*, 239 (2), pp.313–324. [Online]. Available at: doi:10.1007/s00425-013-1977-y.

Azameti, M. K. et al. (2022). Transcriptome profiling reveals the genes and pathways involved in thermo-tolerance in wheat (Triticum aestivum L.) genotype Raj 3765. *Scientific reports*, 12 (1), p.14831. [Online]. Available at: doi:10.1038/s41598-022-18625-7.

Bader, Z. E. et al. (2023). GIGANTEA-ENHANCED EM LEVEL complex initiates drought escape response via dual function of ABA synthesis and flowering promotion. *Plant signaling & behavior*, 18 (1), p.2180056. [Online]. Available at: doi:10.1080/15592324.2023.2180056.

Bahieldin, A. et al. (2005). Field evaluation of transgenic wheat plants stably expressing the HVA1 gene for drought tolerance. *Physiologia plantarum*, 123 (4), pp.421–427. [Online]. Available at: doi:10.1111/j.1399-3054.2005.00470.x.

Bai, C., Liang, Y. and Hawkesford, M. J. (2013). Identification of QTLs associated with seedling root traits and their correlation with plant height in wheat. *Journal of experimental botany*, 64 (6), pp.1745–1753. [Online]. Available at: doi:10.1093/jxb/ert041.

Bakhshi, B. and Shahmoradi, S. S. (2023). Simultaneous selection of high-yielding and drought-tolerant barley landraces using GT, GYT and GYSI methodologies. *Cereal research communications*, 51 (1), pp.237–248. [Online]. Available at: doi:10.1007/s42976-022-00290-1.

Balasubramanian, S. et al. (2006). Potent induction of Arabidopsis thaliana flowering by elevated growth temperature. *PLoS genetics*, 2 (7), p.e106. [Online]. Available at: doi:10.1371/journal.pgen.0020106.

Balfagón, D. et al. (2019). Jasmonic Acid Is Required for Plant Acclimation to a Combination of High Light and Heat Stress. *Plant physiology*, 181 (4), pp.1668–1682. [Online]. Available at: doi:10.1104/pp.19.00956.

Balla, K. et al. (2011). Quality of winter wheat in relation to heat and drought shock after anthesis. *Czech Journal of Food Science*, 29 (2), pp.117–128. [Online]. Available at: doi:10.17221/227/2010-cjfs.

Balla, K. et al. (2019). Heat stress responses in a large set of winter wheat cultivars (Triticum aestivum L.) depend on the timing and duration of stress. *PLOS ONE*, 14 (9), p.e0222639. [Online]. Available at: doi:10.1371/journal.pone.0222639.

Bastow, R. et al. (2004). Vernalization requires epigenetic silencing of FLC by histone methylation. *Nature*, 427 (6970), pp.164–167. [Online]. Available at: doi:10.1038/nature02269.

Beena, R. et al. (2021). Association mapping of drought tolerance and agronomic traits in rice (Oryza sativa L.) landraces. *BMC plant biology*, 21 (1), p.484. [Online]. Available at: doi:10.1186/s12870-021-03272-3.

Begcy, K. and Walia, H. (2015). Drought stress delays endosperm development and misregulates genes associated with cytoskeleton organization and grain quality proteins in developing wheat seeds. *Plant science: an international journal of experimental plant biology*, 240, pp.109–119. [Online]. Available at: doi:10.1016/j.plantsci.2015.08.024.

Beillouin, D. et al. (2020). Impact of extreme weather conditions on European crop production in 2018. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 375 (1810), p.20190510. [Online]. Available at: doi:10.1098/rstb.2019.0510.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B, Statistical methodology*, 57 (1), pp.289–300. [Online]. Available at: http://www.jstor.org/stable/2346101.

Bergkamp, B. et al. (2018). Prominent winter wheat varieties response to post-flowering heat stress under controlled chambers and field based heat tents. *Field crops research*, 222, pp.143–152. [Online]. Available at: doi:10.1016/j.fcr.2018.03.009.

Bertolino, L. T., Caine, R. S. and Gray, J. E. (2019). Impact of Stomatal Density and Morphology on Water-Use Efficiency in a Changing World. *Frontiers in plant science*, 10, p.225. [Online]. Available at: doi:10.3389/fpls.2019.00225.

Bhat, J. Y. et al. (2017). Mechanism of Enzyme Repair by the AAA+ Chaperone Rubisco Activase. *Molecular cell*, 67 (5), pp.744-756.e6. [Online]. Available at: doi:10.1016/j.molcel.2017.07.004.

Bi, H. et al. (2020). Wheat Heat Shock Factor TaHsfA6f Increases ABA Levels and Enhances Tolerance to Multiple Abiotic Stresses in Transgenic Plants. *International journal of molecular sciences*, 21 (9). [Online]. Available at: doi:10.3390/ijms21093121.

Bi, H. et al. (2022). Characterization of the Wheat Heat Shock Factor TaHsfA2e-5D Conferring Heat and Drought Tolerance in Arabidopsis. *International journal of molecular sciences*, 23 (5). [Online]. Available at: doi:10.3390/ijms23052784.

Blum, A., Klueva, N. and Nguyen, H. T. (2001). Wheat cellular thermotolerance is related to yield under heat stress. *Euphytica/ Netherlands journal of plant breeding*, 117 (2), pp.117–123. [Online]. Available at: doi:10.1023/A:1004083305905.

Boddington, K. F. and Graether, S. P. (2019). Binding of a Vitis riparia dehydrin to DNA. *Plant science: an international journal of experimental plant biology*, 287, p.110172. [Online]. Available at: doi:10.1016/j.plantsci.2019.110172.

Bokshi, A. I. et al. (2021). Impact of elevated CO2 and heat stress on wheat pollen viability and grain production. *Functional plant biology: FPB*, 48 (5), pp.503–514. [Online]. Available at: doi:10.1071/FP20187.

Bolger, A. M., Lohse, M. and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* , 30 (15), pp.2114–2120. [Online]. Available at: doi:10.1093/bioinformatics/btu170.

Bolt, S. et al. (2017). ERF105 is a transcription factor gene of Arabidopsis thaliana required for freezing tolerance and cold acclimation. *Plant, cell & environment*, 40 (1), pp.108–120. [Online]. Available at: doi:10.1111/pce.12838.

Borovskii, G. B. et al. (2002). Accumulation of Dehydrins and ABA-Inducible Proteins in Wheat Seedlings during Low-Temperature Acclimation. *Russian journal of plant physiology: a comprehensive Russian journal on modern phytophysiology*, 49 (2), pp.229–234. [Online]. Available at: doi:10.1023/A:1014857708301.

Borrill, P. et al. (2019). Identification of Transcription Factors Regulating Senescence in Wheat through Gene Regulatory Network Modelling. *Plant physiology*, 180 (3), pp.1740–1755. [Online]. Available at: doi:10.1104/pp.19.00380.

Borrill, P., Harrington, S. A. and Uauy, C. (2019). Applying the latest advances in genomics and phenomics for trait discovery in polyploid wheat. *The Plant journal: for cell and molecular biology*, 97 (1), pp.56–72. [Online]. Available at: doi:10.1111/tpj.14150.

Bota, J., Medrano, H. and Flexas, J. (2004). Is photosynthesis limited by decreased Rubisco activity and RuBP content under progressive water stress? *The New phytologist*, 162 (3), pp.671–681. [Online]. Available at: doi:10.1111/j.1469-8137.2004.01056.x.

Boudiar, R. et al. (2020). Effects of Low Water Availability on Root Placement and Shoot Development in Landraces and Modern Barley Cultivars. *Agronomy*, 10 (1), p.134. [Online]. Available at: doi:10.3390/agronomy10010134 [Accessed 12 May 2023].

Boulanger, H. G. et al. (2023). Co-expression network of heat-response transcripts: A glimpse into how splicing factors impact rice basal thermotolerance. *Frontiers in molecular biosciences*, 10, p.1122201. [Online]. Available at: doi:10.3389/fmolb.2023.1122201.

Bourgine, B. and Guihur, A. (2021). Heat Shock Signaling in Land Plants: From Plasma Membrane Sensing to the Transcription of Small Heat Shock Proteins. *Frontiers in plant science*, 12, p.710801. [Online]. Available at: doi:10.3389/fpls.2021.710801.

Bowles, S. and Choi, J.-K. (2019). The Neolithic Agricultural Revolution and the Origins of Private Property. *The journal of political economy*, 127 (5), pp.2186–2228. [Online]. Available at: doi:10.1086/701789.

Boyer, J. S. (1982). Plant productivity and environment. *Science*, 218 (4571), pp.443–448. [Online]. Available at: doi:10.1126/science.218.4571.443.

Brini, F. et al. (2007). Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in Arabidopsis thaliana. *Plant cell reports*, 26 (11), pp.2017–2026. [Online]. Available at: doi:10.1007/s00299-007-0412-x.

Byerlee, D. and Moya, P. (1993). *Impacts of International Wheat Breeding Research in the Developing World, 1966-1990*. CIMMYT. [Online]. Available at: https://play.google.com/store/books/details?id=uetLJfZr3xEC.

Caarls, L. et al. (2017). Arabidopsis JASMONATE-INDUCED OXYGENASES down-regulate plant immunity by hydroxylation and inactivation of the hormone jasmonic acid. *Proceedings of the National Academy of Sciences of the United States of America*, 114 (24), pp.6388–6393. [Online]. Available at: doi:10.1073/pnas.1701101114.

Cai, W. et al. (2018). Overexpression of a wheat (Triticum aestivum L.) bZIP transcription factor gene, TabZIP6, decreased the freezing tolerance of transgenic Arabidopsis seedlings by down-regulating the expression of CBFs. *Plant physiology and biochemistry: PPB / Societe*

francaise de physiologie vegetale, 124, pp.100–111. [Online]. Available at: doi:10.1016/j.plaphy.2018.01.008.

Cai, Z. et al. (2020). Transcriptomic Analysis Reveals Important Roles of Lignin and Flavonoid Biosynthetic Pathways in Rice Thermotolerance During Reproductive Stage. *Frontiers in genetics*, 11, p.562937. [Online]. Available at: doi:10.3389/fgene.2020.562937.

Calvin, K. et al. (2023). *IPCC, 2023: Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [core writing team, H. lee and J. romero (eds.)]. IPCC, Geneva, Switzerland*. Arias, P. et al. (Eds). Intergovernmental Panel on Climate Change (IPCC). [Online]. Available at: doi:10.59327/ipcc/ar6-9789291691647.

Cameron, K. D., Teece, M. A. and Smart, L. B. (2006). Increased accumulation of cuticular wax and expression of lipid transfer protein in response to periodic drying events in leaves of tree tobacco. *Plant physiology*, 140 (1), pp.176–183. [Online]. Available at: doi:10.1104/pp.105.069724.

Cantalapiedra, C. P. et al. (2017). Large Differences in Gene Expression Responses to Drought and Heat Stress between Elite Barley Cultivar Scarlett and a Spanish Landrace. *Frontiers in plant science*, 8, p.647. [Online]. Available at: doi:10.3389/fpls.2017.00647.

Caruana, L., Orr, D. J. and Carmo-Silva, E. (2022). Rubiscosome gene expression is balanced across the hexaploid wheat genome. *Photosynthesis research*, 152 (1), pp.1–11. [Online]. Available at: doi:10.1007/s11120-022-00897-9.

Castelán-Muñoz, N. et al. (2019). MADS-Box Genes Are Key Components of Genetic Regulatory Networks Involved in Abiotic Stress and Plastic Developmental Responses in Plants. *Frontiers in plant science*, 10, p.853. [Online]. Available at: doi:10.3389/fpls.2019.00853.

Caverzan, A., Casassola, A. and Brammer, S. P. (2016). Antioxidant responses of wheat plants under stress. *Genetics and molecular biology*, 39 (1), pp.1–6. [Online]. Available at: doi:10.1590/1678-4685-GMB-2015-0109.

Chaichi, M. et al. (2019). Analysis of transcriptional responses in root tissue of bread wheat landrace (Triticum aestivum L.) reveals drought avoidance mechanisms under water scarcity. *PloS one*, 14 (3), p.e0212671. [Online]. Available at: doi:10.1371/journal.pone.0212671.

Chandra Babu, R. et al. (2004). HVA1, a LEA gene from barley confers dehydration tolerance in transgenic rice (Oryza sativa L.) via cell membrane protection. *Plant science: an international journal of experimental plant biology*, 166 (4), pp.855–862. [Online]. Available at: doi:10.1016/j.plantsci.2003.11.023.

Chandrasekaran, U. et al. (2020). Multifaceted Signaling Networks Mediated by Abscisic Acid Insensitive 4. *Plant communications*, 1 (3), p.100040. [Online]. Available at: doi:10.1016/j.xplc.2020.100040.

Chantret, N. et al. (2005). Molecular basis of evolutionary events that shaped the hardness locus in diploid and polyploid wheat species (Triticum and Aegilops). *The Plant cell*, 17 (4), pp.1033–1045. [Online]. Available at: doi:10.1105/tpc.104.029181.

Chaparian, R. R. and van Kessel, J. C. (2021). Promoter Pull-Down Assay: A Biochemical Screen for DNA-Binding Proteins. *Methods in molecular biology* , 2346, pp.165–172. [Online]. Available at: doi:10.1007/7651_2020_307.

Charmet, G. (2011). Wheat domestication: lessons for the future. *Comptes rendus biologies*, 334 (3), pp.212–220. [Online]. Available at: doi:10.1016/j.crvi.2010.12.013.

Chauhan, H. et al. (2012). The wheat chloroplastic small heat shock protein (sHSP26) is involved in seed maturation and germination and imparts tolerance to heat stress. *Plant, cell & environment*, 35 (11), pp.1912–1931. [Online]. Available at: doi:10.1111/j.1365-3040.2012.02525.x.

Chen, A. et al. (2022). Plant root suberin: A layer of defence against biotic and abiotic stresses. *Frontiers in plant science*, 13, p.1056008. [Online]. Available at: doi:10.3389/fpls.2022.1056008.

Chen, D. et al. (2018). Overexpression of a predominantly root-expressed NAC transcription factor in wheat roots enhances root length, biomass and drought tolerance. *Plant cell reports*, 37 (2), pp.225–237. [Online]. Available at: doi:10.1007/s00299-017-2224-y.

Chen, H.-Y. et al. (2016). ORA47 (octadecanoid-responsive AP2/ERF-domain transcription factor 47) regulates jasmonic acid and abscisic acid biosynthesis and signaling through binding to a novel cis-element. *The New phytologist*, 211 (2), pp.599–613. [Online]. Available at: doi:10.1111/nph.13914.

Chen, X. et al. (2014). Expression and interaction of small heat shock proteins (sHsps) in rice in response to heat stress. *Biochimica et biophysica acta*, 1844 (4), pp.818–828. [Online]. Available at: doi:10.1016/j.bbapap.2014.02.010.

Chen, Y. et al. (2019). The Role of the Late Embryogenesis-Abundant (LEA) Protein Family in Development and the Abiotic Stress Response: A Comprehensive Expression Analysis of Potato (Solanum Tuberosum). *Genes*, 10 (2). [Online]. Available at: doi:10.3390/genes10020148.

Chen, Y.-S. et al. (2015). A late embryogenesis abundant protein HVA1 regulated by an inducible promoter enhances root growth and abiotic stress tolerance in rice without yield penalty. *Plant biotechnology journal*, 13 (1), pp.105–116. [Online]. Available at: doi:10.1111/pbi.12241.

Chen, Z. et al. (2020). *Co-expression network analysis of the genes associated with pistillody-stamen development in wheat*. [Online]. Available at: https://europepmc.org/article/ppr/ppr123541.

Cheng, Z. et al. (2011). The bHLH transcription factor MYC3 interacts with the Jasmonate ZIM-domain proteins to mediate jasmonate response in Arabidopsis. *Molecular plant*, 4 (2), pp.279–288. [Online]. Available at: doi:10.1093/mp/ssq073.

Chini, A. et al. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. *Nature*, 448 (7154), pp.666–671. [Online]. Available at: doi:10.1038/nature06006.

Choi, M.-G. et al. (2020). Peptide transporter2 (PTR2) enhances water uptake during early seed germination in Arabidopsis thaliana. *Plant molecular biology*, 102 (6), pp.615–624. [Online]. Available at: doi:10.1007/s11103-020-00967-3.

Choudhary, M. et al. (2022). Heat Stress during Meiosis Has Lasting Impacts on Plant Growth and Reproduction in Wheat (Triticum aestivum L.). *Agronomy*, 12 (5), p.987. [Online]. Available at: doi:10.3390/agronomy12050987 [Accessed 17 August 2023].

Chourey, K., Ramani, S. and Apte, S. K. (2003). Accumulation of LEA proteins in salt (NaCl) stressed young seedlings of rice (Oryza sativa L.) cultivar Bura Rata and their degradation

during recovery from salinity stress. *Journal of plant physiology*, 160 (10), pp.1165–1174. [Online]. Available at: doi:10.1078/0176-1617-00909.

Chowdhury, S. I. and Wardlaw, I. F. (1978). The effect of temperature on kernel development in cereals. *Australian journal of agricultural research*, 29 (2), pp.205–223. [Online]. Available at: doi:10.1071/ar9780205 [Accessed 15 September 2022].

Chu, C. et al. (2021). RNA-seq analysis reveals different drought tolerance mechanisms in two broadly adapted wheat cultivars “TAM 111” and “TAM 112.” *Scientific reports*, 11 (1), p.4301. [Online]. Available at: doi:10.1038/s41598-021-83372-0.

Chung, H. S. et al. (2008). Regulation and function of Arabidopsis JASMONATE ZIM-domain genes in response to wounding and herbivory. *Plant physiology*, 146 (3), pp.952–964. [Online]. Available at: doi:10.1104/pp.107.115691.

Clarke, S. M. et al. (2009). Jasmonates act with salicylic acid to confer basal thermotolerance in Arabidopsis thaliana. *The New phytologist*, 182 (1), pp.175–187. [Online]. Available at: doi:10.1111/j.1469-8137.2008.02735.x.

Collins, M. et al. (2013). Long-term climate change: projections, commitments and irreversibility. In: *Climate change 2013-The physical science basis: Contribution of working group I to the fifth assessment report of the intergovernmental panel on climate change*. Cambridge University Press. pp.1029–1136. [Online]. Available at: https://research.monash.edu/en/publications/long-term-climate-change-projections-commitments-and-irreversibil.

Corrado, G. and Rao, R. (2017). Towards the Genomic Basis of Local Adaptation in Landraces. *Diversity*, 9 (4), p.51. [Online]. Available at: doi:10.3390/d9040051 [Accessed 25 October 2022].

Crafts-Brandner, S. J. and Salvucci, M. E. (2000). Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO2. *Proceedings of the National Academy of Sciences of the United States of America*, 97 (24), pp.13430–13435. [Online]. Available at: doi:10.1073/pnas.230451497.

Cruz de Carvalho, M. H. (2008). Drought stress and reactive oxygen species: Production, scavenging and signaling. *Plant signaling & behavior*, 3 (3), pp.156–165. [Online]. Available at: doi:10.4161/psb.3.3.5536.

Cseh, A. et al. (2021). Exploring the legacy of Central European historical winter wheat landraces. *Scientific reports*, 11 (1), p.23915. [Online]. Available at: doi:10.1038/s41598-021-03261-4.

Csiszár, J. et al. (2012). Different peroxidase activities and expression of abiotic stress-related peroxidases in apical root segments of wheat genotypes with different drought stress tolerance under osmotic stress. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 52, pp.119–129. [Online]. Available at: doi:10.1016/j.plaphy.2011.12.006.

Cuevas-Velazquez, C. L., Rendón-Luna, D. F. and Covarrubias, A. A. (2014). Dissecting the cryoprotection mechanisms for dehydrins. *Frontiers in plant science*, 5, p.583. [Online]. Available at: doi:10.3389/fpls.2014.00583.

Cui, Y. et al. (2021). In vivo single-particle tracking of the aquaporin AtPIP2;1 in stomata reveals cell type-specific dynamics. *Plant physiology*, 185 (4), pp.1666–1681. [Online]. Available at: doi:10.1093/plphys/kiab007.

Cutler, S. R. et al. (2010). Abscisic acid: emergence of a core signaling network. *Annual review of plant biology*, 61, pp.651–679. [Online]. Available at: doi:10.1146/annurev-arplant-042809-112122.

Dalton, T. P., Shertzer, H. G. and Puga, A. (1999). Regulation of gene expression by reactive oxygen. *Annual review of pharmacology and toxicology*, 39, pp.67–101. [Online]. Available at: doi:10.1146/annurev.pharmtox.39.1.67.

Danyluk, J. et al. (1994). Differential expression of a gene encoding an acidic dehydrin in chilling sensitive and freezing tolerant gramineae species. *FEBS letters*, 344 (1), pp.20–24. [Online]. Available at: doi:10.1016/0014-5793(94)00353-x.

Danyluk, J. et al. (1998). Accumulation of an acidic dehydrin in the vicinity of the plasma membrane during cold acclimation of wheat. *The Plant cell*, 10 (4), pp.623–638. [Online]. Available at: doi:10.1105/tpc.10.4.623.

Daszkowska-Golec, A. et al. (2019). Genetic and Physiological Dissection of Photosynthesis in Barley Exposed to Drought Stress. *International journal of molecular sciences*, 20 (24). [Online]. Available at: doi:10.3390/ijms20246341.

Dbira, S. et al. (2018). Variable Levels of Tolerance to Water Stress (Drought) and Associated Biochemical Markers in Tunisian Barley Landraces. *Molecules* , 23 (3). [Online]. Available at: doi:10.3390/molecules23030613.

De Costa, W. A. J. M. (2011). A review of the possible impacts of climate change on forests in the humid tropics. *Journal of the National Science Foundation of Sri Lanka*, 39 (4), p.281. [Online]. Available at: doi:10.4038/jnsfsr.v39i4.3879.

De Vega, J. J. et al. (2021). Physiological and transcriptional response to drought stress among bioenergy grass Miscanthus species. *Biotechnology for biofuels*, 14 (1), p.60. [Online]. Available at: doi:10.1186/s13068-021-01915-z.

Degen, G. E., Orr, D. J. and Carmo-Silva, E. (2021). Heat-induced changes in the abundance of wheat Rubisco activase isoforms. *The New phytologist*, 229 (3), pp.1298–1311. [Online]. Available at: doi:10.1111/nph.16937.

Dempewolf, H. et al. (2017). Past and future use of wild relatives in crop breeding. *Crop science*, 57 (3), pp.1070–1082. [Online]. Available at: doi:10.2135/cropsci2016.10.0885.

Deng, Y.-S. et al. (2014). Heterology expression of the tomato LeLhcb2 gene confers elevated tolerance to chilling stress in transgenic tobacco. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 80, pp.318–327. [Online]. Available at: doi:10.1016/j.plaphy.2014.04.017.

Devi, R., Kaur, N. and Gupta, A. K. (2012). Potential of antioxidant enzymes in depicting drought tolerance of wheat (Triticum aestivum L.). *Indian journal of biochemistry & biophysics*, 49 (4), pp.257–265. [Online]. Available at: https://www.ncbi.nlm.nih.gov/pubmed/23077787.

Dhanda, S. S. and Sethi, G. S. (2002). Tolerance to drought stress among selected Indian wheat cultivars. *The Journal of agricultural science*, 139 (3), pp.319–326. [Online]. Available at: doi:10.1017/S0021859602002526 [Accessed 18 August 2023].

Diallo, A. O. et al. (2014). Transcriptome analysis of an mvp mutant reveals important changes in global gene expression and a role for methyl jasmonate in vernalization and flowering in wheat. *Journal of experimental botany*, 65 (9), pp.2271–2286. [Online]. Available at: doi:10.1093/jxb/eru102.

Dias, A. S. and Lidon, F. C. (2009). Evaluation of grain filling rate and duration in bread and durum wheat, under heat stress after anthesis. *J. Agron. Crop Sci.* [Online]. Available at: doi:10.1111/j.1439-037X.2008.00347.x.

Díaz, A. et al. (2012). Copy number variation affecting the Photoperiod-B1 and Vernalization-A1 genes is associated with altered flowering time in wheat (Triticum aestivum). *PloS one*, 7 (3), p.e33234. [Online]. Available at: doi:10.1371/journal.pone.0033234.

Ding, L. and Chaumont, F. (2020a). Aquaporin mediating stomatal closure is associated with water conservation under mild water deficit. *bioRxiv*, p.2020.04.15.042234. [Online]. Available at: doi:10.1101/2020.04.15.042234 [Accessed 24 October 2022].

Ding, L. and Chaumont, F. (2020b). Are Aquaporins Expressed in Stomatal Complexes Promising Targets to Enhance Stomatal Dynamics? *Frontiers in plant science*, 11, p.458. [Online]. Available at: doi:10.3389/fpls.2020.00458.

Ding, Y., Avramova, Z. and Fromm, M. (2011). The Arabidopsis trithorax-like factor ATX1 functions in dehydration stress responses via ABA-dependent and ABA-independent pathways. *The Plant journal: for cell and molecular biology*, 66 (5), pp.735–744. [Online]. Available at: doi:10.1111/j.1365-313X.2011.04534.x.

Distelfeld, A., Li, C. and Dubcovsky, J. (2009). Regulation of flowering in temperate cereals. *Current opinion in plant biology*, 12 (2), pp.178–184. [Online]. Available at: doi:10.1016/j.pbi.2008.12.010.

Djanaguiraman, M. et al. (2018). Decreased photosynthetic rate under high temperature in wheat is due to lipid desaturation, oxidation, acylation, and damage of organelles. *BMC plant biology*, 18 (1), p.55. [Online]. Available at: doi:10.1186/s12870-018-1263-z.

Djanaguiraman, M. et al. (2020). Effects of high temperature stress during anthesis and grain filling periods on photosynthesis, lipids and grain yield in wheat. *BMC plant biology*, 20 (1), p.268. [Online]. Available at: doi:10.1186/s12870-020-02479-0.

Do, J.-M. et al. (2023). OsHSP 17.9, a Small Heat Shock Protein, Confers Improved Productivity and Tolerance to High Temperature and Salinity in a Natural Paddy Field in Transgenic Rice Plants. *Collection FAO: Agriculture*, 13 (5), p.931. [Online]. Available at: doi:10.3390/agriculture13050931 [Accessed 5 June 2023].

Dodd, I. C. and Davies, W. J. (1994). Leaf growth responses to ABA are temperature dependent. *Journal of experimental botany*, 45 (7), pp.903–907. [Online]. Available at: doi:10.1093/jxb/45.7.903 [Accessed 22 August 2023].

