Futureproofing Our Food: Dissecting the Drought Regulome of Bread Wheat

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Abstract

Hexaploid bread wheat (*Triticum aestivum* L.) supplies over half the world with a substantial portion of its daily calories, but a changing climate threatens to disrupt supply. Climate change endangers wheat production through massive perturbations in weather patterns, intensifying and increasing the frequency of droughts, and ultimately jeopardising food security and farmer livelihoods, especially in low- and middle-income regions. Projected population growth to over 9.7 billion people by 2050 compounds these issues, further increasing demand. Our understanding of the regulatory basis of wheat drought responses is limited, especially at the early vegetative stages of growth, and so dissecting this complex response is essential for futureproofing our wheat.

Using the YoGI panel, a collection of over 340 landraces, this thesis seeks to dissect the molecular and physiological responses of wheat seedlings to harsh drought exposure at the transcriptomic and epigenetic levels. Through phenotypic analyses, comparative transcriptomics, weighted gene co-expression network analysis (WGCNA), and whole-genome bisulphite sequencing (WGBS), this thesis: characterises the impact of early drought on the wheat transcriptome; explores candidate master regulators of the wheat drought response; identifies candidate master regulators of drought tolerance across wheat landraces; examines the role of drought-responsive DNA methylation in modulating gene expression and transposable element activity; and evaluates the potential effects of within- and across-generation drought priming treatments on drought tolerance in wheat landraces.

By detailing potential mechanisms, key transcriptomic master regulators, and epigenetic signatures associated with the drought response and drought tolerance, this thesis aims to inform future molecular crop breeding programmes that seek to develop more drought-tolerant wheat cultivars.

Author’s Declaration

This thesis is a body of original work and has not previously been presented for a degree or other qualification at this University or elsewhere. All sources have been acknowledged as references. Unless otherwise stated at the relevant sections, I, Isaac J. Reynolds, am the sole author.

The entirety of the work detailed in **Chapter 2** has been published in the journal *Frontiers in Plant Science* (doi.org/10.3389/fpls.2023.1212559), and was written in collaboration with other authors, mainly Liam J. Barratt with whom I share first authorship. Plant growth experiments, RNA extraction, quality control, and sample preparation was conducted in collaboration with the co-author. RNA sequencing quality control and alignment mapping was conducted by myself and Dr Sara Franco Ortega, another co-author, and comparative transcriptomic analyses and were carried out by myself, alongside its accompanying text, while co-expression network construction and subsequent analysis was carried out by the co-author, alongside its accompanying text. I created all figures. Slight changes from the original have been made to the text and to figures to improve readability, ensure that formatting and style are consistent across all chapters, and to clarify points where necessary. Original text is available at the published version (above) and original figures are available in the Supplementary Data.

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This thesis would not have been finished without the love and support of so many people; for them I am forever grateful.

Abbreviations

ABA	*Abscisic Acid*
AD	*After Drought*
ANOVA	*Analysis of Variance*
ART-ANOVA	*Aligned Rank Transformed Analysis of Variance*
ATAC-seq	*Assay for Transposase-Accessible Chromatin Sequencing*
BCE	*Before Common Era*
BD	*Before Drought*
CAD	*Cadenza*
CDS	*Coding Sequence*
ChIP-seq	*Chromatin-immunoprecipitation Sequencing*
Co-IP	*Co-immunoprecipitation Sequencing*
DEA	*Differential Expression Analysis*
DEG	*Differentially Expressed Gene*
DIN	*DNA Integrity Score*
DMA	*Differential Methylation Analysis*
DMG	*Differentially Methylated Gene*
DML	*Differentially Methylated Loci*
DMR	*Differentially Methylated Region*
DMTE	*Differentially Methylated Transposable element*
DNA	*Deoxyribonucleic Acid*
DS	*Drought Susceptible*
dsRNA	*Double Stranded RNA*
DT	*Drought Tolerant*
EMS	*Ethyl methanesulfonate*
epiQTL	*Epigenetic Quantitative Trait Locus*
ET	*Ethylene*
FDR	*False Discovery Rate*
FITC-Dextran	*Fluorescein isothiocyanate-dextran*
GA	*Gibberellic Acid*
GFP	*Green Fluorescent Protein*
GMO	*Genetically Modified Organism*
GO	*Gene Ontology*
GSxx	*Zadoks Growth Stage XX*
HMW	*High Molecular Weight*
HPS	*High Pressure Spraying*
HYV	*High Yielding Varieties*
IAA	*Indole-3-Acetic Acid; Auxin*
IWGSC	*International Wheat Genome Sequencing Consortium*
JA	*Jasmonic Acid*
JAZ	*Jasmonate ZIM-Domain*
lncRNA	*Long noncoding RNA*
log2FC/ LFC	*log2FoldChange*
LTR	*Long Terminal Repeat*
m6A	*N6-Methyladenosine*
MeDIP	*Methylated DNA Immunoprecipitation*
MENA	*Middle East and North Africa*
miRNA	*Micro RNA*
mRNA	*Messenger RNA*
MSAP	*Methylation-Sensitive Amplification Polymorphism*
MUT	*Mutant*
ncRNA	*Noncoding RNA*
nDW	*Normalised Dry Weight (Dry Biomass)*
OE	*Overexpression*
PCA	*Principal Component Analysis*