Dodig, D. et al. (2012). Comparison of responses to drought stress of 100 wheat accessions and landraces to identify opportunities for improving wheat drought resistance. *Plant breeding = Zeitschrift fur Pflanzenzuchtung*, 131 (3), pp.369–379. [Online]. Available at: doi:10.1111/j.1439-0523.2011.01941.x.

Domergue, F. et al. (2010). Three Arabidopsis fatty acyl-coenzyme A reductases, FAR1, FAR4, and FAR5, generate primary fatty alcohols associated with suberin deposition. *Plant physiology*, 153 (4), pp.1539–1554. [Online]. Available at: doi:10.1104/pp.110.158238.

Dorion, S., Lalonde, S. and Saini, H. S. (1996). Induction of Male Sterility in Wheat by Meiotic-Stage Water Deficit Is Preceded by a Decline in Invertase Activity and Changes in Carbohydrate Metabolism in Anthers. *Plant physiology*, 111 (1), pp.137–145. [Online]. Available at: doi:10.1104/pp.111.1.137.

Du, L. et al. (2022). TaERF87 and TaAKS1 synergistically regulate TaP5CS1/TaP5CR1-mediated proline biosynthesis to enhance drought tolerance in wheat. *The New phytologist*. [Online]. Available at: doi:10.1111/nph.18549.

Du, L., Ma, Z. and Mao, H. (2023). Duplicate Genes Contribute to Variability in Abiotic Stress Resistance in Allopolyploid Wheat. *Plants*, 12 (13). [Online]. Available at: doi:10.3390/plants12132465.

Du, Z. et al. (2010). agriGO: a GO analysis toolkit for the agricultural community. *Nucleic acids research*, 38 (Web Server issue), pp.W64-70. [Online]. Available at: doi:10.1093/nar/gkq310.

Duan, H. et al. (2017). Effects of drought stress on growth and development of wheat seedlings. *International journal of agriculture and biology*, 19 (05), pp.1119–1124. [Online]. Available at: doi:10.17957/ijab/15.0393.

Duan, H. et al. (2020). Responsive changes of DNA methylation in wheat (Triticum aestivum) under water deficit. *Scientific reports*, 10 (1), p.7938. [Online]. Available at: doi:10.1038/s41598-020-64660-7.

Duan, S. et al. (2019). Genome-wide identification and abiotic stress-responsive pattern of heat shock transcription factor family in Triticum aestivum L. *BMC genomics*, 20 (1), p.257. [Online]. Available at: doi:10.1186/s12864-019-5617-1.

Duarte, K. E. et al. (2019). Identification and characterization of core abscisic acid (ABA) signaling components and their gene expression profile in response to abiotic stresses in Setaria viridis. *Scientific reports*, 9 (1), p.4028. [Online]. Available at: doi:10.1038/s41598-019-40623-5.

Dubcovsky, J. and Dvorak, J. (2007). Genome plasticity a key factor in the success of polyploid wheat under domestication. *Science*, 316 (5833), pp.1862–1866. [Online]. Available at: doi:10.1126/science.1143986.

Dugasa, M. T. et al. (2021). Comparative transcriptome and tolerance mechanism analysis in the two contrasting wheat (Triticum aestivum L.) cultivars in response to drought and salinity stresses. *Plant growth regulation*, 94 (1), pp.101–114. [Online]. Available at: doi:10.1007/s10725-021-00699-4.

Dunn, J. et al. (2019). Reduced stomatal density in bread wheat leads to increased water-use efficiency. *Journal of experimental botany*, 70 (18), pp.4737–4748. [Online]. Available at: doi:10.1093/jxb/erz248.

Easterling, D. R. et al. (2000). Climate extremes: observations, modeling, and impacts. *Science*, 289 (5487), pp.2068–2074. [Online]. Available at: doi:10.1126/science.289.5487.2068.

Edrisi Maryan, K. et al. (2019). Analysis of Brassica napus dehydrins and their Co-Expression regulatory networks in relation to cold stress. *Gene expression patterns: GEP*, 31, pp.7–17. [Online]. Available at: doi:10.1016/j.gep.2018.10.002.

El Keroumi, I. O. A., Dihazi, A. and Naamani, K. (2019). Use of Morpho-physiological parameters and biochemical markers to select drought tolerant genotypes of durum wheat. *Journal of plant stress physiology*, pp.01–07. [Online]. Available at: doi:10.25081/jpsp.2019.v5.3700.

El-Basyoni, I. et al. (2017). Cell Membrane Stability and Association Mapping for Drought and Heat Tolerance in a Worldwide Wheat Collection. *Sustainability: Science Practice and Policy*, 9 (9), p.1606. [Online]. Available at: doi:10.3390/su9091606 [Accessed 17 August 2023].

Engineer, C. B. et al. (2014). Carbonic anhydrases, EPF2 and a novel protease mediate CO2 control of stomatal development. *Nature*, 513 (7517), pp.246–250. [Online]. Available at: doi:10.1038/nature13452.

Erdayani, E. et al. (2020). Genome-wide analysis of the HSP101/CLPB gene family for heat tolerance in hexaploid wheat. *Scientific reports*, 10 (1), p.3948. [Online]. Available at: doi:10.1038/s41598-020-60673-4.

Eskandari, H. and Kazemi, K. K. (2010). Response of Different Bread Wheat (Triticum aestivum L.) Genotypes to Post-Anthesis Water Deficit. *Notulae Scientia Biologicae*, 2 (4), pp.49–52. [Online]. Available at: doi:10.15835/nsb245002 [Accessed 18 August 2023].

Evenson, R. E. and Gollin, D. (2003). Assessing the impact of the green revolution, 1960 to 2000. *Science*, 300 (5620), pp.758–762. [Online]. Available at: doi:10.1126/science.1078710.

Fábián, A. et al. (2019). Stigma Functionality and Fertility Are Reduced by Heat and Drought Co-stress in Wheat. *Frontiers in plant science*, 10, p.244. [Online]. Available at: doi:10.3389/fpls.2019.00244.

Fahad, S. et al. (2017). Crop Production under Drought and Heat Stress: Plant Responses and Management Options. *Frontiers in plant science*, 8, p.1147. [Online]. Available at: doi:10.3389/fpls.2017.01147.

Fan, Y. et al. (2015). Using QTL mapping to investigate the relationships between abiotic stress tolerance (drought and salinity) and agronomic and physiological traits. *BMC genomics*, 16 (1), p.43. [Online]. Available at: doi:10.1186/s12864-015-1243-8.

Farber, M., Attia, Z. and Weiss, D. (2016). Cytokinin activity increases stomatal density and transpiration rate in tomato. *Journal of experimental botany*, 67 (22), pp.6351–6362. [Online]. Available at: doi:10.1093/jxb/erw398.

Farhad, M. et al. (2023). Heat stress in wheat: a global challenge to feed billions in the current era of the changing climate. *Frontiers in sustainable food systems*, 7. [Online]. Available at: doi:10.3389/fsufs.2023.1203721.

Farooq, M. et al. (2018). Desi chickpea genotypes tolerate drought stress better than kabuli types by modulating germination metabolism, trehalose accumulation, and carbon assimilation. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 126, pp.47–54. [Online]. Available at: doi:10.1016/j.plaphy.2018.02.020.

Farooq, M., Hussain, M. and Siddique, K. H. M. (2014). Drought Stress in Wheat during Flowering and Grain-filling Periods. *Critical reviews in plant sciences*, 33 (4), pp.331–349. [Online]. Available at: doi:10.1080/07352689.2014.875291.

Faye, I. et al. (2006). Characterizing root responses to low phosphorus in pearl millet [*Pennisetum glaucum* (L.) R. br.]. *Agronomy journal*, 98 (5), pp.1187–1194. [Online]. Available at: doi:10.2134/agronj2005.0197.

Fedoroff, N. V. (2012). Presidential address. Transposable elements, epigenetics, and genome evolution. *Science*, 338 (6108), pp.758–767. [Online]. Available at: doi:10.1126/science.338.6108.758.

Feng, B. et al. (2014). Effect of heat stress on the photosynthetic characteristics in flag leaves at the grain-filling stage of different heat-resistant winter wheat varieties. *Journal of agronomy and crop science*, 200 (2), pp.143–155. [Online]. Available at: doi:10.1111/jac.12045.

Feng, Q. et al. (2023). Repressive ZINC FINGER OF ARABIDOPSIS THALIANA proteins promote programmed cell death in the Arabidopsis columella root cap. *Plant physiology*. [Online]. Available at: doi:10.1093/plphys/kiad130.

Fernández-Calvo, P. et al. (2011). The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. *The Plant cell*, 23 (2), pp.701–715. [Online]. Available at: doi:10.1105/tpc.110.080788.

Fernández-Pérez, F. et al. (2015). Peroxidase 4 is involved in syringyl lignin formation in Arabidopsis thaliana. *Journal of plant physiology*, 175, pp.86–94. [Online]. Available at: doi:10.1016/j.jplph.2014.11.006.

Flexas, J. et al. (2004). Diffusive and metabolic limitations to photosynthesis under drought and salinity in C(3) plants. *Plant biology* , 6 (3), pp.269–279. [Online]. Available at: doi:10.1055/s-2004-820867.

Food and Agriculture Organization of the United Nations. (2004). *The State of Food and Agriculture 2003-04*. Food & Agriculture Org. [Online]. Available at: https://play.google.com/store/books/details?id=l6mkaoZgpWYC.

Food and Agriculture Organization of the United Nations et al. (2018). *The State of Food Security and Nutrition in the World 2018: Building climate resilience for food security and nutrition*. Food & Agriculture Org. [Online]. Available at: https://play.google.com/store/books/details?id=LC9uDwAAQBAJ.

Food and Agriculture Organization of the United Nations. (2023). *FAOSTAT: Crops and livestock products*. [Online]. Available at: https://www.fao.org/faostat/en/#data/QCL [Accessed 31 March 2023].

Förster, S. et al. (2019). Wounding-Induced Stomatal Closure Requires Jasmonate-Mediated Activation of GORK K+ Channels by a Ca2+ Sensor-Kinase CBL1-CIPK5 Complex. *Developmental cell*, 48 (1), pp.87-99.e6. [Online]. Available at: doi:10.1016/j.devcel.2018.11.014.

Fu, D. et al. (2005). Large deletions within the first intron in VRN-1 are associated with spring growth habit in barley and wheat. *Molecular genetics and genomics: MGG*, 273 (1), pp.54–65. [Online]. Available at: doi:10.1007/s00438-004-1095-4.

Fu, Y.-B. (2015). Understanding crop genetic diversity under modern plant breeding. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik*, 128 (11), pp.2131–2142. [Online]. Available at: doi:10.1007/s00122-015-2585-y.

Fuller, D. Q. (2007). Contrasting patterns in crop domestication and domestication rates: recent archaeobotanical insights from the Old World. *Annals of botany*, 100 (5), pp.903–924. [Online]. Available at: doi:10.1093/aob/mcm048.

Gabriel, K., Egan, B. and Lithgow, T. (2003). Tom40, the import channel of the mitochondrial outer membrane, plays an active role in sorting imported proteins. *The EMBO journal*, 22 (10), pp.2380–2386. [Online]. Available at: doi:10.1093/emboj/cdg229.

Gaju, O. et al. (2016). Leaf photosynthesis and associations with grain yield, biomass and nitrogen-use efficiency in landraces, synthetic-derived lines and cultivars in wheat. *Field crops research*, 193, pp.1–15. [Online]. Available at: doi:10.1016/j.fcr.2016.04.018.

Galbiati, F. et al. (2016). Hd3a, RFT1 and Ehd1 integrate photoperiodic and drought stress signals to delay the floral transition in rice. *Plant, cell & environment*, 39 (9), pp.1982–1993. [Online]. Available at: doi:10.1111/pce.12760.

Gale, M. D. and Gregory, R. S. (1977). A rapid method for early generation selection of dwarf genotypes in wheat. *Euphytica/ Netherlands journal of plant breeding*, 26 (3), pp.733–738. [Online]. Available at: doi:10.1007/BF00021699.

Gan, P. et al. (2019). Chloroplasts- beyond energy capture and carbon fixation: Tuning of photosynthesis in response to chilling stress. *International journal of molecular sciences*, 20 (20), p.5046. [Online]. Available at: doi:10.3390/ijms20205046 [Accessed 12 July 2023].

Gao, C. et al. (2012). Overexpression of a heat shock protein (ThHSP18.3) from Tamarix hispida confers stress tolerance to yeast. *Molecular biology reports*, 39 (4), pp.4889–4897. [Online]. Available at: doi:10.1007/s11033-011-1284-2.

Gaoshan, Y. et al. (2018). Functional characterization of the Wrab17 gene in the interaction process between wheat and Puccinia triticina. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 133, pp.100–106. [Online]. Available at: doi:10.1016/j.plaphy.2018.10.018.

Gautier, L. et al. (2004). affy--analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* , 20 (3), pp.307–315. [Online]. Available at: doi:10.1093/bioinformatics/btg405.

Ghuge, S. A. et al. (2015a). The Apoplastic Copper AMINE OXIDASE1 Mediates Jasmonic Acid-Induced Protoxylem Differentiation in Arabidopsis Roots. *Plant physiology*, 168 (2), pp.690–707. [Online]. Available at: doi:10.1104/pp.15.00121.

Ghuge, S. A. et al. (2015b). The MeJA-inducible copper amine oxidase AtAO1 is expressed in xylem tissue and guard cells. *Plant signaling & behavior*, 10 (10), p.e1073872. [Online]. Available at: doi:10.1080/15592324.2015.1073872.

Gil, K.-E. et al. (2017). ZEITLUPE Contributes to a Thermoresponsive Protein Quality Control System in Arabidopsis. *The Plant cell*, 29 (11), pp.2882–2894. [Online]. Available at: doi:10.1105/tpc.17.00612.

Girousse, C. et al. (2018). Coexpression network and phenotypic analysis identify metabolic pathways associated with the effect of warming on grain yield components in wheat. *PloS one*, 13 (6), p.e0199434. [Online]. Available at: doi:10.1371/journal.pone.0199434 [Accessed 4 January 2022].

Gitlin-Domagalska, A., Maciejewska, A. and Dębowski, D. (2020). Bowman-Birk Inhibitors: Insights into Family of Multifunctional Proteins and Peptides with Potential Therapeutical Applications. *Pharmaceuticals* , 13 (12). [Online]. Available at: doi:10.3390/ph13120421.

Glover, N. M. et al. (2015). Small-scale gene duplications played a major role in the recent evolution of wheat chromosome 3B. *Genome biology*, 16 (1), p.188. [Online]. Available at: doi:10.1186/s13059-015-0754-6.

Godfray, H. C. J. et al. (2010). Food security: the challenge of feeding 9 billion people. *Science*, 327 (5967), pp.812–818. [Online]. Available at: doi:10.1126/science.1185383.

Gómez-Espejo, A. L. et al. (2022). Worldwide Selection Footprints for Drought and Heat in Bread Wheat (Triticum aestivum L.). *Plants*, 11 (17). [Online]. Available at: doi:10.3390/plants11172289.

Gomez-Pastor, R., Burchfiel, E. T. and Thiele, D. J. (2018). Regulation of heat shock transcription factors and their roles in physiology and disease. *Nature reviews. Molecular cell biology*, 19 (1), pp.4–19. [Online]. Available at: doi:10.1038/nrm.2017.73.

Gordon, A. , Hannon, G. J. (2010). *FASTX-toolkit - FASTQ/A short-reads preprocessing tools*. [Online]. Available at: http://hannonlab.cshl.edu/fastx_toolkit/index.html.

Götz, S. et al. (2008). High-throughput functional annotation and data mining with the Blast2GO suite. *Nucleic acids research*, 36 (10), pp.3420–3435. [Online]. Available at: doi:10.1093/nar/gkn176.

Greenshields, D. L. et al. (2005). Differential regulation of wheat quinone reductases in response to powdery mildew infection. *Planta*, 222 (5), pp.867–875. [Online]. Available at: doi:10.1007/s00425-005-0029-7.

Grondin, A. et al. (2015). Aquaporins Contribute to ABA-Triggered Stomatal Closure through OST1-Mediated Phosphorylation. *The Plant cell*, 27 (7), pp.1945–1954. [Online]. Available at: doi:10.1105/tpc.15.00421.

Guihur, A. et al. (2020). Quantitative proteomic analysis to capture the role of heat-accumulated proteins in moss plant acquired thermotolerance. *Plant, cell & environment*, 44 (7), pp.2117–2133. [Online]. Available at: doi:10.1111/pce.13975.

Guo, J. et al. (2016a). Cuticular Wax Accumulation Is Associated with Drought Tolerance in Wheat Near-Isogenic Lines. *Frontiers in plant science*, 7, p.1809. [Online]. Available at: doi:10.3389/fpls.2016.01809.

Guo, L.-M. et al. (2020a). A class I cytosolic HSP20 of rice enhances heat and salt tolerance in different organisms. *Scientific reports*, 10 (1), p.1383. [Online]. Available at: doi:10.1038/s41598-020-58395-8.

Guo, M. et al. (2016b). The Plant Heat Stress Transcription Factors (HSFs): Structure, Regulation, and Function in Response to Abiotic Stresses. *Frontiers in plant science*, 7, p.114. [Online]. Available at: doi:10.3389/fpls.2016.00114.

Guo, Q. et al. (2017). Alleviation of adverse effects of drought stress on wheat seed germination using atmospheric dielectric barrier discharge plasma treatment. *Scientific reports*, 7 (1), p.16680. [Online]. Available at: doi:10.1038/s41598-017-16944-8.

Guo, Q. et al. (2018). JAZ repressors of metabolic defense promote growth and reproductive fitness in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, 115 (45), pp.E10768–E10777. [Online]. Available at: doi:10.1073/pnas.1811828115.

Guo, Y. et al. (2020b). OsFPFL4 is Involved in the Root and Flower Development by Affecting Auxin Levels and ROS Accumulation in Rice (Oryza sativa). *Rice* , 13 (1), p.2. [Online]. Available at: doi:10.1186/s12284-019-0364-0.

Guóth, A. et al. (2009). Comparison of the Drought Stress Responses of Tolerant and Sensitive Wheat Cultivars During Grain Filling: Changes in Flag Leaf Photosynthetic Activity, ABA Levels, and Grain Yield. *Journal of plant growth regulation*, 28 (2), pp.167–176. [Online]. Available at: doi:10.1007/s00344-009-9085-8.

Gupta, N. K. et al. (2013). Effect of short-term heat stress on growth, physiology and antioxidative defence system in wheat seedlings. *Acta physiologiae plantarum / Polish Academy of Sciences, Committee of Plant Physiology Genetics and Breeding*, 35 (6), pp.1837–1842. [Online]. Available at: doi:10.1007/s11738-013-1221-1.

Gupta, N. K., Gupta, S. and Kumar, A. (2001). Effect of water stress on physiological attributes and their relationship with growth and yield of wheat cultivars at different stages. *Journal of agronomy and crop science*, 186 (1), pp.55–62. [Online]. Available at: doi:10.1046/j.1439-037x.2001.00457.x.

Guyer, L. et al. (2018). Catalytic and structural properties of pheophytinase, the phytol esterase involved in chlorophyll breakdown. *Journal of experimental botany*, 69 (4), pp.879–889. [Online]. Available at: doi:10.1093/jxb/erx326.

Hahn, A. et al. (2011). Crosstalk between Hsp90 and Hsp70 chaperones and heat stress transcription factors in tomato. *The Plant cell*, 23 (2), pp.741–755. [Online]. Available at: doi:10.1105/tpc.110.076018.

HajSalah El Beji, I. et al. (2019). Expansion and Functional Diversification of SKP1-Like Genes in Wheat (Triticum aestivum L.). *International journal of molecular sciences*, 20 (13). [Online]. Available at: doi:10.3390/ijms20133295.

Hakim, M. A. et al. (2012). Protein and Starch Content of 20 Wheat (*Triticum aestivum* L.) Genotypes Exposed to High Temperature Under Late Sowing Conditions. *Warsan wichai witthayasat / Khana Witthayasat Chulalongkon Mahawitthayalai = The Journal of scientific research / Faculty of Science, Chulalongkorn University*, 4 (2), pp.477–477. [Online]. Available at: doi:10.3329/jsr.v4i2.8679 [Accessed 17 August 2023].

Han, S.-H. et al. (2021). Safeguarding genome integrity under heat stress in plants. *Journal of experimental botany*. [Online]. Available at: doi:10.1093/jxb/erab355.

Han, S.-H., Park, Y.-J. and Park, C.-M. (2020). HOS1 activates DNA repair systems to enhance plant thermotolerance. *Nature plants*, 6 (12), pp.1439–1446. [Online]. Available at: doi:10.1038/s41477-020-00809-6.

Hanin, M. et al. (2011). Plant dehydrins and stress tolerance: versatile proteins for complex mechanisms. *Plant signaling & behavior*, 6 (10), pp.1503–1509. [Online]. Available at: doi:10.4161/psb.6.10.17088.

Hansen, J. et al. (2006). Global temperature change. *Proceedings of the National Academy of Sciences of the United States of America*, 103 (39), pp.14288–14293. [Online]. Available at: doi:10.1073/pnas.0606291103.

Hao, Y. et al. (2022). Genome-wide survey of the dehydrin genes in bread wheat (Triticum aestivum L.) and its relatives: identification, evolution and expression profiling under various abiotic stresses. *BMC genomics*, 23 (1), p.73. [Online]. Available at: doi:10.1186/s12864-022-08317-x.

Haokip, S. W., Shankar, K. and Lalrinngheta, J. (2020). Climate change and its impact on fruit crops. *Journal of pharmacognosy and phytochemistry*, 9 (1), pp.435–438. [Online]. Available at: https://www.phytojournal.com/archives/2020.v9.i1.10464/climate-change-and-its-impact-on-fruit-crops [Accessed 30 March 2023].

Hara, M. et al. (2009). DNA binding of citrus dehydrin promoted by zinc ion. *Plant, cell & environment*, 32 (5), pp.532–541. [Online]. Available at: doi:10.1111/j.1365-3040.2009.01947.x.

Harberd, N. P., Flavell, R. B. and Thompson, R. D. (1987). Identification of a transposon-like insertion in a Glu-1 allele of wheat. *Molecular & general genetics: MGG*, 209 (2), pp.326–332. [Online]. Available at: doi:10.1007/BF00329661.

Harper, A. L. et al. (2016). Genome distribution of differential homoeologue contributions to leaf gene expression in bread wheat. *Plant biotechnology journal*, 14 (5), pp.1207–1214. [Online]. Available at: doi:10.1111/pbi.12486.

Hassan, N. M. et al. (2015). Roles of dehydrin genes in wheat tolerance to drought stress. *Journal of advertising research*, 6 (2), pp.179–188. [Online]. Available at: doi:10.1016/j.jare.2013.11.004.

Hawkesford, M. J. et al. (2013). Prospects of doubling global wheat yields. *Food and energy security*, 2 (1), pp.34–48. [Online]. Available at: doi:10.1002/fes3.15.

Hays, D. B. et al. (2007). Heat stress induced ethylene production in developing wheat grains induces kernel abortion and increased maturation in a susceptible cultivar. *Plant science: an international journal of experimental plant biology*, 172 (6), pp.1113–1123. [Online]. Available at: doi:10.1016/j.plantsci.2007.03.004.

He, Q. et al. (2021). OsbHLH6 interacts with OsSPX4 and regulates the phosphate starvation response in rice. *The Plant journal: for cell and molecular biology*, 105 (3), pp.649–667. [Online]. Available at: doi:10.1111/tpj.15061.

He, Z. et al. (2017). Extensive homoeologous genome exchanges in allopolyploid crops revealed by mRNAseq-based visualization. *Plant biotechnology journal*, 15 (5), pp.594–604. [Online]. Available at: doi:10.1111/pbi.12657.

Hedden, P. (2003). The genes of the Green Revolution. *Trends in genetics: TIG*, 19 (1), pp.5–9. [Online]. Available at: doi:10.1016/s0168-9525(02)00009-4.

van Heerden, P. D. R. and de Villiers, O. T. (1996). Evaluation of proline accumulation as an indicator of drought tolerance in spring wheat cultivars. *South African Journal of Plant and Soil/Suid-Afrikaanse Tydskrift Plant en Grond*, 13 (1), pp.17–21. [Online]. Available at: doi:10.1080/02571862.1996.10634368.

Hemming, M. N. et al. (2012). Identification of high-temperature-responsive genes in cereals. *Plant physiology*, 158 (3), pp.1439–1450. [Online]. Available at: doi:10.1104/pp.111.192013.

Henson, I. E., Jensen, C. R. and Turner, N. C. (1989). Leaf Gas Exchange and Water Relations of Lupins and Wheat. III. Abscisic Acid and Drought-Induced Stomatal Closure. *Functional plant biology: FPB*, 16 (5), pp.429–442. [Online]. Available at: doi:10.1071/pp9890429 [Accessed 22 August 2023].

Hertz, M. I. et al. (2013). Ribosomal protein S25 dependency reveals a common mechanism for diverse internal ribosome entry sites and ribosome shunting. *Molecular and cellular biology*, 33 (5), pp.1016–1026. [Online]. Available at: doi:10.1128/MCB.00879-12.

Hetherington, A. M. and Woodward, F. I. (2003). The role of stomata in sensing and driving environmental change. *Nature*, 424 (6951), pp.901–908. [Online]. Available at: doi:10.1038/nature01843.

Higashi, Y. et al. (2013). HsfA1d, a protein identified via FOX hunting using Thellungiella salsuginea cDNAs improves heat tolerance by regulating heat-stress-responsive gene expression. *Molecular plant*, 6 (2), pp.411–422. [Online]. Available at: doi:10.1093/mp/sst024.

Higgins, J. et al. (2012). Use of mRNA-seq to discriminate contributions to the transcriptome from the constituent genomes of the polyploid crop species Brassica napus. *BMC genomics*, 13, p.247. [Online]. Available at: doi:10.1186/1471-2164-13-247.

Himi, E. et al. (2011). Development of PCR markers for Tamyb10 related to R-1, red grain color gene in wheat. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik*, 122 (8), pp.1561–1576. [Online]. Available at: doi:10.1007/s00122-011-1555-2.

Hoang, G. T. et al. (2019). Genome-wide Association Study of a Panel of Vietnamese Rice Landraces Reveals New QTLs for Tolerance to Water Deficit During the Vegetative Phase. *Rice* , 12 (1), p.4. [Online]. Available at: doi:10.1186/s12284-018-0258-6.

Hoekstra, A. Y. and Mekonnen, M. M. (2012). The water footprint of humanity. *Proceedings of the National Academy of Sciences of the United States of America*, 109 (9), pp.3232–3237. [Online]. Available at: doi:10.1073/pnas.1109936109.

Hong, S. W. and Vierling, E. (2001). Hsp101 is necessary for heat tolerance but dispensable for development and germination in the absence of stress. *The Plant journal: for cell and molecular biology*, 27 (1), pp.25–35. [Online]. Available at: doi:10.1046/j.1365-313x.2001.01066.x.

Hong-Bo, S., Zong-Suo, L. and Ming-An, S. (2005). LEA proteins in higher plants: structure, function, gene expression and regulation. *Colloids and surfaces. B, Biointerfaces*, 45 (3–4), pp.131–135. [Online]. Available at: doi:10.1016/j.colsurfb.2005.07.017.

Hossain, A. et al. (2012). The effect of high temperature stress on the phenology, growth and yield of five wheat (Triticum aestivum L.) varieties. *The Asian and Australasian journal of plant science and biotechnology*, 6 (1), pp.14–23. [Online]. Available at: http://www.globalsciencebooks.info/Online/GSBOnline/images/2012/AAJPSB_6(1)/AAJPSB_6(1)14-23o.pdf.

Hossain, A. et al. (2013). Effect of temperature on yield and some agronomic characters of spring wheat (Triticum aestivum L.) genotypes. *International Journal of Agricultural Research Innovation and Technology*, 1 (1–2), pp.44–54. [Online]. Available at: doi:10.3329/ijarit.v1i1-2.13932.

Hossain, A. and da Silva, J. A. T. (2012). Phenology, Growth and Yield of Three Wheat (Triticum aestivum L.) Varieties as Affected by High Temperature Stress. *Notulae Scientia Biologicae*, 4 (3), pp.97–109. [Online]. Available at: doi:10.15835/nsb437879 [Accessed 17 August 2023].

Hossain, M. A. et al. (2011). Involvement of endogenous abscisic acid in methyl jasmonate-induced stomatal closure in Arabidopsis. *Plant physiology*, 156 (1), pp.430–438. [Online]. Available at: doi:10.1104/pp.111.172254.

Houde, M. et al. (1992). Cloning, characterization, and expression of a cDNA encoding a 50-kilodalton protein specifically induced by cold acclimation in wheat. *Plant physiology*, 99 (4), pp.1381–1387. [Online]. Available at: doi:10.1104/pp.99.4.1381.

Hu, H. et al. (2010). Carbonic anhydrases are upstream regulators of CO2-controlled stomatal movements in guard cells. *Nature cell biology*, 12 (1), pp.87–93; sup pp 1-18. [Online]. Available at: doi:10.1038/ncb2009.

Hu, S., Ding, Y. and Zhu, C. (2020). Sensitivity and Responses of Chloroplasts to Heat Stress in Plants. *Frontiers in plant science*, 11, p.375. [Online]. Available at: doi:10.3389/fpls.2020.00375.

Hu, X.-J. et al. (2018). Heat shock factor C2a serves as a proactive mechanism for heat protection in developing grains in wheat via an ABA-mediated regulatory pathway. *Plant, cell & environment*, 41 (1), pp.79–98. [Online]. Available at: doi:10.1111/pce.12957.

Hu, Y. and Schmidhalter, U. (2005). Drought and salinity: A comparison of their effects on mineral nutrition of plants. *Journal of Plant Nutrition and Soil Science*, 168 (4), pp.541–549. [Online]. Available at: doi:10.1002/jpln.200420516.

Huang, H. et al. (2019). Mechanisms of ROS Regulation of Plant Development and Stress Responses. *Frontiers in plant science*, 10, p.800. [Online]. Available at: doi:10.3389/fpls.2019.00800.

Huang, Q. et al. (2015). TaNAC29, a NAC transcription factor from wheat, enhances salt and drought tolerance in transgenic Arabidopsis. *BMC plant biology*, 15, p.268. [Online]. Available at: doi:10.1186/s12870-015-0644-9.

Iehisa, J. C. M. and Takumi, S. (2012). Variation in abscisic acid responsiveness of Aegilops tauschii and hexaploid wheat synthetics due to the D-genome diversity. *Genes & genetic systems*, 87 (1), pp.9–18. [Online]. Available at: doi:10.1266/ggs.87.9.

Iizumi, T. et al. (2018). Uncertainties of potentials and recent changes in global yields of major crops resulting from census- and satellite-based yield datasets at multiple resolutions. *PloS one*, 13 (9), p.e0203809. [Online]. Available at: doi:10.1371/journal.pone.0203809.

Intergovernmental Panel on Climate Change (IPCC). (2014). Long-term Climate Change: Projections, Commitments and Irreversibility. In: *Climate Change 2013 – The Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press. pp.1029–1136. [Online]. Available at: doi:10.1017/CBO9781107415324.024 [Accessed 15 August 2023].

International Wheat Genome Sequencing Consortium (IWGSC). (2014). A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. *Science*, 345 (6194), p.1251788. [Online]. Available at: doi:10.1126/science.1251788.

International Wheat Genome Sequencing Consortium (IWGSC). (2018). Shifting the limits in wheat research and breeding using a fully annotated reference genome. *Science*, 361 (6403). [Online]. Available at: doi:10.1126/science.aar7191.

Iqbal, N. et al. (2022). Nitric Oxide and Abscisic Acid Mediate Heat Stress Tolerance through Regulation of Osmolytes and Antioxidants to Protect Photosynthesis and Growth in Wheat Plants. *Antioxidants (Basel, Switzerland)*, 11 (2). [Online]. Available at: doi:10.3390/antiox11020372.

Iquebal, M. A. et al. (2019). RNAseq analysis reveals drought-responsive molecular pathways with candidate genes and putative molecular markers in root tissue of wheat. *Scientific reports*, 9 (1), p.13917. [Online]. Available at: doi:10.1038/s41598-019-49915-2.

Irizarry, R. A. et al. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* , 4 (2), pp.249–264. [Online]. Available at: doi:10.1093/biostatistics/4.2.249.

Islam, A. U. et al. (2017). Cell membrane stability- an important criterion for selection of heat tolerant genotypes in wheat (Triticum aestivum L.). *Journal of Applied and Natural Science*, 9 (4), pp.1894–1900. [Online]. Available at: doi:10.31018/jans.v9i4.1458 [Accessed 17 August 2023].

Ito, S. et al. (2012). FLOWERING BHLH transcriptional activators control expression of the photoperiodic flowering regulator CONSTANS in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 109 (9), pp.3582–3587. [Online]. Available at: doi:10.1073/pnas.1118876109.

Jagadish, S. V. K. et al. (2016). Implications of High Temperature and Elevated CO2 on Flowering Time in Plants. *Frontiers in plant science*, 7, p.913. [Online]. Available at: doi:10.3389/fpls.2016.00913.

Janka, E. et al. (2013). High temperature stress monitoring and detection using chlorophyll a fluorescence and infrared thermography in chrysanthemum (Dendranthema grandiflora). *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 67, pp.87–94. [Online]. Available at: doi:10.1016/j.plaphy.2013.02.025.

Jansson, S. (1994). The light-harvesting chlorophyll ab-binding proteins. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1184 (1), pp.1–19. [Online]. Available at: doi:10.1016/0005-2728(94)90148-1.

Jansson, S. (1999). A guide to the Lhc genes and their relatives in Arabidopsis/IT>. *Trends in plant science*, 4 (6), pp.236–240. [Online]. Available at: doi:10.1016/s1360-1385(99)01419-3.

Jaspert, N., Throm, C. and Oecking, C. (2011). Arabidopsis 14-3-3 proteins: fascinating and less fascinating aspects. *Frontiers in plant science*, 2, p.96. [Online]. Available at: doi:10.3389/fpls.2011.00096.

Jatoi, W. A. et al. (2011). Effect of water stress on physiological and yield parameters at anthesis stage in elite spring wheat cultivars. *Sarhad Journal of Agriculture*, 27 (1), pp.59–65. [Accessed 18 August 2023].

Jeon, J. et al. (2010). A subset of cytokinin two-component signaling system plays a role in cold temperature stress response in Arabidopsis. *The Journal of biological chemistry*, 285 (30), pp.23371–23386. [Online]. Available at: doi:10.1074/jbc.M109.096644.

Jeong, J. S. et al. (2017). The Deubiquitinating Enzymes UBP12 and UBP13 Positively Regulate MYC2 Levels in Jasmonate Responses. *The Plant cell*, 29 (6), pp.1406–1424. [Online]. Available at: doi:10.1105/tpc.17.00216.

Ji, X. et al. (2010). Importance of pre-anthesis anther sink strength for maintenance of grain number during reproductive stage water stress in wheat. *Plant, cell & environment*, 33 (6), pp.926–942. [Online]. Available at: doi:10.1111/j.1365-3040.2010.02130.x.

Ji, X. et al. (2011). Control of abscisic acid catabolism and abscisic acid homeostasis is important for reproductive stage stress tolerance in cereals. *Plant physiology*, 156 (2), pp.647–662. [Online]. Available at: doi:10.1104/pp.111.176164.

Jia, F. et al. (2014). Overexpression of Late Embryogenesis Abundant 14 enhances Arabidopsis salt stress tolerance. *Biochemical and biophysical research communications*, 454 (4), pp.505–511. [Online]. Available at: doi:10.1016/j.bbrc.2014.10.136.

Jiang, C. et al. (2020). A small heat shock protein gene (RcHSP17.8) from Chinese rose confers resistance to various abiotic stresses in transgenic tobacco. *Plant cell, tissue and organ culture*, 141 (2), pp.407–415. [Online]. Available at: doi:10.1007/s11240-020-01798-2.

Jin, J. et al. (2020). Comparative transcriptome analysis uncovers different heat stress responses in heat-resistant and heat-sensitive jujube cultivars. *PloS one*, 15 (9), p.e0235763. [Online]. Available at: doi:10.1371/journal.pone.0235763.

Joshi, C. P. et al. (1997). Expression of a unique plastid-localized heat-shock protein is genetically linked to acquired thermotolerance in wheat. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik*, 95 (5), pp.834–841. [Online]. Available at: doi:10.1007/s001220050633.

Kadam, S. et al. (2012). Genomic associations for drought tolerance on the short arm of wheat chromosome 4B. *Functional & integrative genomics*, 12 (3), pp.447–464. [Online]. Available at: doi:10.1007/s10142-012-0276-1.

Kamruzzaman, M. et al. (2023). Genetic mapping of candidate loci for water-deficit stress-induced proline accumulation in bread wheat (*Triticum aestivum*). *Plant breeding = Zeitschrift fur Pflanzenzuchtung*, 142 (4), pp.449–462. [Online]. Available at: doi:10.1111/pbr.13096.

Kang, G. Z. et al. (2013). Exogenous salicylic acid enhances wheat drought tolerance by influence on the expression of genes related to ascorbate-glutathione cycle. *Biologia plantarum*, 57 (4), pp.718–724. [Online]. Available at: doi:10.1007/s10535-013-0335-z.

Kang, K. et al. (2009). Senescence-induced serotonin biosynthesis and its role in delaying senescence in rice leaves. *Plant physiology*, 150 (3), pp.1380–1393. [Online]. Available at: doi:10.1104/pp.109.138552.

Kang, N. Y. et al. (2012). Cytokinin receptor-dependent and receptor-independent pathways in the dehydration response of Arabidopsis thaliana. *Journal of plant physiology*, 169 (14), pp.1382–1391. [Online]. Available at: doi:10.1016/j.jplph.2012.05.007.

Kanneganti, V. and Gupta, A. K. (2008). Wall associated kinases from plants - an overview. *Physiology and molecular biology of plants: an international journal of functional plant biology*, 14 (1–2), pp.109–118. [Online]. Available at: doi:10.1007/s12298-008-0010-6.

Kantidze, O. L. et al. (2016). Heat Stress-Induced DNA Damage. *Acta naturae*, 8 (2), pp.75–78. [Online]. Available at: doi:10.32607/20758251-2016-8-2-75-78.

Kappes, F. et al. (2004). Functional domains of the ubiquitous chromatin protein DEK. *Molecular and cellular biology*, 24 (13), pp.6000–6010. [Online]. Available at: doi:10.1128/MCB.24.13.6000-6010.2004.

Kariola, T. et al. (2006). *EARLY RESPONSIVE TO DEHYDRATION 15*, a Negative Regulator of Abscisic Acid Responses in Arabidopsis. *Plant Physiology*, 142 (4), pp.1559–1573. [Online]. Available at: doi:10.1104/pp.106.086223.

Karlson, D. et al. (2002). A cold-regulated nucleic acid-binding protein of winter wheat shares a domain with bacterial cold shock proteins. *The Journal of biological chemistry*, 277 (38), pp.35248–35256. [Online]. Available at: doi:10.1074/jbc.M205774200.

Kaur, A. et al. (2022). OSCA Genes in Bread Wheat: Molecular Characterization, Expression Profiling, and Interaction Analyses Indicated Their Diverse Roles during Development and Stress Response. *International journal of molecular sciences*, 23 (23). [Online]. Available at: doi:10.3390/ijms232314867.

Kaur, A., Grewal, A. and Sharma, P. (2018). Comparative analysis of DNA methylation changes in two contrasting wheat genotypes under water deficit. *Biologia plantarum*, 62 (3), pp.471–478. [Online]. Available at: doi:10.1007/s10535-018-0786-3.

Kazan, K. and Manners, J. M. (2012). JAZ repressors and the orchestration of phytohormone crosstalk. *Trends in plant science*, 17 (1), pp.22–31. [Online]. Available at: doi:10.1016/j.tplants.2011.10.006.

Keneni, G. et al. (2012). Genetic Vulnerability of Modern Crop Cultivars: Causes, Mechanism and Remedies. *International Journal of Plant Research*, 2 (3), pp.69–79. [Online]. Available at: http://article.sapub.org/10.5923.j.plant.20120203.05.html [Accessed 26 September 2022].

Kershaw, I. (1973). The Great Famine and Agrarian Crisis in England 1315-1322. *Past & present*, (59), pp.3–50. [Online]. Available at: http://www.jstor.org/stable/650378.

Key, J. L., Lin, C. Y. and Chen, Y. M. (1981). Heat shock proteins of higher plants. *Proceedings of the National Academy of Sciences of the United States of America*, 78 (6), pp.3526–3530. [Online]. Available at: doi:10.1073/pnas.78.6.3526.

Khan, M. I. et al. (2022). Genome-wide association for heat tolerance at seedling stage in historical spring wheat cultivars. *Frontiers in plant science*, 13, p.972481. [Online]. Available at: doi:10.3389/fpls.2022.972481.

Khatun, S. et al. (2018). Physiological Mechanism of Thermotolerance in Wheat (Triticum aestivum Lin.) Seedlings. *American journal of plant sciences*, 09 (13), pp.2719–2727. [Online]. Available at: doi:10.4236/ajps.2018.913198.

Khodaeiaminjan, M. et al. (2023). Genome-wide association study in two-row spring barley landraces identifies QTL associated with plantlets root system architecture traits in well-watered and osmotic stress conditions. *Frontiers in plant science*, 14, p.1125672. [Online]. Available at: doi:10.3389/fpls.2023.1125672.

Khurana, N., Chauhan, H. and Khurana, P. (2013). Wheat chloroplast targeted sHSP26 promoter confers heat and abiotic stress inducible expression in transgenic Arabidopsis Plants. *PloS one*, 8 (1), p.e54418. [Online]. Available at: doi:10.1371/journal.pone.0054418.

Kibbe, W. A. (2007). OligoCalc: an online oligonucleotide properties calculator. *Nucleic acids research*, 35 (Web Server issue), pp.W43-6. [Online]. Available at: doi:10.1093/nar/gkm234.

Kidwell, M. G. and Lisch, D. (1997). Transposable elements as sources of variation in animals and plants. *Proceedings of the National Academy of Sciences of the United States of America*, 94 (15), pp.7704–7711. [Online]. Available at: doi:10.1073/pnas.94.15.7704.

Kim, D., Alptekin, B. and Budak, H. (2018). CRISPR/Cas9 genome editing in wheat. *Functional & integrative genomics*, 18 (1), pp.31–41. [Online]. Available at: doi:10.1007/s10142-017-0572-x.

Kim, J.-M. et al. (2008). Alterations of lysine modifications on the histone H3 N-tail under drought stress conditions in Arabidopsis thaliana. *Plant & cell physiology*, 49 (10), pp.1580–1588. [Online]. Available at: doi:10.1093/pcp/pcn133.

Kim, J.-M. et al. (2012a). Transition of chromatin status during the process of recovery from drought stress in Arabidopsis thaliana. *Plant & cell physiology*, 53 (5), pp.847–856. [Online]. Available at: doi:10.1093/pcp/pcs053.

Kim, K.-H. et al. (2012b). Overexpression of a chloroplast-localized small heat shock protein OsHSP26 confers enhanced tolerance against oxidative and heat stresses in tall fescue. *Biotechnology letters*, 34 (2), pp.371–377. [Online]. Available at: doi:10.1007/s10529-011-0769-3.

Kim, S. L. et al. (2007). OsMADS51 is a short-day flowering promoter that functions upstream of Ehd1, OsMADS14, and Hd3a. *Plant physiology*, 145 (4), pp.1484–1494. [Online]. Available at: doi:10.1104/pp.107.103291.

Kim, W., Iizumi, T. and Nishimori, M. (2019). Global Patterns of Crop Production Losses Associated with Droughts from 1983 to 2009. *Journal of Applied Meteorology and Climatology*, 58 (6), pp.1233–1244. [Online]. Available at: doi:10.1175/JAMC-D-18-0174.1 [Accessed 23 August 2021].

Kim, Y. Y. et al. (2011). A stress-responsive caleosin-like protein, AtCLO4, acts as a negative regulator of ABA responses in Arabidopsis. *Plant & cell physiology*, 52 (5), pp.874–884. [Online]. Available at: doi:10.1093/pcp/pcr039.

Kiribuchi, K. et al. (2004). RERJ1, a jasmonic acid-responsive gene from rice, encodes a basic helix-loop-helix protein. *Biochemical and biophysical research communications*, 325 (3), pp.857–863. [Online]. Available at: doi:10.1016/j.bbrc.2004.10.126.

Kiyosue, T. et al. (1998). ERD6, a cDNA clone for an early dehydration-induced gene of Arabidopsis, encodes a putative sugar transporter1The nucleotide sequence reported in this paper has been submitted to DDBJ with the accession number of D89051.1. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1370 (2), pp.187–191. [Online]. Available at: doi:10.1016/S0005-2736(98)00007-8.

Kobza, J. and Edwards, G. E. (1987). Influences of leaf temperature on photosynthetic carbon metabolism in wheat. *Plant physiology*, 83 (1), pp.69–74. [Online]. Available at: doi:10.1104/pp.83.1.69.

Kollist, H., Nuhkat, M. and Roelfsema, M. R. G. (2014). Closing gaps: linking elements that control stomatal movement. *The New phytologist*, 203 (1), pp.44–62. [Online]. Available at: doi:10.1111/nph.12832.

Komiya, R. et al. (2008). Hd3a and RFT1 are essential for flowering in rice. *Development* , 135 (4), pp.767–774. [Online]. Available at: doi:10.1242/dev.008631.

Komiya, R., Yokoi, S. and Shimamoto, K. (2009). A gene network for long-day flowering activates RFT1 encoding a mobile flowering signal in rice. *Development* , 136 (20), pp.3443–3450. [Online]. Available at: doi:10.1242/dev.040170.

Kosakivska, I. V. et al. (2022). Changes in hormonal status of winter wheat (Triticum aestivum L.) and spelt wheat (Triticum spelta L.) after heat stress and in recovery period. *Cereal research communications*, 50 (4), pp.821–830. [Online]. Available at: doi:10.1007/s42976-021-00206-5.

Kosova, K., Vitamvas, P. and Prasil, I. T. (2007). The role of dehydrins in plant response to cold. *Biologia plantarum*, 51 (4), pp.601–617. [Online]. Available at: doi:10.1007/s10535-007-0133-6.

Kosová, K., Vítámvás, P. and Prášil, I. T. (2014). Wheat and barley dehydrins under cold, drought, and salinity - what can LEA-II proteins tell us about plant stress response? *Frontiers in plant science*, 5, p.343. [Online]. Available at: doi:10.3389/fpls.2014.00343.

Kotak, S. et al. (2007). Complexity of the heat stress response in plants. *Current opinion in plant biology*, 10 (3), pp.310–316. [Online]. Available at: doi:10.1016/j.pbi.2007.04.011.

Krishnamurthy, P. et al. (2009). The role of root apoplastic transport barriers in salt tolerance of rice (Oryza sativa L.). *Planta*, 230 (1), pp.119–134. [Online]. Available at: doi:10.1007/s00425-009-0930-6.

Krishnamurthy, P. et al. (2011). Root apoplastic barriers block Na+ transport to shoots in rice (Oryza sativa L.). *Journal of experimental botany*, 62 (12), pp.4215–4228. [Online]. Available at: doi:10.1093/jxb/err135.

Krugman, T. et al. (2011). Alteration in expression of hormone-related genes in wild emmer wheat roots associated with drought adaptation mechanisms. *Functional & integrative genomics*, 11 (4), pp.565–583. [Online]. Available at: doi:10.1007/s10142-011-0231-6.

Kumar, A. et al. (2020). Genome-wide Identification and Characterization of Heat Shock Protein Family Reveals Role in Development and Stress Conditions in Triticum aestivum L. *Scientific reports*, 10 (1), p.7858. [Online]. Available at: doi:10.1038/s41598-020-64746-2.

Kumar, K. P. et al. (2019a). Rapid screening for drought tolerance in traditional landraces of rice (Oryza sativa L.) at seedling stage under hydroponics. *Electronic journal of plant breeding*, 10 (2), p.636. [Online]. Available at: doi:10.5958/0975-928x.2019.00080.2.

Kumar, M. et al. (2014). Over-expression of dehydrin gene, OsDhn1, improves drought and salt stress tolerance through scavenging of reactive oxygen species in rice (Oryza sativa L.). *Journal of plant biology = Singmul Hakhoe chi*, 57 (6), pp.383–393. [Online]. Available at: doi:10.1007/s12374-014-0487-1.

Kumar R R et al. (2013). Transcript profiling and biochemical characterization of mitochondrial superoxide dismutase (mtSOD) in Wheat (Triticum aestivum) under different exogenous stresses. *Australian journal of crop science*, 7 (3), pp.414–424. [Online]. Available at: doi:10.3316/informit.261218180083582.

Kumar, R. R. et al. (2018). Characterization of novel heat-responsive transcription factor (TaHSFA6e) gene involved in regulation of heat shock proteins (HSPs) - A key member of heat stress-tolerance network of wheat. *Journal of biotechnology*, 279, pp.1–12. [Online]. Available at: doi:10.1016/j.jbiotec.2018.05.008.

Kumar, R. R. et al. (2019b). RuBisCo activase—a catalytic chaperone involved in modulating the RuBisCo activity and heat stress-tolerance in wheat. *Journal of plant biochemistry and biotechnology*, 28 (1), pp.63–75. [Online]. Available at: doi:10.1007/s13562-018-0463-9.

Kumar, R. R. and Rai, R. D. (2014). Can Wheat Beat the Heat: Understanding the Mechanism of Thermotolerance in Wheat (Triticum aestivum L.). *Cereal research communications*, 42 (1), pp.1–18. [Online]. Available at: doi:10.1556/CRC.42.2014.1.1.

Kumimoto, R. W. et al. (2008). The Nuclear Factor Y subunits NF-YB2 and NF-YB3 play additive roles in the promotion of flowering by inductive long-day photoperiods in Arabidopsis. *Planta*, 228 (5), pp.709–723. [Online]. Available at: doi:10.1007/s00425-008-0773-6.

Kurahashi, Y., Terashima, A. and Takumi, S. (2009). Variation in dehydration tolerance, ABA sensitivity and related gene expression patterns in D-genome progenitor and synthetic hexaploid wheat lines. *International journal of molecular sciences*, 10 (6), pp.2733–2751. [Online]. Available at: doi:10.3390/ijms10062733.

Lal, M. K. et al. (2022). Physiological and molecular insights on wheat responses to heat stress. *Plant cell reports*, 41 (3), pp.501–518. [Online]. Available at: doi:10.1007/s00299-021-02784-4.

Lalonde, S., Beebe, D. U. and Saini, H. S. (1997). Early signs of disruption of wheat anther development associated with the induction of male sterility by meiotic-stage water deficit. *Sexual plant reproduction*, 10 (1), pp.40–48. [Online]. Available at: doi:10.1007/s004970050066.

Lan, Y. et al. (2022). Climate Change Impact on Wheat Performance—Effects on Vigour, Plant Traits and Yield from Early and Late Drought Stress in Diverse Lines. *International journal of molecular sciences*, 23 (6), p.3333. [Online]. Available at: doi:10.3390/ijms23063333 [Accessed 8 June 2022].

Lang, V. et al. (1994). Alterations in Water Status, Endogenous Abscisic Acid Content, and Expression of rab18 Gene during the Development of Freezing Tolerance in Arabidopsis thaliana. *Plant physiology*, 104 (4), pp.1341–1349. [Online]. Available at: doi:10.1104/pp.104.4.1341.

Lång, V. and Palva, E. T. (1992). The expression of a rab-related gene, rab18, is induced by abscisic acid during the cold acclimation process of Arabidopsis thaliana (L.) Heynh. *Plant molecular biology*, 20 (5), pp.951–962. [Online]. Available at: doi:10.1007/BF00027165.

Langer, S. M., Longin, C. F. H. and Würschum, T. (2014). Flowering time control in European winter wheat. *Frontiers in plant science*, 5, p.537. [Online]. Available at: doi:10.3389/fpls.2014.00537.

Langfelder, P. and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. *BMC bioinformatics*, 9, p.559. [Online]. Available at: doi:10.1186/1471-2105-9-559.

Langfelder, P. and Horvath, S. (2012). Fast R Functions for Robust Correlations and Hierarchical Clustering. *Journal of statistical software*, 46 (11). [Online]. Available at: doi:10.18637/jss.v046.i11.

Le Gall, H. et al. (2015). Cell Wall Metabolism in Response to Abiotic Stress. *Plants*, 4 (1), pp.112–166. [Online]. Available at: doi:10.3390/plants4010112.

Lee, C. et al. (2019). Genetic interactions reveal the antagonistic roles of FT/TSF and TFL1 in the determination of inflorescence meristem identity in Arabidopsis. *The Plant journal: for cell and molecular biology*, 99 (3), pp.452–464. [Online]. Available at: doi:10.1111/tpj.14335.