PCR	*Polymerase Chain Reaction*
PEG	*Polyethylene Glycol*
PSII	*Photosystem II*
PTGS	*Post-transcriptional Gene Silencing*
QC	*Quality Control*
QTL	*Quantitative Trait Loci*
RdDM	*RNA-directed DNA Methylation*
RIN	*RNA Integrity Score*
RNA	*Ribonucleic Acid*
RNAi	*RNA Interference*
RNA-seq	*RNA Sequencing*
ROS	*Reactive Oxygen Species*
RRBS	*Reduced Representation Bisulphite Sequencing*
SA	*Salicylic Acid*
scRNA-seq	*Single Cell RNA Sequencing*
siRNA	*Small interfering RNA*
SMC	*Soil Moisture Content*
SNP	*Single Nucleotide Polymorphism*
sRNA	*Small RNA*
STRING	*Search Tool for the Retrieval of Interacting Genes/Proteins*
TAD	*Topologically Associated Domain*
T-DNA	*Transfer DNA*
TE	*Transposable Element*
TF	*Transcription Factor*
TFBM	*Transcription Factor Binding Motif*
TFBS	*Transcription Factor Binding Site*
TILLING	*Targeting Induced Local Lesions in Genomes*
TPM	*Transcripts per Million*
TSS	*Transcription Start Site*
TTS	*Transcription Termination Site*
WGBS	*Whole Genome Bisulphite Sequencing*
WGCNA	*Weighted Gene Co-expression Network Analysis*
WT	*Wild Type*

Key Definitions in this Thesis

Drought response here refers to the coordinated reactions of a plant employed to survive water-deficit conditions at various physiological, morphological, and molecular levels.

Drought tolerance in this thesis refers to a plant's inherent ability to survive and sustain growth under water-deficit conditions.

Drought priming in this thesis is used to describe a mechanism in which an initial exposure to stress (priming treatment) can induce an acquired enhanced tolerance to that stress in the future, or to induce an acquired enhanced tolerance in its progeny.

Co-expression network modules in WGCNA are clusters of genes that show substantial co-expression with each other. Genes within a module are thought to often share regulatory mechanisms or have similar molecular functions.

Module Eigengenes are defined as the first principal component of a module within a given network, and does not refer to a single extant gene, but rather a representative expression profile for its associated module.

Hub genes are highly connected genes within a module that may act as key transcriptional regulators – they are 'large effect' individuals that show high levels of expression correlation with the other genes in the module. There are many ways of identifying potentially biologically relevant hub genes, though in this thesis hub genes that are further explored are selected based on their number of connections to other genes (degree score), differential expression status, expression profile, and putative function.

Gene connectivity refers to how connected a gene is to other genes. In this thesis a connectivity threshold is used to remove very weak correlation connections. After filtering this using **edge weights**, gene connectivity is calculated as the total number of genes that a given gene is connected to in an undirected manner, and this is referred to as the '**degree score'**.

An **edge weight** quantifies the strength of the co-expression correlation relationship between two genes, scaled between 0 and 1 – for example, an edge weight of 1 may represent the strongest possible connection and correlation between two genes, suggesting that they have identical expression profiles.

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1. Introduction

1.1. Wheat, drought, and early development

Modern bread wheat (*Triticum aestivum)* is an incredibly important crop, consumed across the globe by humans and animals alike, and is the most widely used member of the *Triticeae*, a tribe of the wider grass family (*Poaceae*). Its importance to our diets cannot be overstated, evidenced by its prevalence in civilisations across history, and the billions of people who rely on it daily.

Like any facet of agriculture, wheat is exposed to environmental hurdles – heat, drought, salinity, among others – exacerbated by increasingly unpredictable and harsh climate conditions in much of the world. Further understanding how our crops respond to stresses, and how we might improve them, especially at conventionally 'safe' periods of growth like the seedling stage, is paramount to achieving wheat's continued success and meeting the nutrient demand of the future.

As a globally grown crop, wheat spans an enormous geographical range, and this has given rise to many landraces (locally bred domesticates), characterised by their adaptations to the specific environmental hurdles of their locales (Cheng et al., 2024; Korpetis et al., 2023; Zeven, 1998). In turn, this has enabled landrace collections to possess a considerable amount of genetic and phenotypic diversity that can be exploited for studying the genetic basis of traits and for the identification of novel alleles, mechanisms, and traits that can be used to improve the elite germplasm.

In this thesis, I will examine drought responses at the seedling stage, leveraging the broad genetic and phenotypic diversity of wheat landraces to explore stress regulation at the early stages of development. This introductory chapter will detail the climatic challenges that threaten our crop production, a brief evolutionary history of wheat and the benefits and traps of its complex genome, what we know about how wheat crops respond to stress, and how we might study this response in more depth to ultimately develop more resilient wheat crops.

1.2. A hotter planet: current and future impacts of climate change on wheat

Climate change poses an enormous challenge for agriculture as we progress through the 21st century – stable growing conditions that farmers have relied upon for centuries are expected to shift, and many are expected to rethink land use (Bousfield, Morton and Edwards, 2024; Plumpton and Wentworth, 2019). Climate change, in this thesis, refers to the long-term shifts in average global temperatures and weather patterns that have defined our climates, from the local to the continental and global levels.