Lee, J. H. et al. (2013). Regulation of temperature-responsive flowering by MADS-box transcription factor repressors. *Science*, 342 (6158), pp.628–632. [Online]. Available at: doi:10.1126/science.1241097.

Lee, J. W. et al. (2020). Early Light-Inducible Protein (ELIP) Can Enhance Resistance to Cold-Induced Photooxidative Stress in Chlamydomonas reinhardtii. *Frontiers in physiology*, 11, p.1083. [Online]. Available at: doi:10.3389/fphys.2020.01083.

Lee, M. H. et al. (2022). Comparison of Gene Expression Changes in Three Wheat Varieties with Different Susceptibilities to Heat Stress Using RNA-Seq Analysis. *International journal of molecular sciences*, 23 (18). [Online]. Available at: doi:10.3390/ijms231810734.

Lee, S. et al. (2000). The yeast heat shock transcription factor changes conformation in response to superoxide and temperature. *Molecular biology of the cell*, 11 (5), pp.1753–1764. [Online]. Available at: doi:10.1091/mbc.11.5.1753.

Levy, A. A. and Feldman, M. (2022). Evolution and origin of bread wheat. *The Plant cell*, 34 (7), pp.2549–2567. [Online]. Available at: doi:10.1093/plcell/koac130.

Lévy, E. et al. (2019). Causative Links between Protein Aggregation and Oxidative Stress: A Review. *International journal of molecular sciences*, 20 (16). [Online]. Available at: doi:10.3390/ijms20163896.

Li, C., Lin, H. and Dubcovsky, J. (2015). Factorial combinations of protein interactions generate a multiplicity of florigen activation complexes in wheat and barley. *The Plant journal: for cell and molecular biology*, 84 (1), pp.70–82. [Online]. Available at: doi:10.1111/tpj.12960.

Li, H., Ruan, J. and Durbin, R. (2008). Mapping short DNA sequencing reads and calling variants using mapping quality scores. *Genome research*, 18 (11), pp.1851–1858. [Online]. Available at: doi:10.1101/gr.078212.108.

Li, J. et al. (2013). Wheat 14-3-3 Protein Conferring Growth Retardation in Arabidopsis. *Journal of integrative agriculture*, 12 (2), pp.209–217. [Online]. Available at: doi:10.1016/S2095-3119(13)60220-8.

Li, J. et al. (2014). A MITE insertion into the 3′-UTR regulates the transcription of TaHSP16.9 in common wheat. *The Crop Journal*, 2 (6), pp.381–387. [Online]. Available at: doi:10.1016/j.cj.2014.07.001.

Li, J. et al. (2016). The Populus trichocarpa PtHSP17.8 involved in heat and salt stress tolerances. *Plant cell reports*, 35 (8), pp.1587–1599. [Online]. Available at: doi:10.1007/s00299-016-1973-3.

Li, L. et al. (2019). Genetic dissection of drought and heat-responsive agronomic traits in wheat. *Plant, cell & environment*, 42 (9), pp.2540–2553. [Online]. Available at: doi:10.1111/pce.13577.

Li, N. et al. (2020). Plant Hormone-Mediated Regulation of Heat Tolerance in Response to Global Climate Change. *Frontiers in plant science*, 11, p.627969. [Online]. Available at: doi:10.3389/fpls.2020.627969.

Li, Q. et al. (2017). Physcomitrella Patens Dehydrins (PpDHNA and PpDHNC) Confer Salinity and Drought Tolerance to Transgenic Arabidopsis Plants. *Frontiers in plant science*, 8, p.1316. [Online]. Available at: doi:10.3389/fpls.2017.01316.

Li, Q. et al. (2023). DNA methylation levels of TaP5CS and TaBADH are associated with enhanced tolerance to PEG-induced drought stress triggered by drought priming in wheat. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 200, p.107769. [Online]. Available at: doi:10.1016/j.plaphy.2023.107769.

Li, Y. et al. (2021). Dual roles of the serine/arginine-rich splicing factor SR45a in promoting and interacting with nuclear cap-binding complex to modulate the salt-stress response in Arabidopsis. *The New phytologist*, 230 (2), pp.641–655. [Online]. Available at: doi:10.1111/nph.17175.

Liang, Y. et al. (2021). Transcriptome profiling of two rice genotypes under mild field drought stress during grain-filling stage. *AoB plants*, 13 (4), p.lab043. [Online]. Available at: doi:10.1093/aobpla/plab043.

Lim, J., Lim, C. W. and Lee, S. C. (2018). The Pepper Late Embryogenesis Abundant Protein, CaDIL1, Positively Regulates Drought Tolerance and ABA Signaling. *Frontiers in plant science*, 9, p.1301. [Online]. Available at: doi:10.3389/fpls.2018.01301.

Lima, R. B. et al. (2013). Heat stress causes alterations in the cell-wall polymers and anatomy of coffee leaves (Coffea arabica L.). *Carbohydrate polymers*, 93 (1), pp.135–143. [Online]. Available at: doi:10.1016/j.carbpol.2012.05.015.

Lin, Y. et al. (2019). Dissection of Phenotypic and Genetic Variation of Drought-Related Traits in Diverse Chinese Wheat Landraces. *The plant genome*, 12 (3), pp.1–14. [Online]. Available at: doi:10.3835/plantgenome2019.03.0025.

Ling, Y., Mahfouz, M. M. and Zhou, S. (2021). Pre-mRNA alternative splicing as a modulator for heat stress response in plants. *Trends in plant science*, 26 (11), pp.1153–1170. [Online]. Available at: doi:10.1016/j.tplants.2021.07.008.

Líška, D. et al. (2016). Asymmetrical development of root endodermis and exodermis in reaction to abiotic stresses. *Annals of botany*, 118 (4), pp.667–674. [Online]. Available at: doi:10.1093/aob/mcw047.

Liu, B. et al. (2016). Testing the responses of four wheat crop models to heat stress at anthesis and grain filling. *Global change biology*, 22 (5), pp.1890–1903. [Online]. Available at: doi:10.1111/gcb.13212.

Liu, B. et al. (2021). Functional specificity, diversity, and redundancy of Arabidopsis JAZ family repressors in jasmonate and COI1-regulated growth, development, and defense. *The New phytologist*, 231 (4), pp.1525–1545. [Online]. Available at: doi:10.1111/nph.17477.

Liu, G. et al. (2022). The deubiquitinases UBP12 and UBP13 integrate with the E3 ubiquitin ligase XBAT35.2 to modulate VPS23A stability in ABA signaling. *Science advances*, 8 (14), p.eabl5765. [Online]. Available at: doi:10.1126/sciadv.abl5765.

Liu, H. et al. (2019). Genome-wide identification of and functional insights into the late embryogenesis abundant (LEA) gene family in bread wheat (Triticum aestivum). *Scientific reports*, 9 (1), p.13375. [Online]. Available at: doi:10.1038/s41598-019-49759-w.

Liu, H. et al. (2020a). Transcription factor TabHLH49 positively regulates dehydrin WZY2 gene expression and enhances drought stress tolerance in wheat. *BMC plant biology*, 20 (1), p.259. [Online]. Available at: doi:10.1186/s12870-020-02474-5.

Liu, J. et al. (2012a). PsbP-domain protein1, a nuclear-encoded thylakoid lumenal protein, is essential for photosystem I assembly in Arabidopsis. *The Plant cell*, 24 (12), pp.4992–5006. [Online]. Available at: doi:10.1105/tpc.112.106542.

Liu, L., Wang, X. and Chang, C. (2022). Toward a smart skin: Harnessing cuticle biosynthesis for crop adaptation to drought, salinity, temperature, and ultraviolet stress. *Frontiers in plant science*, 13, p.961829. [Online]. Available at: doi:10.3389/fpls.2022.961829.

Liu, R. et al. (2013). Light-harvesting chlorophyll a/b-binding proteins, positively involved in abscisic acid signalling, require a transcription repressor, WRKY40, to balance their function. *Journal of experimental botany*, 64 (18), pp.5443–5456. [Online]. Available at: doi:10.1093/jxb/ert307.

Liu, X. et al. (2012b). AtPP2CG1, a protein phosphatase 2C, positively regulates salt tolerance of Arabidopsis in abscisic acid-dependent manner. *Biochemical and biophysical research communications*, 422 (4), pp.710–715. [Online]. Available at: doi:10.1016/j.bbrc.2012.05.064.

Liu, Y. et al. (2010). Contributions of climatic and crop varietal changes to crop production in the North China Plain, since 1980s. *Global change biology*, 16 (8), pp.2287–2299. [Online]. Available at: doi:10.1111/j.1365-2486.2009.02077.x.

Liu, Z. et al. (2015). Temporal transcriptome profiling reveals expression partitioning of homeologous genes contributing to heat and drought acclimation in wheat (Triticum aestivum

L.). *BMC plant biology*, 15 (1), pp.1–20. [Online]. Available at: doi:10.1186/s12870-015-0511-8 [Accessed 15 March 2023].

Liu, Z. et al. (2017). Plasma Membrane CRPK1-Mediated Phosphorylation of 14-3-3 Proteins Induces Their Nuclear Import to Fine-Tune CBF Signaling during Cold Response. *Molecular cell*, 66 (1), pp.117-128.e5. [Online]. Available at: doi:10.1016/j.molcel.2017.02.016.

Liu, Z. et al. (2020b). TaHsfA2-1, a new gene for thermotolerance in wheat seedlings: Characterization and functional roles. *Journal of plant physiology*, 246–247, p.153135. [Online]. Available at: doi:10.1016/j.jplph.2020.153135.

Lohani, N., Singh, M. B. and Bhalla, P. L. (2022). Rapid Transcriptional Reprogramming Associated With Heat Stress-Induced Unfolded Protein Response in Developing Brassica napus Anthers. *Frontiers in plant science*, 13, p.905674. [Online]. Available at: doi:10.3389/fpls.2022.905674.

Lopes, M. S. et al. (2015). Exploiting genetic diversity from landraces in wheat breeding for adaptation to climate change. *Journal of experimental botany*, 66 (12), pp.3477–3486. [Online]. Available at: doi:10.1093/jxb/erv122.

Lopez, C. G. et al. (2003). Dehydrin expression and drought tolerance in seven wheat cultivars. *Crop science*, 43 (2), pp.577–582. [Online]. Available at: doi:10.2135/cropsci2003.5770.

Lorenzo, O. et al. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *The Plant cell*, 15 (1), pp.165–178. [Online]. Available at: doi:10.1105/tpc.007468.

Loukoianov, A. et al. (2005). Regulation of VRN-1 vernalization genes in normal and transgenic polyploid wheat. *Plant physiology*, 138 (4), pp.2364–2373. [Online]. Available at: doi:10.1104/pp.105.064287.

Love, M. I., Huber, W. and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology*, 15 (12), p.550. [Online]. Available at: doi:10.1186/s13059-014-0550-8.

Loveys, B. R. and Kriedemann, P. E. (1974). Internal Control of Stomatal Physiology and Photosynthesis. I. Stomatal Regulation and Associated Changes in Endogenous Levels of Abscisic and Phaseic Acids. *Functional plant biology: FPB*, 1 (3), pp.407–415. [Online]. Available at: doi:10.1071/pp9740407 [Accessed 22 August 2023].

Lu, L. et al. (2022). Wheat genotypes tolerant to heat at seedling stage tend to be also tolerant at adult stage: The possibility of early selection for heat tolerance breeding. *The Crop Journal*, 10 (4), pp.1006–1013. [Online]. Available at: doi:10.1016/j.cj.2022.01.005.

Luna, C. M. et al. (2005). Drought controls on H2O2 accumulation, catalase (CAT) activity and CAT gene expression in wheat. *Journal of experimental botany*, 56 (411), pp.417–423. [Online]. Available at: doi:10.1093/jxb/eri039.

Luo, R. et al. (2022). The barley DIR gene family: An expanded gene family that is involved in stress responses. *Frontiers in genetics*, 13, p.1042772. [Online]. Available at: doi:10.3389/fgene.2022.1042772.

Lv, L. et al. (2020). Gene co-expression network analysis to identify critical modules and candidate genes of drought-resistance in wheat. *PloS one*, 15 (8), p.e0236186. [Online]. Available at: doi:10.1371/journal.pone.0236186.

Lv, L. et al. (2022). Transcription-associated metabolomic profiling reveals the critical role of frost tolerance in wheat. *BMC plant biology*, 22 (1), p.333. [Online]. Available at: doi:10.1186/s12870-022-03718-2.

Lv, S. et al. (2018). Comparative Analysis of the Transcriptional Response of Tolerant and Sensitive Wheat Genotypes to Drought Stress in Field Conditions. *Agronomy*, 8 (11), p.247. [Online]. Available at: doi:10.3390/agronomy8110247 [Accessed 23 August 2021].

Ma, J. et al. (2017). Transcriptomics Analyses Reveal Wheat Responses to Drought Stress during Reproductive Stages under Field Conditions. *Frontiers in plant science*, 8, p.592. [Online]. Available at: doi:10.3389/fpls.2017.00592.

Ma, J. et al. (2022). A NAC transcription factor, TaNAC5D-2, acts as a positive regulator of drought tolerance through regulating water loss in wheat (Triticum aestivum L.). *Environmental and experimental botany*, 196, p.104805. [Online]. Available at: doi:10.1016/j.envexpbot.2022.104805.

Ma, L. and Li, G. (2018). FAR1-RELATED SEQUENCE (FRS) and FRS-RELATED FACTOR (FRF) Family Proteins in Arabidopsis Growth and Development. *Frontiers in plant science*, 9, p.692. [Online]. Available at: doi:10.3389/fpls.2018.00692.

Magwanga, R. O. et al. (2018). Characterization of the late embryogenesis abundant (LEA) proteins family and their role in drought stress tolerance in upland cotton. *BMC genetics*, 19 (1), p.6. [Online]. Available at: doi:10.1186/s12863-017-0596-1.

Mahajan, S. and Tuteja, N. (2005). Cold, salinity and drought stresses: an overview. *Archives of biochemistry and biophysics*, 444 (2), pp.139–158. [Online]. Available at: doi:10.1016/j.abb.2005.10.018.

Mahpara, S. et al. (2022). The impact of PEG-induced drought stress on seed germination and seedling growth of different bread wheat (Triticum aestivum L.) genotypes. *PloS one*, 17 (2), p.e0262937. [Online]. Available at: doi:10.1371/journal.pone.0262937.

Majid, S. A., Asghar, R. and Murtaza, G. (2007). Yield stability analysis conferring adaptation of wheat to pre-and post-anthesis drought conditions. *Pakistan journal of botany*, 39 (5), pp.1623–1637. [Online]. Available at: https://www.academia.edu/download/41681924/PJB3951623.pdf [Accessed 18 August 2023].

Major, I. T. et al. (2017). Regulation of growth-defense balance by the JASMONATE ZIM-DOMAIN (JAZ)-MYC transcriptional module. *The New phytologist*, 215 (4), pp.1533–1547. [Online]. Available at: doi:10.1111/nph.14638.

Malefo, M. B. et al. (2020). Investigating the role of Bowman-Birk serine protease inhibitor in Arabidopsis plants under drought stress. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 149, pp.286–293. [Online]. Available at: doi:10.1016/j.plaphy.2020.02.007.

Manès, Y. et al. (2012). Genetic yield gains of the CIMMYT international semi-arid wheat yield trials from 1994 to 2010. *Crop science*, 52 (4), pp.1543–1552. [Online]. Available at: doi:10.2135/cropsci2011.10.0574.

Mantyla, E., Lang, V. and Palva, E. T. (1995). Role of Abscisic Acid in Drought-Induced Freezing Tolerance, Cold Acclimation, and Accumulation of LT178 and RAB18 Proteins in Arabidopsis thaliana. *Plant physiology*, 107 (1), pp.141–148. [Online]. Available at: doi:10.1104/pp.107.1.141.

Mao, H. et al. (2020). Regulatory changes in TaSNAC8-6A are associated with drought tolerance in wheat seedlings. *Plant biotechnology journal*, 18 (4), pp.1078–1092. [Online]. Available at: doi:10.1111/pbi.13277.

Mao, H. et al. (2022a). The wheat ABA receptor gene TaPYL1-1B contributes to drought tolerance and grain yield by increasing water-use efficiency. *Plant biotechnology journal*, 20 (5), pp.846–861. [Online]. Available at: doi:10.1111/pbi.13764.

Mao, H. et al. (2022b). Variation in cis-regulation of a NAC transcription factor contributes to drought tolerance in wheat. *Molecular plant*, 15 (2), pp.276–292. [Online]. Available at: doi:10.1016/j.molp.2021.11.007.

Mao, X. et al. (2012). TaNAC2, a NAC-type wheat transcription factor conferring enhanced multiple abiotic stress tolerances in Arabidopsis. *Journal of experimental botany*, 63 (8), pp.2933–2946. [Online]. Available at: doi:10.1093/jxb/err462.

Mase, K. and Tsukagoshi, H. (2021). Reactive Oxygen Species Link Gene Regulatory Networks During Arabidopsis Root Development. *Frontiers in plant science*, 12, p.660274. [Online]. Available at: doi:10.3389/fpls.2021.660274.

Mathew, I. et al. (2019). Genome-wide association study of drought tolerance and biomass allocation in wheat. *PloS one*, 14 (12), p.e0225383. [Online]. Available at: doi:10.1371/journal.pone.0225383.

Mathur, S., Agrawal, D. and Jajoo, A. (2014). Photosynthesis: response to high temperature stress. *Journal of photochemistry and photobiology. B, Biology*, 137, pp.116–126. [Online]. Available at: doi:10.1016/j.jphotobiol.2014.01.010.

Matsunaga, S. et al. (2021). Stage-Specific Characterization of Physiological Response to Heat Stress in the Wheat Cultivar Norin 61. *International journal of molecular sciences*, 22 (13). [Online]. Available at: doi:10.3390/ijms22136942.

Matsuoka, D. et al. (2015). An abscisic acid inducible Arabidopsis MAPKKK, MAPKKK18 regulates leaf senescence via its kinase activity. *Plant molecular biology*, 87 (6), pp.565–575. [Online]. Available at: doi:10.1007/s11103-015-0295-0.

Maulana, F. et al. (2020). Genome-Wide Association Mapping of Seedling Drought Tolerance in Winter Wheat. *Frontiers in plant science*, 11, p.573786. [Online]. Available at: doi:10.3389/fpls.2020.573786.

Maurastoni, M. et al. (2023). A Capsid Protein Fragment of a Fusagra-like Virus Found in Carica papaya Latex Interacts with the 50S Ribosomal Protein L17. *Viruses*, 15 (2). [Online]. Available at: doi:10.3390/v15020541.

Maurel, C. et al. (2008). Plant aquaporins: membrane channels with multiple integrated functions. *Annual review of plant biology*, 59, pp.595–624. [Online]. Available at: doi:10.1146/annurev.arplant.59.032607.092734.

Mayfield, J. D. et al. (2007). The 14-3-3 Proteins mu and upsilon influence transition to flowering and early phytochrome response. *Plant physiology*, 145 (4), pp.1692–1702. [Online]. Available at: doi:10.1104/pp.107.108654.

Mazelis, M. and Vennesland, B. (1957). Carbon Dioxide Fixation into Oxalacetate in Higher Plants. *Plant physiology*, 32 (6), pp.591–600. [Online]. Available at: doi:10.1104/pp.32.6.591.

McClellan, A. J. et al. (2005). Protein quality control: chaperones culling corrupt conformations. *Nature cell biology*, 7 (8), pp.736–741. [Online]. Available at: doi:10.1038/ncb0805-736.

McLoughlin, F. et al. (2019). HSP101 Interacts with the Proteasome and Promotes the Clearance of Ubiquitylated Protein Aggregates. *Plant physiology*, 180 (4), pp.1829–1847. [Online]. Available at: doi:10.1104/pp.19.00263.

Medina, J. et al. (1999). The Arabidopsis CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression Is regulated by low temperature but not by abscisic acid or dehydration. *Plant physiology*, 119 (2), pp.463–470. [Online]. Available at: doi:10.1104/pp.119.2.463.

Mei, F. et al. (2021). Overexpression of the wheat NAC transcription factor TaSNAC4-3A gene confers drought tolerance in transgenic Arabidopsis. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 160, pp.37–50. [Online]. Available at: doi:10.1016/j.plaphy.2021.01.004.

Merino, I. et al. (2014). Plantation forestry under global warming: hybrid poplars with improved thermotolerance provide new insights on the in vivo function of small heat shock protein chaperones. *Plant physiology*, 164 (2), pp.978–991. [Online]. Available at: doi:10.1104/pp.113.225730.

Min, J.-H. et al. (2015). The CONSTANS-like 4 transcription factor, AtCOL4, positively regulates abiotic stress tolerance through an abscisic acid-dependent manner in Arabidopsis. *Journal of integrative plant biology*, 57 (3), pp.313–324. [Online]. Available at: doi:10.1111/jipb.12246.

Mishra, D. C. et al. (2021). Weighted gene co-expression analysis for identification of key genes regulating heat stress in wheat. *Cereal research communications*, 49 (1), pp.73–81. [Online]. Available at: doi:10.1007/s42976-020-00072-7.

Mishra, R. C. and Grover, A. (2016). ClpB/Hsp100 proteins and heat stress tolerance in plants. *Critical reviews in biotechnology*, 36 (5), pp.862–874. [Online]. Available at: doi:10.3109/07388551.2015.1051942.

Mishra, S. S., Behera, P. K. and Panda, D. (2019). Genotypic variability for drought tolerance-related morpho-physiological traits among indigenous rice landraces of Jeypore tract of Odisha, India. *Journal of Crop Improvement*, 33 (2), pp.254–278. [Online]. Available at: doi:10.1080/15427528.2019.1579138.

Mitula, F. et al. (2015). Arabidopsis ABA-Activated Kinase MAPKKK18 is Regulated by Protein Phosphatase 2C ABI1 and the Ubiquitin-Proteasome Pathway. *Plant & cell physiology*, 56 (12), pp.2351–2367. [Online]. Available at: doi:10.1093/pcp/pcv146.

Miyamoto, K. et al. (2013). Stress-induced expression of the transcription factor RERJ1 is tightly regulated in response to jasmonic acid accumulation in rice. *Protoplasma*, 250 (1), pp.241–249. [Online]. Available at: doi:10.1007/s00709-012-0400-z.

Mogk, A., Bukau, B. and Kampinga, H. H. (2018). Cellular Handling of Protein Aggregates by Disaggregation Machines. *Molecular cell*, 69 (2), pp.214–226. [Online]. Available at: doi:10.1016/j.molcel.2018.01.004.

Mohanty, S., Grimm, B. and Tripathy, B. C. (2006). Light and dark modulation of chlorophyll biosynthetic genes in response to temperature. *Planta*, 224 (3), pp.692–699. [Online]. Available at: doi:10.1007/s00425-006-0248-6.

Mohi-Ud-Din, M. et al. (2021). Physiological and Biochemical Dissection Reveals a Trade-off Between Antioxidant Capacity and Heat Tolerance in Bread Wheat (Triticum aestivum L.). *Antioxidants (Basel, Switzerland)*, 10 (3). [Online]. Available at: doi:10.3390/antiox10030351.

Møller, S. G. and McPherson, M. J. (1998). Developmental expression and biochemical analysis of the Arabidopsis atao1 gene encoding an H2O2-generating diamine oxidase. *The Plant journal: for cell and molecular biology*, 13 (6), pp.781–791. [Online]. Available at: doi:10.1046/j.1365-313x.1998.00080.x.

Monneveux, P. et al. (2003). Effect of T1BL.1RS chromosome translocation on bread wheat grain yield and physiological related traits in a warm environment. *Cereal research communications*, 31 (3), pp.371–378. [Online]. Available at: doi:10.1007/BF03543367.

Muhammad Aslam, M. et al. (2022). Mechanisms of Abscisic Acid-Mediated Drought Stress Responses in Plants. *International journal of molecular sciences*, 23 (3). [Online]. Available at: doi:10.3390/ijms23031084.

Munasinghe, S. P. et al. (2017). Screening of Sri Lankan rice (Oryza sativa L.) landraces for drought tolerance. *Tropical agricultural research: proceedings of the ... Annual Congress of the Postgraduate Institute for Agriculture, Peradeniya. University of Peradeniya. Postgraduate Institute of Agriculture. Congress*, 28 (2), pp.183–191. [Online]. Available at: https://www.cabdirect.org/cabdirect/abstract/20183099746.

Münch-Garthoff, S. et al. (1997). Expression of beta-1,3-glucanase and chitinase in healthy, stem-rust-affected and elicitor-treated near-isogenic wheat lines showing Sr5-or Sr24-specified race-specific rust resistance. *Planta*, 201 (2), pp.235–244. [Online]. Available at: doi:10.1007/BF01007709.

Munns, R. and Sharp, R. E. (1993). Involvement of Abscisic Acid in Controlling Plant Growth in Soil of Low Water Potential. *Functional plant biology: FPB*, 20 (5), pp.425–437. [Online]. Available at: doi:10.1071/pp9930425 [Accessed 22 August 2023].

Murakami, T. et al. (2004). Over-expression of a small heat shock protein, sHSP17.7, confers both heat tolerance and UV-B resistance to rice plants. *Molecular breeding: new strategies in plant improvement*, 13 (2), pp.165–175. [Online]. Available at: doi:10.1023/B:MOLB.0000018764.30795.c1.

Murphy, J. M. et al. (2004). Quantification of modelling uncertainties in a large ensemble of climate change simulations. *Nature*, 430 (7001), pp.768–772. [Online]. Available at: doi:10.1038/nature02771.

Muvunyi, B. P. et al. (2022). Identification of Potential Zinc Deficiency Responsive Genes and Regulatory Pathways in Rice by Weighted Gene Co-expression Network Analysis. *Rice Science*, 29 (6), pp.545–558. [Online]. Available at: doi:10.1016/j.rsci.2022.04.002.

Naderi, S. et al. (2020). Tolerance responses in wheat landrace Bolani are related to enhanced metabolic adjustments under drought stress. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 150, pp.244–253. [Online]. Available at: doi:10.1016/j.plaphy.2020.03.002.

Nagai, T. and Makino, A. (2009). Differences between rice and wheat in temperature responses of photosynthesis and plant growth. *Plant & cell physiology*, 50 (4), pp.744–755. [Online]. Available at: doi:10.1093/pcp/pcp029.

Nahar, K., Ahamed, K. U. and Fujita, M. (2010). Phenological Variation and its Relation with Yield in several Wheat (Triticum aestivum L.) Cultivars under Normal and Late Sowing

Mediated Heat Stress Condition. *Notulae Scientia Biologicae*, 2 (3), pp.51–56. [Online]. Available at: doi:10.15835/nsb234723 [Accessed 17 August 2023].

Nakajima, Y. and Suzuki, S. (2013). Environmental stresses induce misfolded protein aggregation in plant cells in a microtubule-dependent manner. *International journal of molecular sciences*, 14 (4), pp.7771–7783. [Online]. Available at: doi:10.3390/ijms14047771.

Nardino, M. et al. (2022). Understanding drought response mechanisms in wheat and multi-trait selection. *PloS one*, 17 (4), p.e0266368. [Online]. Available at: doi:10.1371/journal.pone.0266368.

Nieves-Cordones, M. et al. (2010). The Arabidopsis thaliana HAK5 K+ transporter is required for plant growth and K+ acquisition from low K+ solutions under saline conditions. *Molecular plant*, 3 (2), pp.326–333. [Online]. Available at: doi:10.1093/mp/ssp102.

Niu, C.-F. et al. (2012). Wheat WRKY genes TaWRKY2 and TaWRKY19 regulate abiotic stress tolerance in transgenic Arabidopsis plants. *Plant, cell & environment*, 35 (6), pp.1156–1170. [Online]. Available at: doi:10.1111/j.1365-3040.2012.02480.x.

Niu, X. et al. (2020). Identification of wheat DREB genes and functional characterization of TaDREB3 in response to abiotic stresses. *Gene*, 740, p.144514. [Online]. Available at: doi:10.1016/j.gene.2020.144514.

Niu, Y., Figueroa, P. and Browse, J. (2011). Characterization of JAZ-interacting bHLH transcription factors that regulate jasmonate responses in Arabidopsis. *Journal of experimental botany*, 62 (6), pp.2143–2154. [Online]. Available at: doi:10.1093/jxb/erq408.

NOAA National Centers for Environmental Information. (2018). *Monthly National Climate Report for May 2018*. [Online]. Available at: www.ncei.noaa.gov/access/monitoring/monthly-report/national/201805 [Accessed 30 March 2023].

NOAA National Centers for Environmental Information. (2020). *Monthly Global Climate Report for May 2020*. [Online]. Available at: https://www.ncei.noaa.gov/access/monitoring/monthly-report/global/202005 [Accessed 30 March 2023].

NOAA National Centers for Environmental Information. (2022a). *Monthly Global Climate Report for May 2022*. [Online]. Available at: https://www.ncei.noaa.gov/access/monitoring/monthly-report/global/202205 [Accessed 20 March 2023].

NOAA National Centers for Environmental Information. (2022b). *Monthly National Climate Report for May 2022*. [Online]. Available at: https://www.ncei.noaa.gov/access/monitoring/monthly-report/national/202205 [Accessed 30 March 2023].

NOAA National Centers for Environmental Information. (2022c). *State of the Climate: Monthly Global Climate Report for April 2022*. [Online]. Available at: https://www.ncei.noaa.gov/access/monitoring/monthly-report/global/202204 [Accessed 25 October 2022].

NOAA National Centers for Environmental Information. (2022d). *U.S. Drought Monitor Update for April 12, 2022*. [Online]. Available at: https://www.ncei.noaa.gov/news/us-drought-monitor-update-april-12-2022#:~:text=According%20to%20the%20April%2012,decrease%20from%20last%20week's%2048.0%25. [Accessed 25 October 2022].

Nolan, T., Chen, J. and Yin, Y. (2017). Cross-talk of Brassinosteroid signaling in controlling growth and stress responses. *Biochemical Journal*, 474 (16), pp.2641–2661. [Online]. Available at: doi:10.1042/BCJ20160633.

Noman, A. et al. (2018). Sugar beet extract acts as a natural bio-stimulant for physio-biochemical attributes in water stressed wheat (Triticum aestivum L.). *Acta physiologiae plantarum / Polish Academy of Sciences, Committee of Plant Physiology Genetics and Breeding*, 40 (6), p.110. [Online]. Available at: doi:10.1007/s11738-018-2681-0.

Nouraei, S. et al. (2022). Transcriptome Analyses of Near Isogenic Lines Reveal Putative Drought Tolerance Controlling Genes in Wheat. *Frontiers in plant science*, 13, p.857829. [Online]. Available at: doi:10.3389/fpls.2022.857829.

Oblessuc, P. R. et al. (2020). JAZ4 is involved in plant defense, growth, and development in Arabidopsis. *The Plant journal: for cell and molecular biology*, 101 (2), pp.371–383. [Online]. Available at: doi:10.1111/tpj.14548.

Ogiso-Tanaka, E. et al. (2013). Natural variation of the RICE FLOWERING LOCUS T 1 contributes to flowering time divergence in rice. *PloS one*, 8 (10), p.e75959. [Online]. Available at: doi:10.1371/journal.pone.0075959.

Ohama, N. et al. (2017). Transcriptional Regulatory Network of Plant Heat Stress Response. *Trends in plant science*, 22 (1), pp.53–65. [Online]. Available at: doi:10.1016/j.tplants.2016.08.015.

Olvera-Carrillo, Y. et al. (2010). Functional analysis of the group 4 late embryogenesis abundant proteins reveals their relevance in the adaptive response during water deficit in Arabidopsis. *Plant physiology*, 154 (1), pp.373–390. [Online]. Available at: doi:10.1104/pp.110.158964.

Omidi, M. et al. (2014). The Influence of Terminal Heat Stress on Meiosis Abnormalities in Pollen Mother Cells of Wheat. *Cytologia*, 79 (1), pp.49–58. [Online]. Available at: doi:10.1508/cytologia.79.49.

Ooka, H. et al. (2003). Comprehensive analysis of NAC family genes in Oryza sativa and Arabidopsis thaliana. *DNA research: an international journal for rapid publication of reports on genes and genomes*, 10 (6), pp.239–247. [Online]. Available at: doi:10.1093/dnares/10.6.239.

Oury, F.-X. et al. (2012). A study of genetic progress due to selection reveals a negative effect of climate change on bread wheat yield in France. *European journal of agronomy: the journal of the European Society for Agronomy*, 40, pp.28–38. [Online]. Available at: doi:10.1016/j.eja.2012.02.007.

Panikashvili, D. et al. (2009). The Arabidopsis DCR encoding a soluble BAHD acyltransferase is required for cutin polyester formation and seed hydration properties. *Plant physiology*, 151 (4), pp.1773–1789. [Online]. Available at: doi:10.1104/pp.109.143388.

Paradis, S. et al. (2011). Arabidopsis thaliana lipid phosphate phosphatase 2 is involved in abscisic acid signalling in leaves. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 49 (3), pp.357–362. [Online]. Available at: doi:10.1016/j.plaphy.2011.01.010.

Park, J.-E. et al. (2007). GH3-mediated Auxin Homeostasis Links Growth Regulation with Stress Adaptation Response in Arabidopsis*. *The Journal of biological chemistry*, 282 (13), pp.10036–10046. [Online]. Available at: doi:10.1074/jbc.M610524200.

Park, S. J. et al. (2009). Cold shock domain proteins affect seed germination and growth of Arabidopsis thaliana under abiotic stress conditions. *Plant & cell physiology*, 50 (4), pp.869–878. [Online]. Available at: doi:10.1093/pcp/pcp037.

Parry, M. A. J. et al. (2002). Rubisco activity: effects of drought stress. *Annals of botany*, 89 Spec No (7), pp.833–839. [Online]. Available at: doi:10.1093/aob/mcf103.

Parry, M. A. J. et al. (2009). Mutation discovery for crop improvement. *Journal of experimental botany*, 60 (10), pp.2817–2825. [Online]. Available at: doi:10.1093/jxb/erp189.

Pasriga, R. et al. (2019). Overexpression of RICE FLOWERING LOCUS T 1 (RFT1) Induces Extremely Early Flowering in Rice. *Molecules and cells*, 42 (5), pp.406–417. [Online]. Available at: doi:10.14348/molcells.2019.0009.

Patel, J. and Mishra, A. (2021). Plant aquaporins alleviate drought tolerance in plants by modulating cellular biochemistry, root-architecture, and photosynthesis. *Physiologia plantarum*, 172 (2), pp.1030–1044. [Online]. Available at: doi:10.1111/ppl.13324.

Patro, R. et al. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nature methods*, 14 (4), pp.417–419. [Online]. Available at: doi:10.1038/nmeth.4197.

Paull, J. G., Nable, R. O. and Rathjen, A. J. (1992). Physiological and genetic control of the tolerance of wheat to high concentrations of boron and implications for plant breeding. *Plant and soil*, 146 (1), pp.251–260. [Online]. Available at: doi:10.1007/BF00012019.

Pavlopoulos, G. A. et al. (2011). Using graph theory to analyze biological networks. *BioData mining*, 4, p.10. [Online]. Available at: doi:10.1186/1756-0381-4-10.

Peng, J. H., Sun, D. and Nevo, E. (2011). Domestication evolution, genetics and genomics in wheat. *Molecular breeding: new strategies in plant improvement*, 28 (3), pp.281–301. [Online]. Available at: doi:10.1007/s11032-011-9608-4.

Perdomo, J. A. et al. (2017). Rubisco and Rubisco Activase Play an Important Role in the Biochemical Limitations of Photosynthesis in Rice, Wheat, and Maize under High Temperature and Water Deficit. *Frontiers in plant science*, 8, p.490. [Online]. Available at: doi:10.3389/fpls.2017.00490.

Petersen, G. et al. (2006). Phylogenetic relationships of Triticum and Aegilops and evidence for the origin of the A, B, and D genomes of common wheat (Triticum aestivum). *Molecular phylogenetics and evolution*, 39 (1), pp.70–82. [Online]. Available at: doi:10.1016/j.ympev.2006.01.023.

Pfeifer, M. et al. (2014). Genome interplay in the grain transcriptome of hexaploid bread wheat. *Science*, 345 (6194), p.1250091. [Online]. Available at: doi:10.1126/science.1250091.

Pfeifer, W. H. et al. (2005). Breeding for abiotic stress tolerance in wheat. In: *Abiotic Stresses*. CRC Press. pp.423–512. [Online]. Available at: doi:10.1201/9781482293609-21.

Pingali, P. L. (2012). Green revolution: impacts, limits, and the path ahead. *Proceedings of the National Academy of Sciences of the United States of America*, 109 (31), pp.12302–12308. [Online]. Available at: doi:10.1073/pnas.0912953109.

Pinski, A. et al. (2021). Changes in the Cell Wall Proteome of Leaves in Response to High Temperature Stress in Brachypodium distachyon. *International journal of molecular sciences*, 22 (13). [Online]. Available at: doi:10.3390/ijms22136750.

Pirasteh-Anosheh, H. et al. (2016). Stomatal responses to drought stress. In: *Water Stress and Crop Plants*. Chichester, UK: John Wiley & Sons, Ltd. pp.24–40. [Online]. Available at: doi:10.1002/9781119054450.ch3.

Placido, D. F. et al. (2013). Introgression of novel traits from a wild wheat relative improves drought adaptation in wheat. *Plant physiology*, 161 (4), pp.1806–1819. [Online]. Available at: doi:10.1104/pp.113.214262.

Plaut, Z. et al. (2004). Transport of dry matter into developing wheat kernels and its contribution to grain yield under post-anthesis water deficit and elevated temperature. *Field crops research*, 86 (2), pp.185–198. [Online]. Available at: doi:10.1016/j.fcr.2003.08.005.

Pospíšil, P. (2016). Production of Reactive Oxygen Species by Photosystem II as a Response to Light and Temperature Stress. *Frontiers in plant science*, 7, p.1950. [Online]. Available at: doi:10.3389/fpls.2016.01950.

Postiglione, A. E. and Muday, G. K. (2020). The Role of ROS Homeostasis in ABA-Induced Guard Cell Signaling. *Frontiers in plant science*, 11, p.968. [Online]. Available at: doi:10.3389/fpls.2020.00968.

Pradhan, G. P. and Prasad, P. V. V. (2015). Evaluation of wheat chromosome translocation lines for high temperature stress tolerance at grain filling stage. *PloS one*, 10 (2), p.e0116620. [Online]. Available at: doi:10.1371/journal.pone.0116620.

Prasad, P. V. V. and Djanaguiraman, M. (2014). Response of floret fertility and individual grain weight of wheat to high temperature stress: sensitive stages and thresholds for temperature and duration. *Functional plant biology: FPB*, 41 (12), pp.1261–1269. [Online]. Available at: doi:10.1071/FP14061 [Accessed 16 August 2023].

Priest, H. D. et al. (2014). Analysis of global gene expression in Brachypodium distachyon reveals extensive network plasticity in response to abiotic stress. *PloS one*, 9 (1), p.e87499. [Online]. Available at: doi:10.1371/journal.pone.0087499.

Pudelski, B. et al. (2012). The plastid outer envelope protein OEP16 affects metabolic fluxes during ABA-controlled seed development and germination. *Journal of experimental botany*, 63 (5), pp.1919–1936. [Online]. Available at: doi:10.1093/jxb/err375.

Puhakainen, T. et al. (2004). Overexpression of multiple dehydrin genes enhances tolerance to freezing stress in Arabidopsis. *Plant molecular biology*, 54 (5), pp.743–753. [Online]. Available at: doi:10.1023/B:PLAN.0000040903.66496.a4.

Purwestri, Y. A. et al. (2009). The 14-3-3 protein GF14c acts as a negative regulator of flowering in rice by interacting with the florigen Hd3a. *Plant & cell physiology*, 50 (3), pp.429–438. [Online]. Available at: doi:10.1093/pcp/pcp012.

Qaseem, M. F., Qureshi, R. and Shaheen, H. (2019). Effects of Pre-Anthesis Drought, Heat and Their Combination on the Growth, Yield and Physiology of diverse Wheat (Triticum aestivum L.) Genotypes Varying in Sensitivity to Heat and drought stress. *Scientific reports*, 9 (1), p.6955. [Online]. Available at: doi:10.1038/s41598-019-43477-z.

Qin, D. et al. (2008). Heat stress-responsive transcriptome analysis in heat susceptible and tolerant wheat (Triticum aestivum L.) by using Wheat Genome Array. *BMC genomics*, 9, p.432. [Online]. Available at: doi:10.1186/1471-2164-9-432.

Qin, W. et al. (2022). Activation tagging identifies WRKY14 as a repressor of plant thermomorphogenesis in Arabidopsis. *Molecular plant*, 15 (11), pp.1725–1743. [Online]. Available at: doi:10.1016/j.molp.2022.09.018.

Quarrie, S. A. (1980). Genotypic Differences in Leaf Water Potential, Abscisic Acid and Proline Concentrations in Spring Wheat during Drought Stress. *Annals of botany*, 46 (4), pp.383–394. [Online]. Available at: doi:10.1093/oxfordjournals.aob.a085929 [Accessed 22 August 2023].

Queitsch, C. et al. (2000). Heat Shock Protein 101 Plays a Crucial Role in Thermotolerance in Arabidopsis. *The Plant cell*, 12 (4), pp.479–492. [Online]. Available at: doi:10.2307/3871063.

R Core Team. (2021). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. [Online]. Available at: https://www.R-project.org/.

Rahman, A. M. et al. (2009). Growth and yield components of wheat genotypes exposed to high temperature stress under control environment. *Bangladesh Journal of Agricultural Research*, 34 (3), pp.360–372. [Online]. Available at: doi:10.3329/bjar.v34i3.3961 [Accessed 17 August 2023].

Rahman, M. A. et al. (2022). Heat Shock Proteins and Antioxidant Genes Involved in Heat Combined with Drought Stress Responses in Perennial Rye Grass. *Life* , 12 (9). [Online]. Available at: doi:10.3390/life12091426.

Rahman, M. S. and Wilson, J. H. (1978). Determination of spikelet number in wheat. III.* Effect of varying temperature on ear development. *Australian journal of agricultural research*, 29 (3), pp.459–467. [Online]. Available at: doi:10.1071/ar9780459 [Accessed 25 July 2023].

Ramírez-González, R. H. et al. (2018). The transcriptional landscape of polyploid wheat. *Science*, 361 (6403). [Online]. Available at: doi:10.1126/science.aar6089.

Ranathunge, K. et al. (2016). Ammonium-induced architectural and anatomical changes with altered suberin and lignin levels significantly change water and solute permeabilities of rice (Oryza sativa L.) roots. *Planta*, 243 (1), pp.231–249. [Online]. Available at: doi:10.1007/s00425-015-2406-1.

Rangan, P., Furtado, A. and Henry, R. (2020). Transcriptome profiling of wheat genotypes under heat stress during grain-filling. *Journal of cereal science*, 91, p.102895. [Online]. Available at: doi:10.1016/j.jcs.2019.102895.

Rani, S. H. et al. (2010). Defective in Cuticular Ridges (DCR) of Arabidopsis thaliana, a Gene Associated with Surface Cutin Formation, Encodes a Soluble Diacylglycerol Acyltransferase*. *The Journal of biological chemistry*, 285 (49), pp.38337–38347. [Online]. Available at: doi:10.1074/jbc.M110.133116.

Rashid, A. and Deyholos, M. K. (2011). PELPK1 (At5g09530) contains a unique pentapeptide repeat and is a positive regulator of germination in Arabidopsis thaliana. *Plant cell reports*, 30 (9), pp.1735–1745. [Online]. Available at: doi:10.1007/s00299-011-1081-3.

Rasool, F. et al. (2022). Transcriptome unveiled the gene expression patterns of root architecture in drought-tolerant and sensitive wheat genotypes. *Plant physiology and biochemistry: PPB / Societe francaise de physiologie vegetale*, 178, pp.20–30. [Online]. Available at: doi:10.1016/j.plaphy.2022.02.025.

Rativa, A. G. S. et al. (2020). Root responses of contrasting rice genotypes to low temperature stress. *Journal of plant physiology*, 255, p.153307. [Online]. Available at: doi:10.1016/j.jplph.2020.153307.

Ray, D. K. et al. (2013). Yield Trends Are Insufficient to Double Global Crop Production by 2050. *PloS one*, 8 (6), p.e66428. [Online]. Available at: doi:10.1371/journal.pone.0066428.

Ray, D. K. et al. (2019). Climate change has likely already affected global food production. *PloS one*, 14 (5), p.e0217148. [Online]. Available at: doi:10.1371/journal.pone.0217148.

Rebetzke, G. J. et al. (2014). Use of a large multiparent wheat mapping population in genomic dissection of coleoptile and seedling growth. *Plant biotechnology journal*, 12 (2), pp.219–230. [Online]. Available at: doi:10.1111/pbi.12130.

Reddy, S. K. et al. (2014). Physiology and transcriptomics of water-deficit stress responses in wheat cultivars TAM 111 and TAM 112. *Journal of plant physiology*, 171 (14), pp.1289–1298. [Online]. Available at: doi:10.1016/j.jplph.2014.05.005.

Reinert, S. et al. (2016). Genome-Wide Association Mapping in the Global Diversity Set Reveals New QTL Controlling Root System and Related Shoot Variation in Barley. *Frontiers in plant science*, 7, p.1061. [Online]. Available at: doi:10.3389/fpls.2016.01061.

Renkow, M. and Byerlee, D. (2010). The impacts of CGIAR research: A review of recent evidence. *Food policy*, 35 (5), pp.391–402. [Online]. Available at: doi:10.1016/j.foodpol.2010.04.006.

Renny-Byfield, S. and Wendel, J. F. (2014). Doubling down on genomes: polyploidy and crop plants. *American journal of botany*, 101 (10), pp.1711–1725. [Online]. Available at: doi:10.3732/ajb.1400119.

Renzi, J. P. et al. (2022). How Could the Use of Crop Wild Relatives in Breeding Increase the Adaptation of Crops to Marginal Environments? *Frontiers in plant science*, 13, p.886162. [Online]. Available at: doi:10.3389/fpls.2022.886162.

Reynolds, M., Dreccer, F. and Trethowan, R. (2007). Drought-adaptive traits derived from wheat wild relatives and landraces. *Journal of experimental botany*, 58 (2), pp.177–186. [Online]. Available at: doi:10.1093/jxb/erl250.

Riaz, M. W. et al. (2021). Effects of Heat Stress on Growth, Physiology of Plants, Yield and Grain Quality of Different Spring Wheat (Triticum aestivum L.) Genotypes. *Sustainability: Science Practice and Policy*, 13 (5), p.2972. [Online]. Available at: doi:10.3390/su13052972 [Accessed 28 August 2023].

Riboni, M. et al. (2013). GIGANTEA enables drought escape response via abscisic acid-dependent activation of the florigens and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS. *Plant physiology*, 162 (3), pp.1706–1719. [Online]. Available at: doi:10.1104/pp.113.217729.

Riboni, M. et al. (2014). Environmental stress and flowering time: the photoperiodic connection. *Plant signaling & behavior*, 9 (7), p.e29036. [Online]. Available at: doi:10.4161/psb.29036.

Riedesel, L. et al. (2023). Timing and intensity of heat and drought stress determine wheat yield losses in Germany. *PloS one*, 18 (7), p.e0288202. [Online]. Available at: doi:10.1371/journal.pone.0288202.

Ritchie, H. (2017). *Yields vs. Land Use: How the Green Revolution enabled us to feed a growing population*. [Online]. Available at: https://ourworldindata.org/yields-vs-land-use-how-has-the-world-produced-enough-food-for-a-growing-population [Accessed 15 August 2023].

Ritchie, H. and Roser, M. (2023). How has world population growth changed over time? *Our World in Data*. [Online]. Available at: https://ourworldindata.org/population-growth-over-time [Accessed 14 August 2023].

Rodríguez-Valentín, R. et al. (2014). Group 6 Late Embryogenesis Abundant (LEA) Proteins in Monocotyledonous Plants: Genomic Organization and Transcript Accumulation Patterns in Response to Stress in Oryza sativa. *Plant molecular biology reporter / ISPMB*, 32 (1), pp.198–208. [Online]. Available at: doi:10.1007/s11105-013-0641-9.

Rohila, J. S., Jain, R. K. and Wu, R. (2002). Genetic improvement of Basmati rice for salt and drought tolerance by regulated expression of a barley Hva1 cDNA. *Plant science: an international journal of experimental plant biology*, 163 (3), pp.525–532. [Online]. Available at: doi:10.1016/S0168-9452(02)00155-3.

Rong, W. et al. (2014). The ERF transcription factor TaERF3 promotes tolerance to salt and drought stresses in wheat. *Plant biotechnology journal*, 12 (4), pp.468–479. [Online]. Available at: doi:10.1111/pbi.12153.

le Roux, M.-S. L. et al. (2020). Wheat Line “RYNO3936” Is Associated With Delayed Water Stress-Induced Leaf Senescence and Rapid Water-Deficit Stress Recovery. *Frontiers in plant science*, 11, p.1053. [Online]. Available at: doi:10.3389/fpls.2020.01053.

le Roux, M.-S. L. et al. (2021). EMS Derived Wheat Mutant BIG8-1 (Triticum aestivum L.)-A New Drought Tolerant Mutant Wheat Line. *International journal of molecular sciences*, 22 (10). [Online]. Available at: doi:10.3390/ijms22105314.

Roychowdhury, R. et al. (2023). Pre-anthesis spike growth dynamics and its association to yield components among elite bread wheat cultivars (Triticum aestivum L. spp.) under Mediterranean climate. *Field crops research*, 298, p.108948. [Online]. Available at: doi:10.1016/j.fcr.2023.108948.

Runquist, M. and Kruger, N. J. (1999). Control of gluconeogenesis by isocitrate lyase in endosperm of germinating castor bean seedlings. *The Plant journal: for cell and molecular biology*, 19 (4), pp.423–431. [Online]. Available at: doi:10.1046/j.1365-313x.1999.00533.x.

Saad, A. S. I. et al. (2013). A rice stress-responsive NAC gene enhances tolerance of transgenic wheat to drought and salt stresses. *Plant science: an international journal of experimental plant biology*, 203–204, pp.33–40. [Online]. Available at: doi:10.1016/j.plantsci.2012.12.016.

Sabouri, A. et al. (2022). Screening of rice drought-tolerant lines by introducing a new composite selection index and competitive with multivariate methods. *Scientific reports*, 12 (1), p.2163. [Online]. Available at: doi:10.1038/s41598-022-06123-9.

Saini, H. S. (1997). Effects of water stress on male gametophyte development in plants. *Sexual plant reproduction*, 10 (2), pp.67–73. [Online]. Available at: doi:10.1007/s004970050069.

Saini, H. S. and Aspinall, D. (1982). Abnormal Sporogenesis in Wheat (Triticum aestivum L.) Induced by Short Periods of High Temperature. *Annals of botany*, 49 (6), pp.835–846. [Online]. Available at: doi:10.1093/oxfordjournals.aob.a086310 [Accessed 16 August 2023].

Saini, H. S., Sedgley, M. and Aspinall, D. (1983). Effect of Heat Stress During Floral Development on Pollen Tube Growth and Ovary Anatomy in Wheat (Triticum aestivum L.). *Functional plant biology: FPB*, 10 (2), pp.137–144. [Online]. Available at: doi:10.1071/pp9830137 [Accessed 16 August 2023].

Saini, H. S., Sedgley, M. and Aspinall, D. (1984). Development Anatomy in Wheat of Male Sterility Induced by Heat Stress, Water Deficit or Abscisic Acid. *Functional plant biology: FPB*, 11 (4), pp.243–253. [Online]. Available at: doi:10.1071/pp9840243 [Accessed 16 August 2023].

Sairam, R. K., Deshmukh, P. S. and Saxena, D. C. (1998). Role of antioxidant systems in wheat genotypes tolerance to water stress. *Biologia plantarum*, 41 (3), pp.387–394. [Online]. Available at: doi:10.1023/a:1001898310321.

Sakamoto, Y. and Takagi, S. (2013). LITTLE NUCLEI 1 and 4 regulate nuclear morphology in Arabidopsis thaliana. *Plant & cell physiology*, 54 (4), pp.622–633. [Online]. Available at: doi:10.1093/pcp/pct031.

Sallam, A. et al. (2018). Genetic variation in drought tolerance at seedling stage and grain yield in low rainfall environments in wheat (Triticum aestivum L.). *Euphytica/ Netherlands journal of plant breeding*, 214 (9), p.169. [Online]. Available at: doi:10.1007/s10681-018-2245-9.

Sallam, A. et al. (2019). Drought Stress Tolerance in Wheat and Barley: Advances in Physiology, Breeding and Genetics Research. *International journal of molecular sciences*, 20 (13). [Online]. Available at: doi:10.3390/ijms20133137.