The climate has historically been influenced by natural, non-anthropogenic processes: short term shifts in cyclical ocean patterns, such as El Niño and La Niña phenomena, or volcanic eruptions causing atmospheric dimming through particulate release, simultaneously contributing to temperature increases and short-term cooling through reflected sunlight; longer term shifts can be caused by changes to Earth's orbit and wobble (known as Milankovitch cycles), resulting in longer periods of warming or cooling, by tectonic plate movements, or by variations in solar irradiance from the Sun. However, increasing global temperatures have been observed since the onset of the Industrial Revolution, as have increasingly variable weather patterns. Global surface temperatures have increased by ~1.1°C since 1850-1900 (Calvin *et al.*, 2023), with the post-1970 rate of warming high than in any other 50 year period in the last two millennia: the Earth is now likely warmer than at any point in the last 100,000 years (Snyder, 2016; Calvin *et al.*, 2023), and between 1-1.75°C higher than during the pre-industrial era (WMO, 2021). The consequences of increased warming and more extreme, variable climates are threefold for agriculture: longer, hotter, drier weather patterns, disrupted water cycles, and less predictable precipitation, all of which disrupt agricultural water availability.

As temperatures rise and the climate becomes more unstable, patterns of rainfall become more erratic, with wetter wet events and drier dry events (Zaitchik *et al.*, 2023), accompanied by rising sea levels, reduced terrestrial water storage, and changed plant evapotranspiration rates (Seo et al., 2025; Pratap and Markonis, 2022). Weather in the United Kingdom is becoming increasingly extreme: UK winters (2009-2018) were found to be 12% wetter than the average from 1961-1990, while summers were projected to be up to 5.1°C hotter on average by 2070 (Met Office, 2023), increasing the likelihood of frequent and severe periods of high aridity. Regions sensitive to drought are projected to have expanded by 120x104 km^2 between 2003-2020 compared to 1981-2002 (Li et al., 2024c). Soil moisture deficit, which reached up to ~280 km^3 across Europe in 2022, is expected to increase, driven by acute and lagging effects of climate change (Bevacqua *et al.*, 2024). As a result, water shortages are expected to substantially increase (Schewe *et al.*, 2014); 80% of global crop land is expected to see intensified water scarcity between 2026-2050, increasing from 39% in the period between 1981-2005 (Liu et al., 2022d), while the United Nations project that over 5.8 billion could experience water scarcity for at least one month per year (UNESCO World Water Assessment Programme, 2018).

These climatic shifts represent a challenging scenario for agriculture; estimates suggest that even a 1°C increase in temperature can result in a 10-20% reduction on crop yields (Rose et al., 2016; Asseng, Foster and Turner, 2011). Severe droughts have in the past substantially affected food security, with severe water shortages in Russia in 2010 resulting in large reductions in grain harvest and a destabilised market (Wegren, 2011), while spring wheat is considered to be particularly sensitive to drought in many of its primary production zones, especially during critical growth periods (Liu, Lv and Li, 2024).

While the changing climate may present some advantages for domestic wheat production, with a reduction in the probability of unexpected late frosts and heat stress at critical stages alongside increased CO_2 levels promoting growth (Harkness *et al.*, 2020; Slater *et al.*, 2022), drought would still remain a potential problem at the local level, while more extreme weather variations would increase vulnerabilities to stress previously unexperienced by UK agriculture (Slater *et al.*, 2022). Similarly, both climate change-associated cold-wet and hot-dry periods were found to have adverse effects on winter wheat yields, grain filling, grain weight, and grain number in China (Jiang *et al.*, 2025).

Climate change is not expected to just have an impact on yields, with research indicating that agronomic traits like anthesis date and overall plant development in wheat fields are shifting; fields are going to be impacted in different ways at different growth stages than conventionally thought. Meta-analyses suggest that warming may advance anthesis dates by upwards of 3.5%, while reducing the length of the growth cycle by 1.18% (Li et al., 2022b). Some estimates suggest that anthesis could shift earlier by up to 14 days (He et al., 2024b), which could cause substantial shifts in wheat sowing and harvesting time. Grain quality is also expected to affected, with increased CO2 levels causing reduced wheat grain protein content (Fradgley et al., 2023; Taub, Miller and Allen, 2008). Other agronomic traits affected include soil quality: projections suggest that current trends of soil erosion are likely to continue and worsen over the 21st century (Eekhout and de Vente, 2022), while soil-plant-atmospheric interactions are likely to be affected by extreme weather conditions that disrupt soil structure, reduce moisture, and increase salinity and acidification (Oishy *et al.*, 2025). As droughts begin to impact the early growing season more and more in countries like the UK (Environment Agency, 2025; AHDB, 2025), it is paramount that we understand wheat's drought regulome from sowing to seed.

In essence, climate change presents a significant challenge for increasing wheat crop yields – increasing temperatures driven by anthropogenic activity is expected to intensify heatwaves and drought events, cause more perturbations in weather patterns, disrupt the water cycle, and shift the stable seasons that farmers have enjoyed for centuries, requiring substantial adaptation in agricultural practices and in seedstocks. As these seasons shift and droughts further encroach on the early growing periods of wheat, a greater understanding of how these plants respond to water deficit is needed. By further understanding how plants respond to drought stress in their early stages, and by identifying key markers of tolerance in wheat plants at early stages, we may be able to fill critical knowledge gaps and ameliorate some of the worst effects of drought on wheat growth.