Salvucci, M. E. et al. (2001). Exceptional sensitivity of Rubisco activase to thermal denaturation in vitro and in vivo. *Plant physiology*, 127 (3), pp.1053–1064. [Online]. Available at: https://www.ncbi.nlm.nih.gov/pubmed/11706186.

Salvucci, M. E., Portis, A. R., Jr and Ogren, W. L. (1985). A soluble chloroplast protein catalyzes ribulosebisphosphate carboxylase/oxygenase activation in vivo. *Photosynthesis research*, 7 (2), pp.193–201. [Online]. Available at: doi:10.1007/BF00037012.

Sánchez-Rodríguez, E. et al. (2010). Genotypic differences in some physiological parameters symptomatic for oxidative stress under moderate drought in tomato plants. *Plant science: an international journal of experimental plant biology*, 178 (1), pp.30–40. [Online]. Available at: doi:10.1016/j.plantsci.2009.10.001.

Sancho, R. et al. (2022). Patterns of pan-genome occupancy and gene coexpression under water-deficit in Brachypodium distachyon. *Molecular ecology*, 31 (20), pp.5285–5306. [Online]. Available at: doi:10.1111/mec.16661.

Sandhu, J. et al. (2021). Endoplasmic reticulum stress pathway mediates the early heat stress response of developing rice seeds. *Plant, cell & environment*, 44 (8), pp.2604–2624. [Online]. Available at: doi:10.1111/pce.14103.

Santner, A. and Estelle, M. (2007). The JAZ proteins link jasmonate perception with transcriptional changes. *The Plant cell*, 19 (12), pp.3839–3842. [Online]. Available at: doi:10.1105/tpc.107.056960.

Saradadevi, R. et al. (2014). Contrasting stomatal regulation and leaf ABA concentrations in wheat genotypes when split root systems were exposed to terminal drought. *Field crops research*, 162, pp.77–86. [Online]. Available at: doi:10.1016/j.fcr.2014.02.004.

Saradadevi, R. et al. (2015). Root biomass in the upper layer of the soil profile is related to the stomatal response of wheat as the soil dries. *Functional plant biology: FPB*, 43 (1), pp.62–74. [Online]. Available at: doi:10.1071/FP15216.

Sardans, J. and Peñuelas, J. (2004). Increasing drought decreases phosphorus availability in an evergreen Mediterranean forest. *Plant and soil*, 267 (1), pp.367–377. [Online]. Available at: doi:10.1007/s11104-005-0172-8.

Sardans, J., Peñuelas, J. and Ogaya, R. (2008). Drought's impact on Ca, Fe, Mg, Mo and S concentration and accumulation patterns in the plants and soil of a Mediterranean evergreen Quercus ilex forest. *Biogeochemistry*, 87 (1), pp.49–69. [Online]. Available at: doi:10.1007/s10533-007-9167-2.

Sarieva, G. E., Kenzhebaeva, S. S. and Lichtenthaler, H. K. (2010). Adaptation potential of photosynthesis in wheat cultivars with a capability of leaf rolling under high temperature conditions. *Russian journal of plant physiology: a comprehensive Russian journal on modern phytophysiology*, 57 (1), pp.28–36. [Online]. Available at: doi:10.1134/S1021443710010048.

Sarkar, S. et al. (2021). Tolerance mechanisms for breeding wheat against heat stress: A review. *South African journal of botany: official journal of the South African Association of Botanists = Suid-Afrikaanse tydskrif vir plantkunde: amptelike tydskrif van die Suid-Afrikaanse Genootskap van Plantkundiges*, 138, pp.262–277. [Online]. Available at: doi:10.1016/j.sajb.2021.01.003.

Sasaki, K. and Imai, R. (2011). Pleiotropic roles of cold shock domain proteins in plants. *Frontiers in plant science*, 2, p.116. [Online]. Available at: doi:10.3389/fpls.2011.00116.

Sasaki-Sekimoto, Y. et al. (2014). Comprehensive analysis of protein interactions between JAZ proteins and bHLH transcription factors that negatively regulate jasmonate signaling. *Plant signaling & behavior*, 9 (1), p.e27639. [Online]. Available at: doi:10.4161/psb.27639.

Sato, H. et al. (2019). NF-YB2 and NF-YB3 Have Functionally Diverged and Differentially Induce Drought and Heat Stress-Specific Genes. *Plant physiology*, 180 (3), pp.1677–1690. [Online]. Available at: doi:10.1104/pp.19.00391.

Savicka, M. and Škute, N. (2010). Effects of high temperature on malondialdehyde content, superoxide production and growth changes in wheat seedlings (*Triticum aestivum* L.). *Ekologija* , 56 (1), pp.26–33. [Online]. Available at: doi:10.2478/v10055-010-0004-x.

Scarpeci, T. E. et al. (2017). Overexpression of AtERF019 delays plant growth and senescence, and improves drought tolerance in Arabidopsis. *Journal of experimental botany*, 68 (3), pp.673–685. [Online]. Available at: doi:10.1093/jxb/erw429.

Scharf, K.-D. et al. (2012). The plant heat stress transcription factor (Hsf) family: structure, function and evolution. *Biochimica et biophysica acta*, 1819 (2), pp.104–119. [Online]. Available at: doi:10.1016/j.bbagrm.2011.10.002.

Schilling, S. et al. (2020). Genome-wide analysis of MIKC-type MADS-box genes in wheat: pervasive duplications, functional conservation and putative neofunctionalization. *The New phytologist*, 225 (1), pp.511–529. [Online]. Available at: doi:10.1111/nph.16122.

Schittenhelm, S. et al. (2020). Effect of two-week heat stress during grain filling on stem reserves, senescence, and grain yield of European winter wheat cultivars. *Journal of agronomy and crop science*, 206 (6), pp.722–733. [Online]. Available at: doi:10.1111/jac.12410.

Schmidt, J. et al. (2019). Novel Alleles for Combined Drought and Heat Stress Tolerance in Wheat. *Frontiers in plant science*, 10, p.1800. [Online]. Available at: doi:10.3389/fpls.2019.01800.

Schmidt, J. W. (1983). Drought resistance and wheat breeding. *Agricultural water management*, 7 (1), pp.181–194. [Online]. Available at: doi:10.1016/0378-3774(83)90082-3.

Senapati, N. et al. (2019). Drought tolerance during reproductive development is important for increasing wheat yield potential under climate change in Europe. *Journal of experimental botany*, 70 (9), pp.2549–2560. [Online]. Available at: doi:10.1093/jxb/ery226.

Seo, P. J. et al. (2011). An Arabidopsis senescence-associated protein SAG29 regulates cell viability under high salinity. *Planta*, 233 (1), pp.189–200. [Online]. Available at: doi:10.1007/s00425-010-1293-8.

Seo, P. J. and Park, C.-M. (2011). Cuticular wax biosynthesis as a way of inducing drought resistance. *Plant signaling & behavior*, 6 (7), pp.1043–1045. [Online]. Available at: doi:10.4161/psb.6.7.15606.

Shaar-Moshe, L., Blumwald, E. and Peleg, Z. (2017). Unique Physiological and Transcriptional Shifts under Combinations of Salinity, Drought, and Heat. *Plant physiology*, 174 (1), pp.421–434. [Online]. Available at: doi:10.1104/pp.17.00030.

Shah, N. H. and Paulsen ., G. M. (2004). Injury to Photosynthesis and Productivity from Interaction Between High Temperature and Drought During Maturation of Wheat. *Asian Journal of Plant Sciences*, 4 (1), pp.67–74. [Online]. Available at: doi:10.3923/ajps.2005.67.74.

Shamsi, K. and Kobraee, S. (2011). Bread wheat production under drought stress conditions. *Annals of biological research*, 2 (3), pp.352–358. [Online]. Available at: https://www.cabdirect.org/cabdirect/abstract/20113237958.

Shan, C., Zhou, Y. and Liu, M. (2015). Nitric oxide participates in the regulation of the ascorbate-glutathione cycle by exogenous jasmonic acid in the leaves of wheat seedlings under drought stress. *Protoplasma*, 252 (5), pp.1397–1405. [Online]. Available at: doi:10.1007/s00709-015-0756-y.

Shanahan, J. F. et al. (1990). Membrane thermostability and heat tolerance of spring wheat. *Crop science*, 30 (2), p.247. [Online]. Available at: doi:10.2135/cropsci1990.0011183x003000020001x.

Shannon, P. et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research*, 13 (11), pp.2498–2504. [Online]. Available at: doi:10.1101/gr.1239303.

Sharifi, P. and Mohammadkhani, N. (2016). Effects of drought stress on photosynthesis factors in wheat genotypes during anthesis. *Cereal research communications*, 44 (2), pp.229–239. [Online]. Available at: doi:10.1556/0806.43.2015.054.

Sharma, P. et al. (2012). Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of botany*, 2012. [Online]. Available at: doi:10.1155/2012/217037 [Accessed 14 August 2020].

Sharma, V. et al. (2022). Response of Wheat Genotypes to Drought Stress Stimulated by PEG. *Stresses*, 2 (1), pp.26–51. [Online]. Available at: doi:10.3390/stresses2010003 [Accessed 2 December 2022].

Shavrukov, Y. et al. (2017). Early Flowering as a Drought Escape Mechanism in Plants: How Can It Aid Wheat Production? *Frontiers in plant science*, 8, p.1950. [Online]. Available at: doi:10.3389/fpls.2017.01950.

Shaw, L. M. et al. (2020). Epistatic interactions between PHOTOPERIOD1, CONSTANS1 and CONSTANS2 modulate the photoperiodic response in wheat. *PLoS genetics*, 16 (7), p.e1008812. [Online]. Available at: doi:10.1371/journal.pgen.1008812.

Shen, Q. and Ho, T. H. (1995). Functional dissection of an abscisic acid (ABA)-inducible gene reveals two independent ABA-responsive complexes each containing a G-box and a novel cis-acting element. *The Plant cell*, 7 (3), pp.295–307. [Online]. Available at: doi:10.1105/tpc.7.3.295.

Shenoda, J. E. et al. (2021). Effect of long-term heat stress on grain yield, pollen grain viability and germinability in bread wheat (Triticum aestivum L.) under field conditions. *Heliyon*, 7 (6), p.e07096. [Online]. Available at: doi:10.1016/j.heliyon.2021.e07096.

Shepherd, T. and Wynne Griffiths, D. (2006). The effects of stress on plant cuticular waxes. *The New phytologist*, 171 (3), pp.469–499. [Online]. Available at: doi:10.1111/j.1469-8137.2006.01826.x.

Shewry, P. R. (2009). Wheat. *Journal of experimental botany*, 60 (6), pp.1537–1553. [Online]. Available at: doi:10.1093/jxb/erp058.

Shewry, P. R. and Hey, S. J. (2015). The contribution of wheat to human diet and health. *Food and energy security*, 4 (3), pp.178–202. [Online]. Available at: doi:10.1002/fes3.64.

Shi, H. et al. (2020). Constitutive expression of a group 3 LEA protein from Medicago falcata (MfLEA3) increases cold and drought tolerance in transgenic tobacco. *Plant cell reports*, 39 (7), pp.851–860. [Online]. Available at: doi:10.1007/s00299-020-02534-y.

Shi, S.-Y. et al. (2016). Expression pattern and function analyses of the MADS thranscription factor genes in wheat (Triticum aestivum L.) under phosphorus-starvation condition. *Journal of integrative agriculture*, 15 (8), pp.1703–1715. [Online]. Available at: doi:10.1016/S2095-3119(15)61167-4.

Shin, N.-H. et al. (2019). Rice Senescence-Induced Receptor-Like Kinase (OsSRLK) Is Involved in Phytohormone-Mediated Chlorophyll Degradation. *International journal of molecular sciences*, 21 (1). [Online]. Available at: doi:10.3390/ijms21010260.

Shukla, V. et al. (2021). Suberin plasticity to developmental and exogenous cues is regulated by a set of MYB transcription factors. *Proceedings of the National Academy of Sciences of the United States of America*, 118 (39). [Online]. Available at: doi:10.1073/pnas.2101730118.

Sierla, M. et al. (2016). Reactive Oxygen Species in the Regulation of Stomatal Movements. *Plant physiology*, 171 (3), pp.1569–1580. [Online]. Available at: doi:10.1104/pp.16.00328.

de Silva, N. D. G. et al. (2021). Root Suberin Plays Important Roles in Reducing Water Loss and Sodium Uptake in Arabidopsis thaliana. *Metabolites*, 11 (11). [Online]. Available at: doi:10.3390/metabo11110735.

Simkin, A. J., López-Calcagno, P. E. and Raines, C. A. (2019). Feeding the world: improving photosynthetic efficiency for sustainable crop production. *Journal of experimental botany*, 70 (4), pp.1119–1140. [Online]. Available at: doi:10.1093/jxb/ery445.

Singh, A. and Desgagné-Penix, I. (2017). Transcriptome and metabolome profiling of Narcissus pseudonarcissus “King Alfred” reveal components of Amaryllidaceae alkaloid metabolism. *Scientific reports*, 7 (1), p.17356. [Online]. Available at: doi:10.1038/s41598-017-17724-0.

Sirohi, P. et al. (2020). Identification of drought stress-responsive genes in rice (Oryza sativa) by meta-analysis of microarray data. *Journal of genetics*, 99. [Online]. Available at: doi:10.1007/s12041-020-01195-w.

Sivamani, E. et al. (2000). Improved biomass productivity and water use efficiency under water deficit conditions in transgenic wheat constitutively expressing the barley HVA1 gene. *Plant science: an international journal of experimental plant biology*, 155 (1), pp.1–9. [Online]. Available at: doi:10.1016/s0168-9452(99)00247-2.

Slafer, G. A. (1996). Differences in phasic development rate amongst wheat cultivars independent of responses to photoperiod and vernalization. A viewpoint of the intrinsic earliness hypothesis. *The Journal of agricultural science*, 126 (4), pp.403–419. [Online]. Available at: doi:10.1017/S0021859600075493 [Accessed 19 July 2023].

Smita, S. et al. (2013). Identification of conserved drought stress responsive gene-network across tissues and developmental stages in rice. *Bioinformation*, 9 (2), pp.72–78. [Online]. Available at: doi:10.6026/97320630009072.

Snape, J. W. et al. (2001). Waiting for fine times: genetics of flowering time in wheat. *Euphytica/ Netherlands journal of plant breeding*, 119 (1), pp.185–190. [Online]. Available at: doi:10.1023/A:1017594422176.

Soltani, A., Gholipoor, M. and Zeinali, E. (2006). Seed reserve utilization and seedling growth of wheat as affected by drought and salinity. *Environmental and experimental botany*, 55 (1), pp.195–200. [Online]. Available at: doi:10.1016/j.envexpbot.2004.10.012.

Sonder, K. (2016). *Global map of wheat mega-environments*. [Online]. Available at: https://hdl.handle.net/11529/10625.

Soneson, C., Love, M. I. and Robinson, M. D. (2015). Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research*, 4, p.1521. [Online]. Available at: doi:10.12688/f1000research.7563.2.

Song, Y., Miao, Y. and Song, C.-P. (2014). Behind the scenes: the roles of reactive oxygen species in guard cells. *The New phytologist*, 201 (4), pp.1121–1140. [Online]. Available at: doi:10.1111/nph.12565.

de Sousa, T. et al. (2021). The 10,000-Year Success Story of Wheat! *Foods (Basel, Switzerland)*, 10 (9). [Online]. Available at: doi:10.3390/foods10092124.

Sperotto, R. A. et al. (2018). Deep RNAseq indicates protective mechanisms of cold-tolerant indica rice plants during early vegetative stage. *Plant cell reports*, 37 (2), pp.347–375. [Online]. Available at: doi:10.1007/s00299-017-2234-9.

Staswick, P. E. et al. (2005). Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. *The Plant cell*, 17 (2), pp.616–627. [Online]. Available at: doi:10.1105/tpc.104.026690.

Steed, A. et al. (2022). Identification of Fusarium Head Blight Resistance in Triticum timopheevii Accessions and Characterization of Wheat-T. timopheevii Introgression Lines for

Enhanced Resistance. *Frontiers in plant science*, 13, p.943211. [Online]. Available at: doi:10.3389/fpls.2022.943211.

Stephens, M. (2017). False discovery rates: a new deal. *Biostatistics* , 18 (2), pp.275–294. [Online]. Available at: doi:10.1093/biostatistics/kxw041.

Stone, P. J. and Nicolas, M. E. (1994). Wheat Cultivars Vary Widely in Their Responses of Grain Yield and Quality to Short Periods of Post-Anthesis Heat Stress. *Functional plant biology: FPB*, 21 (6), pp.887–900. [Online]. Available at: doi:10.1071/pp9940887 [Accessed 13 September 2022].

Stone, P. J. and Nicolas, M. E. (1995). Effect of Timing of Heat Stress During Grain Filling on Two Wheat Varieties Differing in Heat Tolerance. I. Grain Growth. *Functional plant biology: FPB*, 22 (6), pp.927–934. [Online]. Available at: doi:10.1071/pp9950927 [Accessed 13 September 2022].

Stone, P. J. and Nicolas, M. E. (1996). Effect of Timing of Heat Stress During Grain Filling on Two Wheat Varieties Differing in Heat Tolerance. II. Fractional Protein Accumulation. *Functional plant biology: FPB*, 23 (6), pp.739–749. [Online]. Available at: doi:10.1071/pp9960739 [Accessed 13 September 2022].

Subbarao, G. V. et al. (2000). Osmotic adjustment, water relations and carbohydrate remobilization in pigeonpea under water deficits. *Journal of plant physiology*, 157 (6), pp.651–659. [Online]. Available at: doi:10.1016/S0176-1617(00)80008-5.

Sultan, B., Defrance, D. and Iizumi, T. (2019). Evidence of crop production losses in West Africa due to historical global warming in two crop models. *Scientific reports*, 9 (1), p.12834. [Online]. Available at: doi:10.1038/s41598-019-49167-0.

Sun, C. et al. (2012a). The histone methyltransferase SDG724 mediates H3K36me2/3 deposition at MADS50 and RFT1 and promotes flowering in rice. *The Plant cell*, 24 (8), pp.3235–3247. [Online]. Available at: doi:10.1105/tpc.112.101436.

Sun, L. et al. (2012b). ZmHSP16.9, a cytosolic class I small heat shock protein in maize (Zea mays), confers heat tolerance in transgenic tobacco. *Plant cell reports*, 31 (8), pp.1473–1484. [Online]. Available at: doi:10.1007/s00299-012-1262-8.

Sun, Y. et al. (2015). Potassium Retention under Salt Stress Is Associated with Natural Variation in Salinity Tolerance among Arabidopsis Accessions. *PloS one*, 10 (5), p.e0124032. [Online]. Available at: doi:10.1371/journal.pone.0124032.

Sun, Z. et al. (2021). Plant Dehydrins: Expression, Regulatory Networks, and Protective Roles in Plants Challenged by Abiotic Stress. *International journal of molecular sciences*, 22 (23). [Online]. Available at: doi:10.3390/ijms222312619.

Taji, T. et al. (2004). Comparative genomics in salt tolerance between Arabidopsis and aRabidopsis-related halophyte salt cress using Arabidopsis microarray. *Plant physiology*, 135 (3), pp.1697–1709. [Online]. Available at: doi:10.1104/pp.104.039909.

Takagi, H., Hempton, A. K. and Imaizumi, T. (2023). Photoperiodic flowering in Arabidopsis: Multilayered regulatory mechanisms of CONSTANS and the florigen FLOWERING LOCUS T. *Plant communications*, p.100552. [Online]. Available at: doi:10.1016/j.xplc.2023.100552.

Takahagi, K., Inoue, K. and Mochida, K. (2018). Gene Co-expression Network Analysis Suggests the Existence of Transcriptional Modules Containing a High Proportion of

Transcriptionally Differentiated Homoeologs in Hexaploid Wheat. *Frontiers in plant science*, 9, p.1163. [Online]. Available at: doi:10.3389/fpls.2018.01163.

Takahashi, S. et al. (2017). Differential Effects of Phosphatidylinositol 4-Kinase (PI4K) and 3-Kinase (PI3K) Inhibitors on Stomatal Responses to Environmental Signals. *Frontiers in plant science*, 8, p.677. [Online]. Available at: doi:10.3389/fpls.2017.00677.

Tamura, K., Stecher, G. and Kumar, S. (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular biology and evolution*, 38 (7), pp.3022–3027. [Online]. Available at: doi:10.1093/molbev/msab120.

Tang, Y. et al. (2023). Transcriptome and WGCNA reveal hub genes in sugarcane tiller seedlings in response to drought stress. *Scientific reports*, 13 (1), p.12823. [Online]. Available at: doi:10.1038/s41598-023-40006-x.

Tanksley, S. D. and McCouch, S. R. (1997). Seed banks and molecular maps: unlocking genetic potential from the wild. *Science*, 277 (5329), pp.1063–1066. [Online]. Available at: doi:10.1126/science.277.5329.1063.

Tao, Z. et al. (2022). Physiological Mechanism of Abscisic Acid-Induced Heat-Tolerance Responses to Cultivation Techniques in Wheat and Maize—Review. *Agronomy*, 12 (7), p.1579. [Online]. Available at: doi:10.3390/agronomy12071579 [Accessed 22 August 2023].

Taoka, K.-I. et al. (2011). 14-3-3 proteins act as intracellular receptors for rice Hd3a florigen. *Nature*, 476 (7360), pp.332–335. [Online]. Available at: doi:10.1038/nature10272.

Tardy, F., Créach, A. and Havaux, M. (1998). Photosynthetic pigment concentration, organization and interconversions in a pale green Syrian landrace of barley (Hordeum vulgareL., Tadmor) adapted to harsh climatic conditions. *Plant, cell & environment*, 21 (5), pp.479–489. [Online]. Available at: doi:10.1046/j.1365-3040.1998.00293.x.

Tarutani, Y. et al. (2004). Molecular characterization of two highly homologous receptor-like kinase genes, RLK902 and RKL1, in Arabidopsis thaliana. *Bioscience, biotechnology, and biochemistry*, 68 (9), pp.1935–1941. [Online]. Available at: doi:10.1271/bbb.68.1935.

Tehseen, M. M. et al. (2022). Exploring the Genetic Diversity and Population Structure of Wheat Landrace Population Conserved at ICARDA Genebank. *Frontiers in genetics*, 13, p.900572. [Online]. Available at: doi:10.3389/fgene.2022.900572.

Teixeira, M. A. et al. (2018). Classification and phylogenetic analyses of the Arabidopsis and tomato G-type lectin receptor kinases. *BMC genomics*, 19 (1), p.239. [Online]. Available at: doi:10.1186/s12864-018-4606-0.

Tewari, A. K. and Tripathy, B. C. (1998). Temperature-stress-induced impairment of chlorophyll biosynthetic reactions in cucumber and wheat. *Plant physiology*, 117 (3), pp.851–858. [Online]. Available at: doi:10.1104/pp.117.3.851.

Thatcher, L. F. et al. (2016). Characterization of a JAZ7 activation-tagged Arabidopsis mutant with increased susceptibility to the fungal pathogen Fusarium oxysporum. *Journal of experimental botany*, 67 (8), pp.2367–2386. [Online]. Available at: doi:10.1093/jxb/erw040.

Thines, B. et al. (2007). JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. *Nature*, 448 (7154), pp.661–665. [Online]. Available at: doi:10.1038/nature05960.

Thornton, P. K. et al. (2014). Climate variability and vulnerability to climate change: a review. *Global change biology*, 20 (11), pp.3313–3328. [Online]. Available at: doi:10.1111/gcb.12581.

Tian, B. et al. (2018). Expression of a rice soluble starch synthase gene in transgenic wheat improves the grain yield under heat stress conditions. *In vitro cellular & developmental biology. Plant: journal of the Tissue Culture Association*, 54 (3), pp.216–227. [Online]. Available at: doi:10.1007/s11627-018-9893-2.

Tian, F. et al. (2021). Recent Advances in the Roles of HSFs and HSPs in Heat Stress Response in Woody Plants. *Frontiers in plant science*, 12, p.704905. [Online]. Available at: doi:10.3389/fpls.2021.704905.

Tian, G. et al. (2023). Allelic variation of TaWD40-4B.1 contributes to drought tolerance by modulating catalase activity in wheat. *Nature communications*, 14 (1), p.1200. [Online]. Available at: doi:10.1038/s41467-023-36901-6.

Tian, T. et al. (2017). agriGO v2.0: a GO analysis toolkit for the agricultural community, 2017 update. *Nucleic acids research*, 45 (W1), pp.W122–W129. [Online]. Available at: doi:10.1093/nar/gkx382.

Tian, X. et al. (2022). Stress granule-associated TaMBF1c confers thermotolerance through regulating specific mRNA translation in wheat (Triticum aestivum). *The New phytologist*, 233 (4), pp.1719–1731. [Online]. Available at: doi:10.1111/nph.17865.

Tilman, D. et al. (2011). Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences of the United States of America*, 108 (50), pp.20260–20264. [Online]. Available at: doi:10.1073/pnas.1116437108.

Tiwari, L. D., Khungar, L. and Grover, A. (2020). AtHsc70-1 negatively regulates the basal heat tolerance in Arabidopsis thaliana through affecting the activity of HsfAs and Hsp101. *The Plant journal: for cell and molecular biology*, 103 (6), pp.2069–2083. [Online]. Available at: doi:10.1111/tpj.14883.

Tiwari, P. et al. (2019). Role of dehydrin-FK506-binding protein complex in enhancing drought tolerance through the ABA-mediated signaling pathway. *Environmental and experimental botany*, 158, pp.136–149. [Online]. Available at: doi:10.1016/j.envexpbot.2018.10.031.

Tiwari, P. and Chakrabarty, D. (2021). Dehydrin in the past four decades: From chaperones to transcription co-regulators in regulating abiotic stress response. *Current Research in Biotechnology*, 3, pp.249–259. [Online]. Available at: doi:10.1016/j.crbiot.2021.07.005.

Tolleter, D., Hincha, D. K. and Macherel, D. (2010). A mitochondrial late embryogenesis abundant protein stabilizes model membranes in the dry state. *Biochimica et biophysica acta*, 1798 (10), pp.1926–1933. [Online]. Available at: doi:10.1016/j.bbamem.2010.06.029.

Tonsor, S. J. et al. (2008). Heat shock protein 101 effects in A. thaliana: genetic variation, fitness and pleiotropy in controlled temperature conditions. *Molecular ecology*, 17 (6), pp.1614–1626. [Online]. Available at: doi:10.1111/j.1365-294X.2008.03690.x.

Tran, L.-S. P. et al. (2007). Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 104 (51), pp.20623–20628. [Online]. Available at: doi:10.1073/pnas.0706547105.

Tran, L.-S. P., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2010). Role of cytokinin responsive two-component system in ABA and osmotic stress signalings. *Plant signaling & behavior*, 5 (2), pp.148–150. [Online]. Available at: doi:10.4161/psb.5.2.10411.

Trnka, M. et al. (2019). Mitigation efforts will not fully alleviate the increase in water scarcity occurrence probability in wheat-producing areas. *Science advances*, 5 (9), p.eaau2406. [Online]. Available at: doi:10.1126/sciadv.aau2406.

Tsuda, K. et al. (2000). New members of a cold-responsive group-3 Lea/Rab-related Cor gene family from common wheat (Triticum aestivum L.). *Genes & genetic systems*, 75 (4), pp.179–188. [Online]. Available at: doi:10.1266/ggs.75.179.

Tukey, J. W. (1977). *Exploratory data analysis*. Reading, Mass. [Online]. Available at: http://theta.edu.pl/wp-content/uploads/2012/10/exploratorydataanalysis_tukey.pdf.

Turpaev, K. T. (2002). Reactive oxygen species and regulation of gene expression. *Biochemistry. Biokhimiia*, 67 (3), pp.281–292. [Online]. Available at: doi:10.1023/a:1014819832003.

Ubi, B. E. et al. (2022). Exploiting the miniature inverted-repeat transposable elements insertion polymorphisms as an efficient DNA marker system for genome analysis and evolutionary studies in wheat and related species. *Frontiers in plant science*, 13, p.995586. [Online]. Available at: doi:10.3389/fpls.2022.995586.

Ueno, N. et al. (2019). Accumulation of protein aggregates induces autolytic programmed cell death in hybrid tobacco cells expressing hybrid lethality. *Scientific reports*, 9 (1), p.10223. [Online]. Available at: doi:10.1038/s41598-019-46619-5.

Ullah, A. et al. (2022). Bread Wheat Genotypes Accumulating Free Proline and Phenolics Can Better Tolerate Drought Stress Through Sustained Rate of Photosynthesis. *Journal of Soil Science and Plant Nutrition*, 22 (1), pp.165–176. [Online]. Available at: doi:10.1007/s42729-021-00641-7.

United States Department of Agriculture - Foreign Agricultural Service. (2023). *Grain: World Markets and Trade*. p.20. [Online]. Available at: usda.library.cornell.edu/concern/publications/zs25x844t?locale=en#release-items [Accessed 29 March 2023].

Vabulas, R. M. et al. (2010). Protein folding in the cytoplasm and the heat shock response. *Cold Spring Harbor perspectives in biology*, 2 (12), p.a004390. [Online]. Available at: doi:10.1101/cshperspect.a004390.

Van Oosterom, E. J., Ceccarelli, S. and Peacock, J. M. (1993). Yield response of barley to rainfall and temperature in Mediterranean environments. *The Journal of agricultural science*, 121 (3), pp.307–313. [Online]. Available at: doi:10.1017/S0021859600085488 [Accessed 12 May 2023].

Velichko, A. K. et al. (2012). Dual effect of heat shock on DNA replication and genome integrity. *Molecular biology of the cell*, 23 (17), pp.3450–3460. [Online]. Available at: doi:10.1091/mbc.E11-12-1009.

van Velthuizen, H. (2007). *Mapping Biophysical Factors that Influence Agricultural Production and Rural Vulnerability*. Food & Agriculture Org. [Online]. Available at: https://play.google.com/store/books/details?id=62JdCQJ7ZyQC.

Venske, E. et al. (2019). Bread wheat: a role model for plant domestication and breeding. *Hereditas*, 156, p.16. [Online]. Available at: doi:10.1186/s41065-019-0093-9.

Verma, G. et al. (2017). Genome-wide analysis of rice dehydrin gene family: Its evolutionary conservedness and expression pattern in response to PEG induced dehydration stress. *PloS one*, 12 (5), p.e0176399. [Online]. Available at: doi:10.1371/journal.pone.0176399.

Vidya, E. and Duchaine, T. F. (2022). Eukaryotic mRNA Decapping Activation. *Frontiers in genetics*, 13, p.832547. [Online]. Available at: doi:10.3389/fgene.2022.832547.

Vignjevic, M. et al. (2015). Traits in spring wheat cultivars associated with yield loss caused by a heat stress episode after anthesis. *Journal of agronomy and crop science*, 201 (1), pp.32–48. [Online]. Available at: doi:10.1111/jac.12085.

Vishwakarma, H. et al. (2018). Heat stress transcripts, differential expression, and profiling of heat stress tolerant gene TaHsp90 in Indian wheat (Triticum aestivum L.) cv C306. *PloS one*, 13 (6), p.e0198293. [Online]. Available at: doi:10.1371/journal.pone.0198293.

Voss-Fels, K. P. et al. (2017). Linkage drag constrains the roots of modern wheat. *Plant, cell & environment*, 40 (5), pp.717–725. [Online]. Available at: doi:10.1111/pce.12888.

Vuković, R. et al. (2022). Physiological, Biochemical and Molecular Response of Different Winter Wheat Varieties under Drought Stress at Germination and Seedling Growth Stage. *Antioxidants (Basel, Switzerland)*, 11 (4). [Online]. Available at: doi:10.3390/antiox11040693.

Wager, A. and Browse, J. (2012). Social Network: JAZ Protein Interactions Expand Our Knowledge of Jasmonate Signaling. *Frontiers in plant science*, 3, p.41. [Online]. Available at: doi:10.3389/fpls.2012.00041.

Waines, J. G. and Ehdaie, B. (2007). Domestication and crop physiology: roots of green-revolution wheat. *Annals of botany*, 100 (5), pp.991–998. [Online]. Available at: doi:10.1093/aob/mcm180.

Waki, T. et al. (2020). A conserved strategy of chalcone isomerase-like protein to rectify promiscuous chalcone synthase specificity. *Nature communications*, 11 (1), p.870. [Online]. Available at: doi:10.1038/s41467-020-14558-9.

Wan, C. et al. (2022). How Does the Environment Affect Wheat Yield and Protein Content Response to Drought? A Meta-Analysis. *Frontiers in plant science*, 13, p.896985. [Online]. Available at: doi:10.3389/fpls.2022.896985.

Wang, G.-F. et al. (2011). Molecular analysis of common wheat genes encoding three types of cytosolic heat shock protein 90 (Hsp90): functional involvement of cytosolic Hsp90s in the control of wheat seedling growth and disease resistance. *The New phytologist*, 191 (2), pp.418–431. [Online]. Available at: doi:10.1111/j.1469-8137.2011.03715.x.

Wang, H. et al. (2017). The bHLH Transcription Factors MYC2, MYC3, and MYC4 Are Required for Jasmonate-Mediated Inhibition of Flowering in Arabidopsis. *Molecular plant*, 10 (11), pp.1461–1464. [Online]. Available at: doi:10.1016/j.molp.2017.08.007.

Wang, J. et al. (2016). Wheat Transcription Factor TaAREB3 Participates in Drought and Freezing Tolerances in Arabidopsis. *International journal of biological sciences*, 12 (2), pp.257–269. [Online]. Available at: doi:10.7150/ijbs.13538.

Wang, J. et al. (2021a). Changing lengths of the Four Seasons by global warming. *Geophysical research letters*, 48 (6). [Online]. Available at: doi:10.1029/2020gl091753.

Wang, L. et al. (2020a). Receptor kinase FERONIA regulates flowering time in Arabidopsis. *BMC plant biology*, 20 (1), p.26. [Online]. Available at: doi:10.1186/s12870-019-2223-y.

Wang, Q. et al. (2021b). The Ghd7 transcription factor represses ARE1 expression to enhance nitrogen utilization and grain yield in rice. *Molecular plant*, 14 (6), pp.1012–1023. [Online]. Available at: doi:10.1016/j.molp.2021.04.012.

Wang, Q.-L. et al. (2018a). Metabolic Reprogramming in Chloroplasts under Heat Stress in Plants. *International journal of molecular sciences*, 19 (3). [Online]. Available at: doi:10.3390/ijms19030849.

Wang, R. et al. (2019). Genome-wide identification and expression profiling of glutathione transferase gene family under multiple stresses and hormone treatments in wheat (Triticum aestivum L.). *BMC genomics*, 20 (1), p.986. [Online]. Available at: doi:10.1186/s12864-019-6374-x.

Wang, W. et al. (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in plant science*, 9 (5), pp.244–252. [Online]. Available at: doi:10.1016/j.tplants.2004.03.006.

Wang, W. et al. (2018b). Gene editing and mutagenesis reveal inter-cultivar differences and additivity in the contribution of TaGW2 homoeologues to grain size and weight in wheat. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik*, 131 (11), pp.2463–2475. [Online]. Available at: doi:10.1007/s00122-018-3166-7.

Wang, X. et al. (2020b). Genome-Wide Identification and Expression Profile Analysis of the Phospholipase C Gene Family in Wheat (Triticum aestivum L.). *Plants*, 9 (7). [Online]. Available at: doi:10.3390/plants9070885.

Wang, X. et al. (2021c). Abscisic acid and jasmonic acid are involved in drought priming-induced tolerance to drought in wheat. *The Crop Journal*, 9 (1), pp.120–132. [Online]. Available at: doi:10.1016/j.cj.2020.06.002.

Wang, X. et al. (2023). Association mapping identifies loci and candidate genes for grain-related traits in spring wheat in response to heat stress. *Plant science: an international journal of experimental plant biology*, 331, p.111676. [Online]. Available at: doi:10.1016/j.plantsci.2023.111676.

Wang, X., Niu, Y. and Zheng, Y. (2021). Multiple Functions of MYB Transcription Factors in Abiotic Stress Responses. *International journal of molecular sciences*, 22 (11). [Online]. Available at: doi:10.3390/ijms22116125.

Wang, Y. et al. (2014). Classification and expression diversification of wheat dehydrin genes. *Plant science: an international journal of experimental plant biology*, 214, pp.113–120. [Online]. Available at: doi:10.1016/j.plantsci.2013.10.005.

Wang, Y. et al. (2022). WGCNA Analysis Identifies the Hub Genes Related to Heat Stress in Seedling of Rice (Oryza sativa L.). *Genes*, 13 (6). [Online]. Available at: doi:10.3390/genes13061020.

Wang, Y., Wang, X. and Paterson, A. H. (2012). Genome and gene duplications and gene expression divergence: a view from plants. *Annals of the New York Academy of Sciences*, 1256, pp.1–14. [Online]. Available at: doi:10.1111/j.1749-6632.2011.06384.x.

Wang, Z. Y. et al. (2020c). Molecular cloning and functional analysis of a cold-regulated gene Wcor18 in wheat. *Cereal research communications*, 48 (1), pp.9–15. [Online]. Available at: doi:10.1007/s42976-019-00007-x.

Wang, Z.-Y. et al. (2008). Soil-Water Threshold Range of Chemical Signals and Drought Tolerance Was Mediated by ROS Homeostasis in Winter Wheat During Progressive Soil Drying. *Journal of plant growth regulation*, 27 (4), pp.309–319. [Online]. Available at: doi:10.1007/s00344-008-9057-4.

Wardlaw, I. F. et al. (1989). The tolerance of wheat to high temperatures during reproductive growth. I. Survey procedures and general response patterns. *Australian journal of agricultural research*, 40 (1), pp.1–13. [Online]. Available at: doi:10.1071/ar9890001 [Accessed 15 September 2022].

Wardlaw, I. F. and Willenbrink, J. (2000). Mobilization of fructan reserves and changes in enzyme activities in wheat stems correlate with water stress during kernel filling. *The New phytologist*, 148 (3), pp.413–422. [Online]. Available at: doi:10.1046/j.1469-8137.2000.00777.x.

Wäspi, U. et al. (1998). The defense-related rice gene Pir7b encodes an alpha/beta hydrolase fold protein exhibiting esterase activity towards naphthol AS-esters. *European journal of biochemistry / FEBS*, 254 (1), pp.32–37. [Online]. Available at: doi:10.1046/j.1432-1327.1998.2540032.x.

Wasternack, C. (2014). Action of jasmonates in plant stress responses and development--applied aspects. *Biotechnology advances*, 32 (1), pp.31–39. [Online]. Available at: doi:10.1016/j.biotechadv.2013.09.009.

Weise, A. et al. (2000). A new subfamily of sucrose transporters, SUT4, with low affinity/high capacity localized in enucleate sieve elements of plants. *The Plant cell*, 12 (8), pp.1345–1355. [Online]. Available at: doi:10.1105/tpc.12.8.1345.

Werner, T. et al. (2003). Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *The Plant cell*, 15 (11), pp.2532–2550. [Online]. Available at: doi:10.1105/tpc.014928.

Werner, T. et al. (2010). Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in Arabidopsis and tobacco. *The Plant cell*, 22 (12), pp.3905–3920. [Online]. Available at: doi:10.1105/tpc.109.072694.

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer Science & Business Media. [Online]. Available at: https://play.google.com/store/books/details?id=bes-AAAAQBAJ.

Wigley, T. M. and Raper, S. C. (2001). Interpretation of high projections for global-mean warming. *Science*, 293 (5529), pp.451–454. [Online]. Available at: doi:10.1126/science.1061604.

Wingen, L. U. et al. (2014). Establishing the A. E. Watkins landrace cultivar collection as a resource for systematic gene discovery in bread wheat. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik*, 127 (8), pp.1831–1842. [Online]. Available at: doi:10.1007/s00122-014-2344-5.

Wu, B. and Guo, W. (2015). The Exocyst at a Glance. *Journal of cell science*, 128 (16), pp.2957–2964. [Online]. Available at: doi:10.1242/jcs.156398.

Wu, B.-B. et al. (2022). Allele mining of wheat ABA receptor at TaPYL4 suggests neo-functionalization among the wheat homoeologs. *Journal of integrative agriculture*, 21 (8), pp.2183–2196. [Online]. Available at: doi:10.1016/S2095-3119(21)63699-7.

Wu, H.-C., Bulgakov, V. P. and Jinn, T.-L. (2018). Pectin Methylesterases: Cell Wall Remodeling Proteins Are Required for Plant Response to Heat Stress. *Frontiers in plant science*, 9, p.1612. [Online]. Available at: doi:10.3389/fpls.2018.01612.

Wu, R. et al. (2023). Genome-Wide Identification of the Light-Harvesting Chlorophyll a/b Binding Protein Gene Family in Pyrus bretschneideri and Their Transcriptomic Features under Drought Stress. *Horticulturae*, 9 (5), p.522. [Online]. Available at: doi:10.3390/horticulturae9050522 [Accessed 12 July 2023].

Xi, W. et al. (2023). Transcriptome Analysis of Roots from Wheat (Triticum aestivum L.) Varieties in Response to Drought Stress. *International journal of molecular sciences*, 24 (8). [Online]. Available at: doi:10.3390/ijms24087245.

Xiao, C. et al. (2021). Expression Pattern and Functional Analyses of Arabidopsis Guard Cell-Enriched GDSL Lipases. *Frontiers in plant science*, 12, p.748543. [Online]. Available at: doi:10.3389/fpls.2021.748543.

Xie, Y., Ravet, K. and Pearce, S. (2021). Extensive structural variation in the Bowman-Birk inhibitor family in common wheat (Triticum aestivum L.). *BMC genomics*, 22 (1), p.218. [Online]. Available at: doi:10.1186/s12864-021-07475-8.

Xu, D. et al. (1996). Expression of a Late Embryogenesis Abundant Protein Gene, HVA1, from Barley Confers Tolerance to Water Deficit and Salt Stress in Transgenic Rice. *Plant physiology*, 110 (1), pp.249–257. [Online]. Available at: doi:10.1104/pp.110.1.249.

Xu, W. et al. (2016). Proteomic comparison reveals the contribution of chloroplast to salt tolerance of a wheat introgression line. *Scientific reports*, 6, p.32384. [Online]. Available at: doi:10.1038/srep32384.

Xue, D. et al. (2017). Molecular and Evolutionary Mechanisms of Cuticular Wax for Plant Drought Tolerance. *Frontiers in plant science*, 8, p.621. [Online]. Available at: doi:10.3389/fpls.2017.00621.

Yadav, M. R. et al. (2022). Impacts, Tolerance, Adaptation, and Mitigation of Heat Stress on Wheat under Changing Climates. *International journal of molecular sciences*, 23 (5). [Online]. Available at: doi:10.3390/ijms23052838.

Yamada, S. et al. (2012). Involvement of OsJAZ8 in jasmonate-induced resistance to bacterial blight in rice. *Plant & cell physiology*, 53 (12), pp.2060–2072. [Online]. Available at: doi:10.1093/pcp/pcs145.

Yamaguchi, A. et al. (2005). TWIN SISTER OF FT (TSF) acts as a floral pathway integrator redundantly with FT. *Plant & cell physiology*, 46 (8), pp.1175–1189. [Online]. Available at: doi:10.1093/pcp/pci151.

Yamaji, N. et al. (2015). Orchestration of three transporters and distinct vascular structures in node for intervascular transfer of silicon in rice. *Proceedings of the National Academy of Sciences of the United States of America*, 112 (36), pp.11401–11406. [Online]. Available at: doi:10.1073/pnas.1508987112.

Yamamoto, Y. (2016). Quality Control of Photosystem II: The Mechanisms for Avoidance and Tolerance of Light and Heat Stresses are Closely Linked to Membrane Fluidity of the

Thylakoids. *Frontiers in plant science*, 7, p.1136. [Online]. Available at: doi:10.3389/fpls.2016.01136.

Yan, L. et al. (2004). Allelic variation at the VRN-1 promoter region in polyploid wheat. *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik*, 109 (8), pp.1677–1686. [Online]. Available at: doi:10.1007/s00122-004-1796-4.

Yan, L. et al. (2006). The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*. *Proceedings of the National Academy of Sciences of the United States of America*, 103 (51), pp.19581–19586. [Online]. Available at: doi:10.1073/pnas.0607142103.

Yang, K. A. et al. (2006). Identification of cell wall genes modified by a permissive high temperature in Chinese cabbage. *Plant science: an international journal of experimental plant biology*, 171 (1), pp.175–182. [Online]. Available at: doi:10.1016/j.plantsci.2006.03.013.

Yang, W. et al. (2015). The K-segments of wheat dehydrin WZY2 are essential for its protective functions under temperature stress. *Frontiers in plant science*, 6, p.406. [Online]. Available at: doi:10.3389/fpls.2015.00406.

Yates, A. D. et al. (2022). Ensembl Genomes 2022: an expanding genome resource for non-vertebrates. *Nucleic acids research*, 50 (D1), pp.D996–D1003. [Online]. Available at: doi:10.1093/nar/gkab1007.

Ye, C. et al. (2021). Initiation and Execution of Programmed Cell Death and Regulation of Reactive Oxygen Species in Plants. *International journal of molecular sciences*, 22 (23). [Online]. Available at: doi:10.3390/ijms222312942.

Yoshida, K. et al. (2003). Molecular biology and application of plant peroxidase genes. *Applied microbiology and biotechnology*, 60 (6), pp.665–670. [Online]. Available at: doi:10.1007/s00253-002-1157-7.

Yoshida, T., Mogami, J. and Yamaguchi-Shinozaki, K. (2014). ABA-dependent and ABA-independent signaling in response to osmotic stress in plants. *Current opinion in plant biology*, 21, pp.133–139. [Online]. Available at: doi:10.1016/j.pbi.2014.07.009.

Yu, A. et al. (2020a). Photosynthetic Phosphoribulokinase Structures: Enzymatic Mechanisms and the Redox Regulation of the Calvin-Benson-Bassham Cycle. *The Plant cell*, 32 (5), pp.1556–1573. [Online]. Available at: doi:10.1105/tpc.19.00642.

Yu, B. et al. (2020b). Weighted gene coexpression network analysis-based identification of key modules and hub genes associated with drought sensitivity in rice. *BMC plant biology*, 20 (1), p.478. [Online]. Available at: doi:10.1186/s12870-020-02705-9.

Yu, H. et al. (2018a). Impact of Droughts on Winter Wheat Yield in Different Growth Stages during 2001–2016 in Eastern China. *International Journal of Disaster Risk Science*, 9 (3), pp.376–391. [Online]. Available at: doi:10.1007/s13753-018-0187-4.

Yu, S. et al. (2012). Identification of rice transcription factors associated with drought tolerance using the Ecotilling method. *PloS one*, 7 (2), p.e30765. [Online]. Available at: doi:10.1371/journal.pone.0030765.

Yu, X. et al. (2018b). The Jasmonate ZIM-domain protein gene SlJAZ2 regulates plant morphology and accelerates flower initiation in Solanum lycopersicum plants. *Plant science: an international journal of experimental plant biology*, 267, pp.65–73. [Online]. Available at: doi:10.1016/j.plantsci.2017.11.008.

Yu, Z., Wang, X. and Zhang, L. (2018). Structural and Functional Dynamics of Dehydrins: A Plant Protector Protein under Abiotic Stress. *International journal of molecular sciences*, 19 (11). [Online]. Available at: doi:10.3390/ijms19113420.

Yuenyong, W. et al. (2019). Isocitrate lyase plays important roles in plant salt tolerance. *BMC plant biology*, 19 (1), p.472. [Online]. Available at: doi:10.1186/s12870-019-2086-2.

Zadoks, J. C., Chang, T. T. and Konzak, C. F. (1974). A decimal code for the growth stages of cereals. *Weed research*, 14 (6), pp.415–421. [Online]. Available at: doi:10.1111/j.1365-3180.1974.tb01084.x.

Zamora, O. et al. (2021). Jasmonic acid and salicylic acid play minor roles in stomatal regulation by CO2 , abscisic acid, darkness, vapor pressure deficit and ozone. *The Plant journal: for cell and molecular biology*, 108 (1), pp.134–150. [Online]. Available at: doi:10.1111/tpj.15430.

Zamora-Briseño, J. A. and de Jiménez, E. S. (2016). A LEA 4 protein up-regulated by ABA is involved in drought response in maize roots. *Molecular biology reports*, 43 (4), pp.221–228. [Online]. Available at: doi:10.1007/s11033-016-3963-5.

Zang, X. et al. (2017). Overexpression of wheat ferritin gene TaFER-5B enhances tolerance to heat stress and other abiotic stresses associated with the ROS scavenging. *BMC plant biology*, 17 (1), p.14. [Online]. Available at: doi:10.1186/s12870-016-0958-2.

Zargar, S. M. et al. (2017). Impact of drought on photosynthesis: Molecular perspective. *Plant Gene*, 11, pp.154–159. [Online]. Available at: doi:10.1016/j.plgene.2017.04.003.

Zeng, H. et al. (2015). Involvement of calmodulin and calmodulin-like proteins in plant responses to abiotic stresses. *Frontiers in plant science*, 6, p.600. [Online]. Available at: doi:10.3389/fpls.2015.00600.

Zeng, Z. et al. (2022). Gene-coexpression network analysis identifies specific modules and hub genes related to cold stress in rice. *BMC genomics*, 23 (1), p.251. [Online]. Available at: doi:10.1186/s12864-022-08438-3.

Zeven, A. C. (1998). Landraces: A review of definitions and classifications. *Euphytica/ Netherlands journal of plant breeding*, 104 (2), pp.127–139. [Online]. Available at: doi:10.1023/A:1018683119237.

Zhai, H. et al. (2021). Wheat heat tolerance is impaired by heightened deletions in the distal end of 4AL chromosomal arm. *Plant biotechnology journal*, 19 (5), pp.1038–1051. [Online]. Available at: doi:10.1111/pbi.13529.

Zhai, M. et al. (2016). Over-expression of JrsHSP17.3 gene from Juglans regia confer the tolerance to abnormal temperature and NaCl stresses. *Journal of plant biology = Singmul Hakhoe chi*, 59 (5), pp.549–558. [Online]. Available at: doi:10.1007/s12374-015-0507-9.

Zhai, Q. et al. (2015). Transcriptional Mechanism of Jasmonate Receptor COI1-Mediated Delay of Flowering Time in Arabidopsis. *The Plant cell*, 27 (10), pp.2814–2828. [Online]. Available at: doi:10.1105/tpc.15.00619.

Zhang, H. et al. (2013a). Persistent whole-chromosome aneuploidy is generally associated with nascent allohexaploid wheat. *Proceedings of the National Academy of Sciences of the United States of America*, 110 (9), pp.3447–3452. [Online]. Available at: doi:10.1073/pnas.1300153110.

Zhang, H. et al. (2019). Phosphoinositide 3-Kinase Promotes Oxidative Burst, Stomatal Closure and Plant Immunity in Bacterial Invasion. *Frontiers in plant science*, 10, p.1740. [Online]. Available at: doi:10.3389/fpls.2019.01740.

Zhang, H. M. et al. (2013b). Changes of dehydrin profiles induced by drought in winter wheat at different developmental stages. *Biologia plantarum*, 57 (4), pp.797–800. [Online]. Available at: doi:10.1007/s10535-013-0361-x.

Zhang, H., Zhao, Y. and Zhu, J.-K. (2020). Thriving under Stress: How Plants Balance Growth and the Stress Response. *Developmental cell*, 55 (5), pp.529–543. [Online]. Available at: doi:10.1016/j.devcel.2020.10.012.

Zhang, H.-M. et al. (2022a). Cloning and Expression Analysis of YSK2 Type Dehydrin Gene WDHN2 in Wheat (Triticum aestivum). *Journal of Agricultural Botany*, 30 (8), pp.1443–1453. [Online]. Available at: doi:10.3969/j.issn.1674-7968.2022.08.001.

Zhang, J. et al. (2018). Effect of Drought on Agronomic Traits of Rice and Wheat: A Meta-Analysis. *International journal of environmental research and public health*, 15 (5). [Online]. Available at: doi:10.3390/ijerph15050839.

Zhang, L. et al. (2015). Three WRKY transcription factors additively repress abscisic acid and gibberellin signaling in aleurone cells. *Plant science: an international journal of experimental plant biology*, 236, pp.214–222. [Online]. Available at: doi:10.1016/j.plantsci.2015.04.014.

Zhang, R. et al. (2022b). Heat Stress Tolerance 2 Confers Basal Heat Stress Tolerance in Allohexaploid Wheat (Triticum aestivum L.). *Journal of experimental botany*. [Online]. Available at: doi:10.1093/jxb/erac297.

Zhang, S.-W. et al. (2009). Altered architecture and enhanced drought tolerance in rice via the down-regulation of indole-3-acetic acid by TLD1/OsGH3.13 activation. *Plant physiology*, 151 (4), pp.1889–1901. [Online]. Available at: doi:10.1104/pp.109.146803.