1.3. A hungry planet: population growth and crop yields

By 2050, feeding a projected 9.4 billion people will place unprecedented strain on global agricultural production. This growth is disproportionately concentrated to the global south, with populations across Central and Southern Africa expected to almost double (United Nations and Department of Economic and Social Affairs Population Division, 2019). Global populations have increased over twofold since 1960 (Lam, 2023) with an average increase by country of ~1% a year (Zahid, Robinson and Kelly, 2016); estimated growth rates were significantly lower during the early Holocene, with only a 0.04% year-on-year increase (Zahid, Robinson and Kelly, 2016; Gurven and Davison, 2019).

Agricultural yields have historically kept pace with steady population growth, largely through improvements to seedstocks, rather than increases in cultivated land, which has not substantially increased globally since 1960 (**Figure 1.1**). China has shown yield increases that vastly outstrip population growth, yet in Pakistan yield growth lags significantly. When demand outstrips production, food scarcity can slow or decline population growth and drive health crises (Lima, 2014; Molotoks, Smith and Dawson, 2021). Scarcity presents a monumental threat to health, disproportionally so in developing nations where it contributes to half of childhood mortality cases and rates of cardiovascular disease and adult mortality (Sun et al., 2020; Bain et al., 2013).

Current agricultural practices have generally been sufficient to support global population growth (**Figure 1.1**), but this is unlikely to be true in the future with current trends. Preventing food scarcity and promoting food security likely requires substantial growth from current production; projected demand necessitates cereal production increases of 70-100% (Roy et al., 2024; Hunter et al., 2017), but even this is dependent on factors like maintaining nutritional content and the amelioration of climate change. Some models project that the yields of staple crops and cereals, including wheat, will decrease by up to 15-30% in some regions by 2050 (Serdeczny et al., 2017; Rippke et al., 2016; Hristov et al., 2020; Jaggard, Qi and Ober, 2010), falling short of rising demand. As mentioned previously, current wheat yield increases are relatively low or stagnant (Ray *et al.*, 2012; Hawkesford *et al.*, 2013), with the current rate of increases in wheat only resulting in an increase of 38% by 2050 (Ray *et al.*, 2013). Achieving higher yields to meet demand requires a change of approach towards improving crop productivity – conventional crop improvement programmes and tinkering with agricultural practices are not achieving the gains required.

Yield gains can be made through modernising farm and agricultural practices, especially in lower and middle-income countries. Greater and optimised use of fertilisers and pesticides, shifting towards irrigation-based systems, and increased mechanisation through the use of tractors and similar equipment can almost double yields by increasing the efficiency of agricultural land (Silva et al., 2023; Thomas, 2020; Epule et al., 2018). In countries with well-established and high-yielding agronomic practices, greater yields are better achieved through improvements to the seed stock, leveraging molecular technologies for the identification and introduction of beneficial alleles to the germplasm, or through amelioration of specific local factors hampering yield growth – for example, soil degradation and desertification across China and India over the last century are known to limit yields (Bhattacharyya *et al.*, 2015; Delang, 2018).

A coordinated strategy pairing environmental policies to reverse detracting factors with the use of genetic improvement strategies should help to raise crop yields in the coming decades. Crop yields must rise to meet growing demand, but the looming environmental stresses of climate change represent a hurdle to obtaining the production increases required.

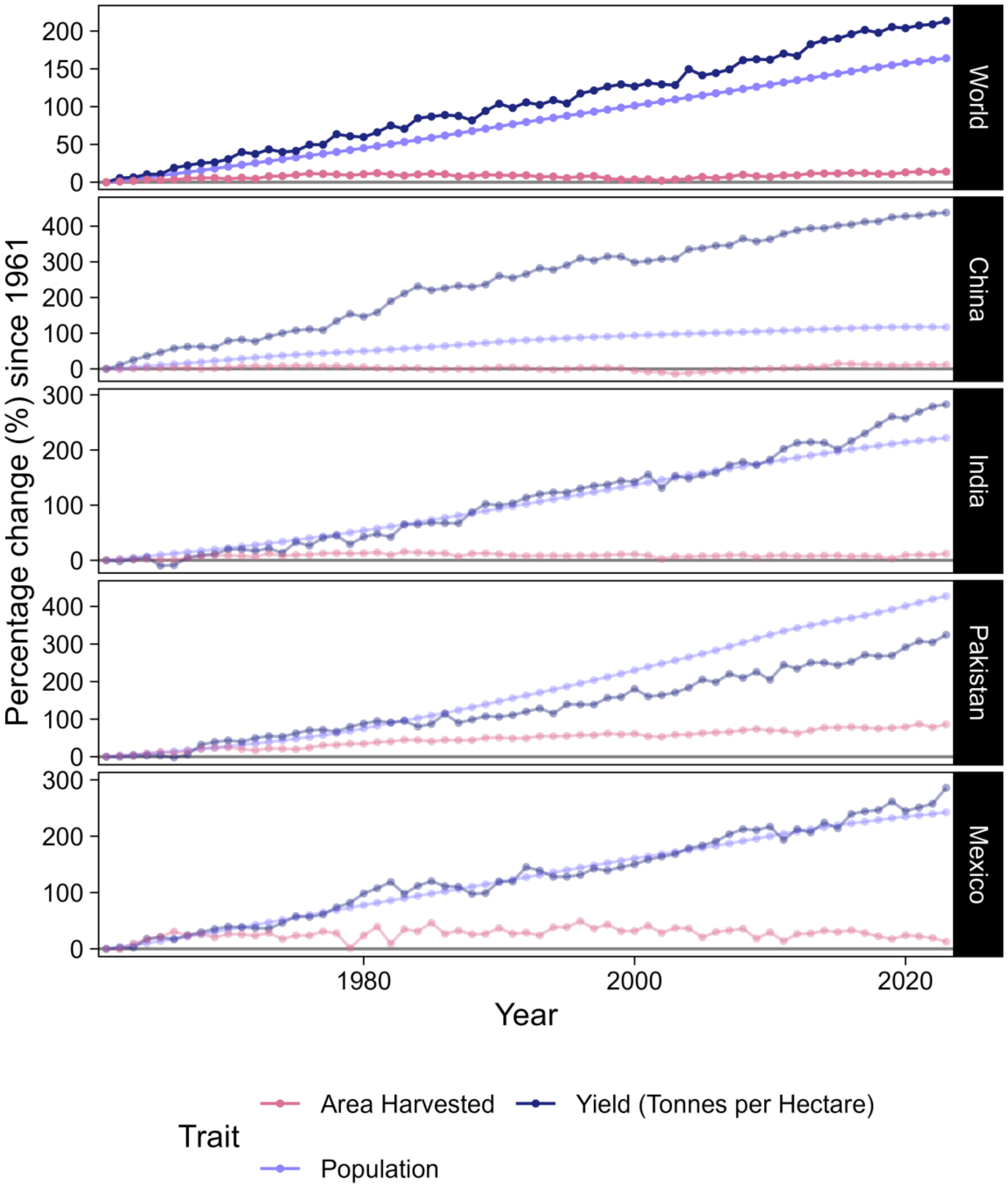

Figure 1.1: Yield has matched or fallen short of population growth in some major wheat-producing countries, with the exception of China. Percentage change in population growth, area of land harvested, and yield (tonnes per hectare) relative to the value in 1961 was compared per year in both global and country-specific contexts. Countries were selected due to their relevance to and uptake of high-yielding varieties during the Green Revolution. Data was sourced from Ritchie (2017).

1.4. Ancestors and agriculture: an evolutionary history of bread wheat

Wheat is now one of the most universally produced cereals on the planet, covering over 545 million acres of farmland, and in 2022 produced 808.4 million tonnes of grain – second only to Maize production (Food and Agriculture Organization of the United Nations (FAO), 2025). Wheat's importance to our diets cannot be overstated, comprising over 20% of global daily protein and calorie intake (Shiferaw *et al.*, 2013; Venske *et al.*, 2019); wheat is also vital as an agricultural feed, with one fifth of global wheat grain production consumed by livestock (Grote *et al.*, 2021). As a result, people's livelihoods and the production of wheat grains are deeply interconnected: in a world where wheat is a dietary keystone, global shifts in wheat production and availability due to war, geopolitical instability, and wider economic forces have both direct and indirect impacts (Kuhla, Puma and Otto, 2024; Gaupp et al., 2020; Distefano et al., 2018). Over half the world relies on uninterrupted wheat production, but the looming environmental, political, and economic challenges of the future strain the delicate relationship between the world and one of its most essential crops.

Since its first cultivation in ~8,000 BCE in the Fertile Crescent of the Levant and Mesopotamia, bread wheat has played a significant role in our lives. Wheat propagated from the Fertile Crescent as early farming communities spread agricultural practices across Africa, Asia, and Europe (de Sousa *et al.*, 2021). Its use has hugely increased in the millennia since; wheat is grown across an enormous range, as far north as 67° N and as far south as 45° S, as low as 2 metres below and as high as 4570 m above sea level, and across temperate, tropical, and sub-tropical environments (De Flaviis et al., 2022; Levy and Feldman, 2022; Food and Agriculture Organization of the United Nations, 2002), exposing it to an enormous range of environmental conditions.

Modern bread wheat emerged between 9,000 - 8,000 BCE through whole genome duplication and hybridisation events between wild progenitors, resulting in the allohexaploid species that we use today. Wheat's progenitors contributed three separate diploid homoeologous sub-genomes that gave rise to an overall genome size of ~15.5-17 Gb comprised of 42 chromosomes ($2n = 6x = 42$ (AABBDD): in a given somatic cell, 2n represents diploid chromosome count, and 6x denotes its hexaploidy, resulting in six sets of seven chromosomes). The development of the hexaploid wheat genome can be traced back to two separate allopolyploidisation hybridisation events; the first between wild diploid wheat (*Triticum uratu;* AA genome progenitor) and an unknown likely extinct species (BB genome progenitor) about 800,000 years ago, producing the allotetraploid *Triticum turgidium* (AABB). The second hybridisation event occurred between *T. turgidum* and *Aegilops tauschii* (DD), approximately 10,000 years ago, ultimately producing the modern *T. aestivum* – this second hybridisation event is believed to have occurred due to the cultivation of tetraploid wheat alongside *A. tauschii*, suggesting that there may be no 'true' wild variety of *T. aestivum* (Levy and Feldman, 2022). The unknown BB genome progenitor is thought to be a relative of another grass, *Aegilops speltoides*, though a specific species is yet to be identified (El Baidouri *et al.*, 2017; Levy and Feldman, 2022). Wheat's complex genome is just one of the reasons that it has been able to establish itself as one of the dominant crops of our time, but it has also given rise to considerable genomic obstacles that have held back molecular breeding programmes and crop improvement against environmental stressors.