Zhang, X. et al. (2022c). Wheat genotypes with higher yield sensitivity to drought overproduced proline and lost minor biomass under severer water stress. *Frontiers in plant science*, 13, p.1035038. [Online]. Available at: doi:10.3389/fpls.2022.1035038.

Zhang, X. et al. (2023). Mitigation of heat stress in wheat (Triticum aestivum L.) via regulation of physiological attributes using sodium nitroprusside and gibberellic acid. *BMC plant biology*, 23 (1), p.302. [Online]. Available at: doi:10.1186/s12870-023-04321-9.

Zhao, C. et al. (2017). Temperature increase reduces global yields of major crops in four independent estimates. *Proceedings of the National Academy of Sciences of the United States of America*, 114 (35), pp.9326–9331. [Online]. Available at: doi:10.1073/pnas.1701762114.

Zhao, W. et al. (2020). Effects of Water Stress on Photosynthesis, Yield, and Water Use Efficiency in Winter Wheat. *WATER*, 12 (8), p.2127. [Online]. Available at: doi:10.3390/w12082127 [Accessed 21 August 2023].

Zheng, R. et al. (2023). OsFTL12, a member of FT-like family, modulates the heading date and plant architecture by florigen repression complex in rice. *Plant biotechnology journal*, 21 (7), pp.1343–1360. [Online]. Available at: doi:10.1111/pbi.14020.

Zheng, S.-Z. et al. (2012). Phosphoinositide-specific phospholipase C9 is involved in the thermotolerance of Arabidopsis. *The Plant journal: for cell and molecular biology*, 69 (4), pp.689–700. [Online]. Available at: doi:10.1111/j.1365-313X.2011.04823.x.

Zheng, T. et al. (2019). Post-transcriptional regulation of Ghd7 protein stability by phytochrome and OsGI in photoperiodic control of flowering in rice. *The New phytologist*, 224 (1), pp.306–320. [Online]. Available at: doi:10.1111/nph.16010.

Zhou, L. et al. (2010). Rice hypersensitive induced reaction protein 1 (OsHIR1) associates with plasma membrane and triggers hypersensitive cell death. *BMC plant biology*, 10, p.290. [Online]. Available at: doi:10.1186/1471-2229-10-290.

Zhou, Y. et al. (2012). NnHSP17.5, a cytosolic class II small heat shock protein gene from Nelumbo nucifera, contributes to seed germination vigor and seedling thermotolerance in transgenic Arabidopsis. *Plant cell reports*, 31 (2), pp.379–389. [Online]. Available at: doi:10.1007/s00299-011-1173-0.

Zhu, M. et al. (2019). WGCNA Analysis of Salt-Responsive Core Transcriptome Identifies Novel Hub Genes in Rice. *Genes*, 10 (9). [Online]. Available at: doi:10.3390/genes10090719.

Zhu, W. et al. (2014). The dehydrin wzy2 promoter from wheat defines its contribution to stress tolerance. *Functional & integrative genomics*, 14 (1), pp.111–125. [Online]. Available at: doi:10.1007/s10142-013-0354-z.

Zhu, X. et al. (2015). The DnaJ OsDjA7/8 is essential for chloroplast development in rice (Oryza sativa). *Gene*, 574 (1), pp.11–19. [Online]. Available at: doi:10.1016/j.gene.2015.07.067.

Zivcak, M. et al. (2013). Photosynthetic electron transport and specific photoprotective responses in wheat leaves under drought stress. *Photosynthesis research*, 117 (1–3), pp.529–546. [Online]. Available at: doi:10.1007/s11120-013-9885-3.

9. Supplementary Material

Table S2.1: Stress-associated hub gene module membership, degree score and module size.

Hub Gene	Module	Module Size	Degree Score
TraesCS5A01G105900.1	Blue	1632	1036
TraesCS7A01G050400.1	Darkgrey	159	158
TraesCS6A01G158100.1	Darkmagenta	110	109
TraesCS5A01G369900.1	Darkorange	164	161
TraesCS5D01G125500.1	Lavenderblush1	20	8
TraesCS1A01G314200.1	Navajowhite3	43	42
TraesCS3B01G270800.1	Purple	407	383
TraesCS3B01G285100.1	Skyblue	154	153
TraesCS2A01G447400.1	Skyblue	154	153
TraesCS2B01G205600.1	Steelblue	84	52
TraesCS4D01G207500.1	Thistle	52	35
TraesCS7B01G149200.1	Thistle	52	31
TraesCS7D01G241100.1	Thistle	52	30

Table S2.2: Normalized mean dry weight loss, and RPKM values for each of the six hub genes, for each accession used in validation experiment.

Accession	Normalized Dry Weight Loss	*TraesCS2B01G205600.1*	*TraesCS4D01G207500.1*	*TraesCS5A01G105900.1*	*TraesCS5D01G125500.1*	*TraesCS7B01G149200.1*	*TraesCS7D01G241100.1*
_033	0.442	1.88	108.6	4.49	14.75	37.11	23.75
_048	0.365	3.07	228.07	11.27	35	49.76	37.43
_050	0.445	6.97	103.37	12.34	38.52	22.36	18.78
_055	0.424	2.88	232.59	10.47	38.42	76.05	47.69
_057	0.136	3.71	435.56	10.15	41.85	131.68	95.82
_062	0.389	3.13	49.19	3.57	20.11	10.86	7.39
_078	0.398	0.46	42.93	5.46	21.73	13.04	10.95
_083	0.266	2.88	227.04	9.94	36.07	81.01	44
_086	0.413	15.4	217.76	10.76	34.57	57.74	41.38
_155	0.135	6.86	186.5	9.41	34.76	49	27.26
_235	0.34	0.7	91.87	8.1	15.61	16.04	13.08
_268	0.513	3.51	55.88	5.15	19.03	12.04	9.7
_292	0.358	1.69	72.19	5.75	24.41	6.22	11.87
_310	0.478	0.68	71.91	5.91	21.09	16.44	14.56
_324	0.365	2.89	51.3	5.82	21.54	14.78	12.47

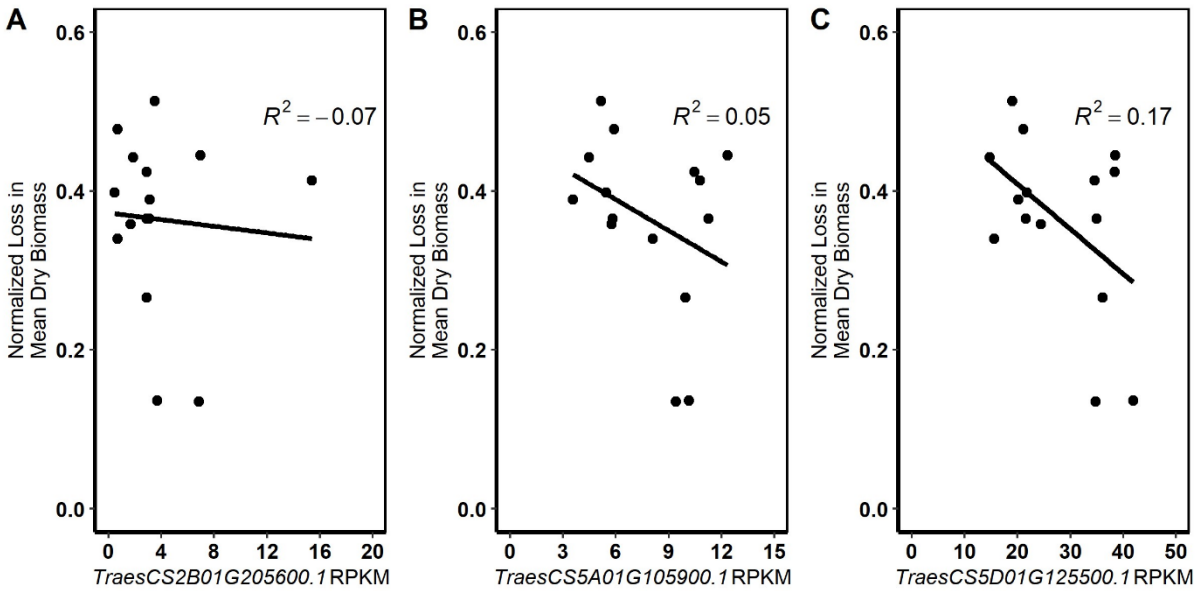

Figure S2.1: Expression of remaining hub genes were not significantly associated with early thermotolerance.

Table S3.1: Information on landrace accessions from the YoGI panel, screened previously for thermotolerance, used for transcriptomic analysis in the present work.

YoGI ID	Heat Tolerance	Accession ID	Accession Name	Origin	Cultivar ID
_033	Susceptible	CWI 9391	PI 250413	Pakistan	CWI 9391_PI 250413
_048	Tolerant	CWI 13434	INDIAN	United States	CWI 13434_INDIAN
_050	Susceptible	CWI 13561	WHITE-RUSSIAN	China	CWI 13561_WHITE-RUSSIAN
_055	Susceptible	CWI 13661	RED EGYPTIAN	Egypt	CWI 13661_RED EGYPTIAN
_057	Tolerant	CWI 13719	PURPLESTRAW	Canada	CWI 13719_PURPLESTRAW
_083	Tolerant	1190004	Rustam Exp Farm 99	Iraq	1190004_Rustam Exp Farm 99
_086	Susceptible	1190021	Boojri	India	1190021_Boojri
_155	Tolerant	1190264		Canary Islands	1190264_
_235	Tolerant	1190627		Iran	1190627_
_268	Susceptible	1190732	Gahu (Nepali) or Kyo (Sikkimese)	India	1190732_Gahu (Nepali) or Kyo (Sikkimese)
_292	Tolerant	1190788		USSR	1190788_
_310	Susceptible	01C0203425	Cordillera 3	Paraguay	01C0203425_ Cordillera 3
_324	Tolerant	01C0202172	MCB 192	Peru	01C0202172_ MCB 192

Table S3.2: Results of the gene ontology term enrichment analysis for each module in the co-expression network, besides the grey pseudo-module. The most significantly enriched GO term is provided, as well as any significantly enriched GO terms related to the stress response. FDR-adjusted *p*-values associated with each significantly enriched term are given in brackets. The number of genes in each module is also provided.

Module	Module Size	Enriched GO Terms
antiquewhite4	87	ADP binding (6e-05)
bisque4	160	NA
black	2415	Regulation of RNA biosynthetic process (6.9e-24), Response to water (0.017)
blue	6821	Prosthetic group metabolic process (0.027)
brown	4023	Amino sugar catabolic process (9e-15), Response to oxidative stress (4.7e-11)
brown4	163	Transferase activtiy (0.045)
coral1	91	NA
coral2	86	Protein heterodimerization activity (6.9e-05)
cyan	1704	L-phenylalanine catabolic process (0.0057)
darkgreen	804	Multi-multicellular organism process (9.5e-13), Response to stress (0.0013)
darkgrey	676	Cellular carbohydrate metabolic process (3.7e-06), Cell wall biogenesis (0.012)
darkmagenta	382	NA
darkolivegreen	384	Sexual reproduction (0.0001), Response to oxidative stress (0.008)
darkorange	637	Protein phosphorylation (5.2e-20)
darkorange2	167	NA
darkred	934	Regulation of RNA biosynthetic process (0.036)
darkseagreen4	96	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (0.0042)
darkslateblue	155	Oxidation-reduction process (0.0028)
darkturquoise	688	Carbohydrate metabolic process (0.0081)
floralwhite	169	Regulation of nucleobase-containing compound metabolic process (5.7e-05)
green	2766	Response to oxidative stress (1.2e-09), Response to stress (0.0032)
greenyellow	2007	Transferase activity, transferring acyl groups other than amino-acyl groups (0.0009)
grey60	1360	Phosphate-containing compound metabolic process (1.8e-07)
honeydew1	96	Iron ion binding (0.013)
indianred4	36	Organic substance biosynthetic process (0.039)
ivory	169	NA
lavenderblush3	98	Protein phosphorylation (3.1e-05)
lightcoral	41	Protein phosphorylation (8.4e-06)
lightcyan	1555	Nucleosome organization (3.4e-09), Response to stress (0.032)
lightcyan1	182	NA
lightgreen	1243	Protein phosphorylation (0.0024)
lightpink4	103	ADP binding (0.00013)
lightsteelblue	49	Organelle (0.045)
lightsteelblue1	186	Small molecule binding (0.012)
lightyellow	1110	Cysteine-type peptidase activity (0.015)

magenta	2176	Protein binding (5e-06)
maroon	111	NA
mediumorchid	85	NA
mediumpurple2	54	Single-organism biosynthetic process (9.6e-06)
mediumpurple3	193	Vesicle-mediated transport (0.018)
midnightblue	1598	Photosynthetic electron transport chain (4.7e-06)
navajowhite2	116	NA
orange	650	Cotranslational protein targeting to membrane (0.0004)
orangered3	57	NA
orangered4	195	Single-organism metabolic process (3.9e-08)
paleturquoise	435	Oxidation-reduction process (0.0019)
palevioletred3	121	Pollen-pistil interaction (0.0054)
pink	2202	Photosynthesis (5.8e-18), Cellular response to stimulus (0.0011)
plum	58	NA
plum1	236	NA
plum2	151	NA
purple	2161	Regulation of primary metabolic process (0.013)
red	2501	Response to biotic stimulus (3.6e-14), Response to stress (1.7e-05)
royalblue	1004	Response to wounding (0.013)
saddlebrown	591	Nucleosome assembly (0.00054)
salmon	1761	Protein phosphorylation (5.9e-08), Response to stress (0.0018)
salmon4	124	NA
sienna3	372	NA
skyblue	614	Nitrogen compound transport (0.00048)
skyblue1	74	Nucleic acid binding (3.2e-07)
skyblue2	85	NA
skyblue3	285	ADP binding (0.042)
steelblue	542	Protein disulfide oxidoreductase activity (0.00037)
tan	1840	Regulation of primary metabolic process (0.0048)
thistle1	128	Photosynthesis (2.5e-13)
thistle2	130	Cellular component assembly (0.034)
turquoise	26420	Translation (9e-128), Cellular response to stress (1.2e-17)
violet	429	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor (0.0045)
white	620	Phosphorylation (0.003)
yellow	3993	Cellular protein localization (1.3e-29), Response to heat (0.021)
yellow4	75	Single-organism metabolic process (0.013)
yellowgreen	310	NA

Table S4.1: Significant markers for flower emergence delay, identified by linear regression analysis. Results of this analysis are given, as well as the BLAST hit for each gene, and their module membership.

Gene	Module	Adjusted R^2	*p*-value	BLAST Hit
TraesCS6A02G139400	black	0.626456	0.00077	*Triticum aestivum cold-responsive protein kinase 1-like*
TraesCS2D02G533000	blue	0.551498	0.002199	*Triticum aestivum isolate R0934F.300k_Assembly_5 7 restorer of fertility-like protein gene*
TraesCS2A02G271700	darkgrey	0.599455	0.001147	*Triticum aestivum transcription factor BHLH6-like*
TraesCS2D02G270300	grey60	0.646682	0.00056	*Triticum aestivum transcription factor BHLH6-like*
TraesCS5D02G219300	lightgreen	0.595335	0.001217	*Triticum aestivum protein TIFY 10c-like*
TraesCS2A02G132300	pink	0.708666	0.000188	*Triticum aestivum protein RICE FLOWERING LOCUS T 1-like*
TraesCS2A02G269700	pink	0.680762	0.000315	*Triticum aestivum flowering-promoting factor 1-like protein 4*
TraesCS2B02G210500	pink	0.655434	0.000486	*Triticum aestivum zinc finger protein 7-like*
TraesCS2B02G154800	pink	0.607252	0.001025	*Triticum aestivum protein RICE FLOWERING LOCUS T 1-like*
TraesCS7A02G233300	pink	0.583062	0.001444	*Triticum aestivum protein RADIALIS-like 4*
TraesCS1B02G136300	purple	0.559116	0.001992	*Triticum aestivum zinc finger CCCH domain-containing protein 34-like*
TraesCS4A02G096300	turquoise	0.588917	0.001332	*Triticum aestivum small polypeptide DEVIL 11-like*
TraesCS4D02G195600	black	0.561254	0.001937	*Triticum aestivum omega-3 fatty acid desaturase, endoplasmic reticulum-like*
TraesCS6D02G232400	black	0.560482	0.001956	*Triticum aestivum acetylglutamate kinase-like*
TraesCS6D02G017800	blue	0.781703	3.70E-05	*Triticum aestivum protein Rf1, mitochondrial-like*
TraesCS3B02G468400	blue	0.65623	0.00048	*Triticum aestivum squamosa promoter-binding-like protein 2*
TraesCS7D02G375600	blue	0.562942	0.001894	*Triticum aestivum C2 and GRAM domain-containing protein At1g03370-like*
TraesCS2D02G224100	brown	0.609744	0.000988	*Triticum aestivum syntaxin-132-like*
TraesCS5D02G463600	darkgrey	0.643438	0.00059	*Triticum aestivum uncharacterized LOC123125834 (LOC123125834)*
TraesCS7D02G186100	darkgrey	0.554176	0.002124	*Triticum aestivum shikimate kinase 2, chloroplastic-like*

TraesCS2D02G452200	darkred	0.552341	0.002175	*Triticum aestivum probable purine permease 11*
TraesCS2A02G161300	grey60	0.641019	0.000614	*Triticum aestivum UMP-CMP kinase 3*
TraesCS7D02G199400	lightcyan	0.551574	0.002196	*Triticum aestivum chlorophyll a-b binding protein of LHCII type 1-like*
TraesCS7B02G462700	lightgreen	0.592737	0.001262	*Triticum aestivum cysteine-rich receptor-like protein kinase 29*
TraesCS1B02G397900	pink	0.711232	0.000179	*Triticum aestivum uncharacterized LOC123097831*
TraesCS3D02G181200	pink	0.708407	0.000189	*Triticum aestivum zinc finger CCCH domain-containing protein 4-like*
TraesCS6B02G127300	pink	0.650305	0.000529	*Triticum aestivum L-aspartate oxidase, chloroplastic-like*
TraesCS1A02G279700	pink	0.601118	0.00112	*Aegilops tauschii subsp. strangulata serine/arginine repetitive matrix protein 2-like*
TraesCS2A02G380100	pink	0.567698	0.001778	*Triticum aestivum uncharacterized LOC123189871 (LOC123189871)*
TraesCS5D02G107200	steelblue	0.659945	0.000451	*Triticum aestivum isolate TcLr26 glutaredoxin C14 protein (GRXC14)*
TraesCS6D02G015000	turquoise	0.756391	6.86E-05	*Triticum aestivum cytochrome P450 709B2-like*
TraesCS4D02G097100	turquoise	0.686091	0.000286	*Triticum aestivum uncharacterized LOC123096298 (LOC123096298)*
TraesCS7A02G053600	turquoise	0.666139	0.000406	*Triticum aestivum pentatricopeptide repeat-containing protein At5g08510-like*
TraesCS7B02G271151	turquoise	0.656493	0.000477	*Triticum aestivum amino acid permease 1-like*
TraesCS2B02G574800	turquoise	0.637816	0.000645	*Triticum aestivum uncharacterized LOC123047293 (LOC123047293)*
TraesCS3B02G442400	turquoise	0.627284	0.00076	*Triticum aestivum ubiquitin carboxyl-terminal hydrolase 21-like (LOC123071829)*
TraesCS2B02G147900	turquoise	0.584516	0.001415	*Triticum aestivum nucleolar protein 56-like*
TraesCS5B02G536900	turquoise	0.57851	0.001537	*Triticum aestivum arginine decarboxylase-like*

Table S5.1: Information of the target mutations present in each TILLING line initially screened for drought tolerance are provided, with the mutated based shown as a capital letter. The effect of these mutations on drought tolerance were predicted, according to the putative function of each hub gene. Cadenza1487 appears twice as it contained two missense mutations in its gene sequence – both causing deleterious base changes (SIFT = 0) and both used for genotyping. Cadenza1487 plants were deemed to be mutants if they possessed the mutant allele at either of these loci.

Probe ID	Gene	Module	TILLING Line	Mutation Effect	Predicted Effect on Drought Tolerance	WT Allele	Mutant Allele
Ta.963.2.A1_at	*TraesCS4D01G050400*	Blue	Cadenza0423	SIFT = 0	Improved	tgaagcacgagccctccC	tgaagcacgagccctccT
Ta.963.2.A1_at	*TraesCS4D01G050400*	Blue	Cadenza1679	STOP	Improved	agacggcgaactggtcctG	agacggcgaactggtcctA
TaAffx.54965.1.S1_x_at	*TraesCS3A01G299200*	Darkgreen	Cadenza0212	STOP	Reduced	cgtcagcgaagaagttgtgG	cgtcagcgaagaagttgtgA
TaAffx.54965.1.S1_x_at	*TraesCS3A01G299200*	Darkgreen	Cadenza0579	SIFT = 0	Reduced	gtattctcaggttctgtggcC	gtattctcaggttctgtggcT
TaAffx.13302.1.S1_at	*TraesCS4A01G204500*	Grey60	Cadenza1245	SIFT = 0	Improved	GtcgagaaaaacaatagtgagatgC	GtcgagaaaaacaatagtgagatgT
TaAffx.39452.2.S1_at	*TraesCS1D01G237300*	Orangered	Cadenza0393	STOP	Uncharacterized	cgtgccacttcctatattcaactG	cgtgccacttcctatattcaactA
TaAffx.39452.2.S1_at	*TraesCS1D01G237300*	Orangered	Cadenza0883	STOP	Uncharacterized	ccttatcaggcagcagttgaaC	ccttatcaggcagcagttgaaT
TaAffx.129139.2.S1_x_at	*TraesCS4D01G115100*	Pink	Cadenza1499	SIFT = 0	Reduced	ggcggcgtcctcgatcaC	ggcggcgtcctcgatcaT
TaAffx.129139.2.S1_x_at	*TraesCS4D01G115100*	Pink	Cadenza1540	STOP	Reduced	ccctcccgcctcgctttC	ccctcccgcctcgctttT
Ta.29814.1.S1_at	*TraesCS7D01G347300*	Purple	Cadenza1687	SIFT = 0	Reduced	cgagaggttcccgtcgcG	cgagaggttcccgtcgcA
Ta.29814.1.S1_at	*TraesCS7D01G347300*	Purple	Cadenza1779	SIFT = 0	Reduced	tcgagaggttcccgtcgC	tcgagaggttcccgtcgT
Ta.11437.1.A1_x_at	*TraesCS5D01G511200*	Red	Cadenza1420	SIFT = 0	Uncharacterized	gcgacgatcatgtcctggC	gcgacgatcatgtcctggT
Ta.11437.1.A1_x_at	*TraesCS5D01G511200*	Red	Cadenza1588	SIFT = 0	Uncharacterized	attgggacacagtcaggtgG	attgggacacagtcaggtgA
Ta.28962.2.S1_at	*TraesCS7D01G376800*	Steelblue	Cadenza1487	STOP	Reduced	gagaaatttgcatctcgtctatgG	gagaaatttgcatctcgtctatgA
Ta.28962.2.S1_at	*TraesCS7D01G376800*	Steelblue	Cadenza1487	STOP	Reduced	ggtaccgtgcctgatagacC	ggtaccgtgcctgatagacT
Ta.28962.2.S1_at	*TraesCS7D01G376800*	Steelblue	Cadenza2074	STOP	Reduced	aataccgtcttcccgtgctG	aataccgtcttcccgtgctA

Table S5.2: Information on the primers and optimized PCR conditions used to genotype each TILLING line used for backcrossing (and then also used during the genotyping of plants in the initial drought tolerance screen). Forward and reverse primer sequences are given, as well as the optimized annealing temperature and PCR extension time.

Gene	TILLING Line	Forward Primer Sequence	Reverse Primer Sequence	PCR Annealing Temperature	PCR Extension Time (seconds)
TraesCS1D01G237300	Cadenza0393	GCCATGCTGAAAGCACCAAC	CCACATACTCTGCTTCAGAGC	55	60
TraesCS4D01G050400	Cadenza0423	AGCGGGTGATGCACGCCGACA	CTTCCCCCGGATGCCAAGG	61.5	45
TraesCS1D01G237300	Cadenza0883	CTATTCTATCTGAAGGGGCTA	CAATCAAGCTCGGCAAATTTG	55	60
TraesCS7D01G376800	Cadenza1487	GGAAAATAGAACACATATACACCGG	GTAAGCCTTGACTCGAGG	57.5	60
TraesCS7D01G376800	Cadenza1487	GCTTTGCGAGTGGTTCAGC	CGGTCAACAAGATTCCTCCAT	60	45
TraesCS7D01G347300	Cadenza1687	ACCCGAGCCTGCGTGGCTTC	CGAAGACATGGTGGTCCTG	63	30
TraesCS7D01G376800	Cadenza2074	CTCGAGAAGCCCATCGTG	CGAGTCGGGGAATGGCAA	55	60

Table S5.3: Details of the basic thermocycling conditions used in PCR reactions are provided, with those steps in bold italics corresponding to the steps that were repeated in a loop 35 times. Ranges are given for steps when optimized values for each reaction varied.

Step	Temperature (°C)	Time (seconds)
Initial Denaturation	98	180
Denaturation	***98***	***10***
Annealing	***55-63***	***30***
Extension	***72***	***30-60***
Final Extension	72	120
Hold	10	Indefinitely

Table S6.1: Information on the landrace accessions used to examine the effect of early drought stress exposure on the wheat transcriptome.

YoGI accession name	Plant ID	Plant Name	Origin	Collection	Habit
_002	BW 7112	RA SHIH PAI Pïl	China	CIMMYT	Spring
_007	BW 19498	LOHARI Y91-92 NO.123	Nepal	CIMMYT	Spring
_010	CWI 2165	K7155.22	Kenya	CIMMYT	Spring
_017	CWI 3924	ROOI INDIES	South Africa	CIMMYT	Spring
_018	CWI 3926	ROOI SPITSKOP	South Africa	CIMMYT	Spring
_021	CWI 6075	KOELZ W 9375:AE	India	CIMMYT	Spring
_026	CWI 6118	KOELZ W 9660:AE	India	CIMMYT	Spring
_047	CWI 13432	WHITE FIFE	Japan	CIMMYT	Spring
_059	CWI 15005	LAGEADINHO	Brazil	CIMMYT	Spring
_145	1190224	Red wheat	China	Watkins	Spring
_153	1190254		Morocco	Watkins	Spring
_161	1190292	Asprokoutsoullon	Cyprus	Watkins	Spring
_164	1190305		Egypt	Watkins	Spring
_261	1190705	Kooseh	Iran	Watkins	Spring