1.5. Challenging genomes: Polyploidisation, genes, and transposable elements

Polyploidisation underpins the agronomic success and resilience of many of today's staple crops by enabling genetic redundancy, novel variation, and increased protein abundance. Through unintentional selection by early farmers, polyploidisation has played a significant role in the domestication of many of today's crops (Akagi *et al.*, 2022); as a result, polyploidy is the basis of many modern crop traits (King, Seppälä and Neiman, 2012; Li et al., 2024d).

Allopolyploidisation hybridisation, a process in which the genomes of two distinct species are incorporated into the same nuclei, can give rise to plants with a greater number of alleles for a given gene. This process has given rise to substantial agronomic benefits through increased gene copy number: polyploidy is associated with increased seedling size, faster germination rate, higher biomass accumulation, and desirable gluten quality (Chan, Ooi and Guja, 2021; Liu et al., 2022b; Feldman et al., 2012). Gene copies across the three genomes, known as homoeologues, make up a large proportion of genes in wheat, with both pairs and triplet homoeologues found to have originated from wheat's common ancestors and brought back together through subsequent speciation and allpolyploidisation events (Pfeifer *et al.*, 2014; Walkowiak *et al.*, 2020).

Polyploidisation can also enable enhanced stress tolerance in plants, displaying greater resistance to parasites (King, Seppälä and Neiman, 2012), abiotic stresses like drought (Jiang et al., 2022; Diallo et al., 2016; del Pozo and Ramirez-Parra, 2014), high salinity (Meng et al., 2016; Yan et al., 2015a), and temperature (Godfree et al., 2017; Nejadsadeghi et al., 2014). Wheat itself is known to be considerably more tolerant to stresses like salinity and drought than its progenitors (Yang *et al.*, 2014; Li, Ma and Xiong, 2018; Gui *et al.*, 2021), something attributed to the wheat genome's high number of alleles and ability to 'generate' diversity through deletions and mutations as a result of recombination (Dubcovsky and Dvorak, 2007). Through polyploidisation events, plant offspring can potentially have a greater chance of acquiring novel adaptations to adverse environmental conditions, especially when the progenitors occupy distinct environmental niches or conditions (Van de Peer et al., 2021; Carlson et al., 2017). These novel adaptations can also emerge as a result of neofunctionalisation due to reduced selection pressures at homoeologous genes across the genomes (Ramanna and Jacobsen, 2003; Heslop-Harrison, Schwarzacher and Liu, 2023).

Transposable elements (TEs) are sequences of DNA able to translocate themselves across the genome using cellular machinery. TEs are in part responsible for wheat's considerable genome complexity, making up ~85% of its overall content, much like in species closely related to its progenitors (Wicker et al., 2018; Zhu et al., 2021a). Polyploidy most likely had little influence on the overall content of TEs as each of the AA, BB, and DD genomes contains similar proportions (Wicker et al., 2018; IWGSC, 2018). These TEs are able to insert themselves into genes, can be expressed as transcripts, and can influence gene expression through both *cis*- and *trans*-regulatory mechanisms, making them volatile genomic elements that can give rise to adaptation and evolution (Bariah, Keidar-Friedman and Kashkush, 2020; Levy and Feldman, 2002; Sabot et al., 2005; Wan et al., 2022b). Their activity is largely regulated through high levels of DNA methylation, which broadly prevents them from transposing and producing transcripts (Miura et al., 2001; He et al., 2022b). Some TEs in wheat appear to be conserved mechanisms for influencing gene expression in *cis* by acting as promoter regions (Wicker *et al.*, 2018), but their ubiquity in genic regions can make these associations hard to dissect. TE activity has previously been implicated in wheat stress responses (Bariah, Gribun and Kashkush, 2022; Sun et al., 2013; Kong et al., 2020), with insertions and TE-derived small RNAs involved in modulating the expression of TEs and genes alike.

Despite the enormous advantages that wheat's complex genome has conveyed, it has also brought about substantial challenges for wheat genomics and molecular crop improvement strategies. Polyploidy and its increase in gene copies can be incredibly useful for improving agricultural productivity (Ramanna and Jacobsen, 2003; Ruiz *et al.*, 2020; Omere, Nwaoguala and Emede, 2023) and is associated with benefits for abiotic stress tolerance (Tossi et al., 2022; Li et al., 2024d, 2021b), disease resistance (Wan et al., 2022b), and reproductive success (Gross and Schiestl, 2015; te Beest et al., 2012), but can introduce significant hurdles for molecular crop improvement strategies. Validation of gene function can take substantially longer with conventional techniques than in diploid species due to the several generations required to generate triple homoeologue knockout lines, while the increased number of chromosomes can lead to greater meiotic instability, negatively affecting offspring (Thriveni *et al.*, 2024). Homoeologues can also be a convoluting factor for CRISPR/Cas9 editing (Schaart, van de Wiel and Smulders, 2021; Ahmad *et al.*, 2023). Large genomes are more costly to sequence and assemble, and require longer computational time for sequencing, alignment, annotation, modelling, and analysis (Claros *et al.*, 2012; Kress *et al.*, 2022) than small genomes like the one found in the model dicot *Arabidopsis thaliana* – wheat's genome clocks in at ~17 Gb (Zhu et al., 2021a) compared to *A. thaliana's* ~125 Mb (Arabidopsis Genome Initiative, 2000), making it ~136x larger – limiting the availability of resources and data. As a result, there is still a lot of work to be done in many complex polyploids, many of which are critically important crops, and this has held back efforts to improve abiotic stress tolerance.

1.6. The Wheat Wild West: From early domesticates to the Green Revolution

Hexaploid bread wheat cultivation and agriculture began in the Fertile Crescent of the Middle East and radiated out to the furthest corners of the world. Wheat is known to have reached Cyprus, Greece, India, and Egypt by 6,000 BCE, followed by its emergence in central Europe by 5,000 BCE and northern Europe by 3,000 BCE (Cooper, 2015; Gohar *et al.*, 2022). Work at archaeological sites like Çatalhöyük (~6,400 BCE; Neolithic settlement, Türkiye) unearthed charred grains were found to display hexaploidy like modern *T. aestivum* (Bilgic *et al.*, 2016), suggesting that they may have come from interstitial varieties that bridge the gap between early and contemporary forms of wheat.

The rapid expansion of wheat from the Fertile Crescent gave rise to an incredible range of phenotypic and genetic diversity as plants adapted to hyperlocal environmental conditions. The Cresent spans temperate Mediterranean lowlands to semi-arid steppes and high mountain ranges (Baird *et al.*, 2018; Castiello *et al.*, 2025), niches that would have driven local wheat adaptations and were likely home to early cultivated wheat. The wheat germplasm has evolved in lockstep with agriculture, though as cultivars became more localised they likely reduced in genetic diversity due to continual selective breeding (Tanksley and McCouch, 1997; Zeibig, Kilian and Frei, 2022; Lopes et al., 2015). Domestication by early farmers would unknowingly take advantage of its large complex genome, rapidly selecting for beneficial traits under specific local environmental conditions: selection for major traits controlled by singular genes may have been possible in <30 years, an incredible rate for domestication (Peng, Sun and Nevo, 2011).

The ability of wheat landraces to adapt to such a vast range of conditions ensured that they would become a major crop for the local communities that relied upon them. Major traits selected for during the domestication of wheat would have ranged from head size, grain yield, more fragile glumes, easily threshed heads, seed content and taste, or architectural traits like the number of productive tillers (de Sousa et al., 2021; Fuller, 2007; Peng, Sun and Nevo, 2011; Lopes et al., 2015).

Since wheat's initial domestication, productivity and yields slowly built alongside agricultural practices and populations. Slow population growth ensured that mild year-on-year yield improvements and agricultural land increases were sufficient to meet demand. However, substantial population increases over the last hundred years – global populations increased by over 4 billion between 1960 and 2011, reaching over 7 bn people (Lam, 2023) – necessitated intervention to dramatically increase yields. By the mid-20th century, yearly yield gains stagnated while demand soared: our grains needed a Green Revolution. From the 1960s-1980s, yields of major crops like maize, wheat, and rice saw huge increases, with production of these crops almost tripling despite land use in area only increasing by 30% (Wik, Pingali and Brocai, 2012; Pingali, 2012; Evenson and Gollin, 2003), reducing the risk of widespread famines. Estimates suggest that 1% yield improvement was achieved per year (Evenson and Gollin, 2003).

One of the most impactful traits introduced to the wheat elite germplasm was the dwarfing trait; American high-yielding varieties (HYVs) were crossed with Japanese semi-dwarf varieties that exhibited reduced height with stronger stems than their landrace relatives. This trait was attributed to mutations at the Reduced height *(Rht)* genes, reducing responsiveness to gibberellic acids (GAs) and stunting growth to allow for resource redistribution to grain development and tillering (Hedden, 2003; Peng et al., 1999; Beharav, Pinthus and Cahaner, 1992).

Adoption of HYVs was rapid and widespread, and by the early 2000s these modern varieties accounted for over 90% of wheat plants sown in both Latin America and Asia, over 70% in the Middle East and North Africa, and almost 60% in non-MENA Africa (Evenson and Gollin, 2003; Renkow and Byerlee, 2010). Global yields raised dramatically, but at a cost: widespread HYV monocultures eroded the genetic diversity of the germplasm as landraces fell out of use (Eliazer Nelson, Ravichandran and Antony, 2019; Newton et al., 2010; Liu et al., 2024b). Reliance on a small selection of cultivars can result in greater vulnerability to adverse conditions (Khoury et al., 2022; Bohra et al., 2022; Keneni et al., 2012; Cheng et al., 2024). Monoculture was in stark contrast to prior agricultural practices; several landraces displaying similar traits were often grown together, conveying diversity that reduced yield losses under adverse conditions (Keneni *et al.*, 2012; Ceccarelli and Grando, 2020). Many Green Revolution-era HYVs and their successors are still in use today, amounting to a vital food resource with enormous vulnerabilities in a world with ever-increasing challenges.

Exploration and reintroduction of landrace variation from collections that span temporal and geographic ranges may provide a path towards ameliorating the narrowing genetic diversity of modern elite cultivars. Landraces possess untapped variation and beneficial alleles (Cheng et al., 2024; Korpetis et al., 2023; Barratt et al., 2023a) that could be introgressed back into elite germplasms to combat modern environmental challenges and mitigate the weaknesses of crop monocultures, as well as increase yields; wheat yield gains have again slowed and stagnated (Ray *et al.*, 2012; Schauberger *et al.*, 2018) and are outpaced by population growth (Ray *et al.*, 2013), highlighting the need for new sources of crop improvement. Landrace panels and their associated genetic diversity may present an opportunity to reintroduce beneficial alleles and develop resistant crops.

1.7. Landraces, diversity, and editing: current and future directions for the development of the wheat elite germplasm

The current elite germplasm is relatively homogenous and presents little genetic diversity relative to wheat landraces and its progenitors, failing to exhibit beneficial tolerance traits to the same degree that would enable them to better survive the stresses of future climates. Wheat breeding strategies must focus on reintroducing lost genetic diversity into desirable high-yielding cultivars, improving their drought tolerance at all stages of growth. This could be induced through introgression of desirable traits from landraces, or be introduced by mutagenesis, gene-editing techniques, stress priming, or by the introduction of transgenes. Current breeding programmes achieve up to 1% grain yield increases per year, but gains have stalled in recent years compared to population growth (Ray *et al.*, 2013; Rahman *et al.*, 2021; Cheng *et al.*, 2024).

Understanding of targets for crossing or gene editing has grown significantly over the last decade, with the advent of fast and cost-effective sequencing. By using phenotypic screening, sequencing, and high-quality genomes, researchers can identify genes and loci that are associated with drought tolerance or govern aspects of wheat's drought response. Some approaches, like Genome-Wide Association Studies (GWAS) and Associative Transcriptomics (AT) take large diverse populations and carry out correlations between genomic diversity and phenotypic traits to identify single nucleotide polymorphisms (SNPs) and gene expression markers (GEMs) that are associated with the phenotype of interest (Rathan et al., 2022; Miller et al., 2016). Quantitative Trait Loci analyses on crossed populations are also useful for identifying trait-associated genomic regions and genes that can subsequently be further explored using methods like GWAS; epiQTLs, where regions of DNA methylation are associated with traits, also presents a potential marker for exploration (Lu *et al.*, 2020).

These methods in concert can pinpoint specific genes with causal relationships to traits (Li et al., 2016; Zhang et al., 2023a), but may present many potential targets, the relationships between which can be difficult to dissect. No singular gene brings about a stress response, instead acting in concert with other genes in a network to effect change: techniques to dissect these relationships, like Weighted Gene Co-Expression Network Analysis (WGCNA) (Langfelder and Horvath, 2008, 2012), can leverage variation between samples and groups to identify the genes with large effects on other genes that display similar expression profiles. Alongside differential expression analysis (DEA), this branch of transcriptomics can help us identify key genes and markers that will have large impacts on drought stress tolerance and other agronomic traits. By utilising the available genomic and transcriptomic resources, researchers or breeders can use marker-assisted crossing and genetic modifications to produce more tolerant, greater-yielding wheat cultivars.

Some breeding approaches have taken to improving the genetic diversity of the elite wheat germplasm to identify new beneficial alleles and enhance abiotic stress tolerance. Wheat landraces – natural reservoirs of novel beneficial alleles and adaptive tolerance traits – are underutilised due to a lack of genetic and phenotypic resources (Wingen *et al.*, 2014). Recent research using the A.E. Watkins Landrace Collection, alongside modern cultivars (Cheng et al., 2024; Wingen et al., 2014), has elucidated the population structure of modern elite wheat, finding that elite cultivars originate from only two of seven ancestral groups, leaving five distinct groups with untapped genetic variation unincorporated into current modern wheat that can be exploited in breeding programmes. Backcrossing landraces with beneficial Watkins QTLs onto the Paragon elite cultivar enabled enhanced phenotypic variation among the progeny, increasing the range of time-to-heading by 8 days and total plant height by 18 cm, and ultimately achieved potential yield increases of almost 1 tonne per hectare (Cheng *et al.*, 2024), suggesting that introgression between landraces and elites can greatly improve agronomic traits in breeding programmes. Despite the benefits, the process of crossing these lines can prove time-consuming and difficult due to linkage drag – where spatially close harmful alleles are introduced alongside the desired QTL – requiring repeated backcrossing and screening or gene editing to remove (Voss-Fels *et al.*, 2017). Selection of varieties to cross together can also prove difficult, with agronomic traits found to be significantly associated with the choice of elite variety (Schneider *et al.*, 2021), indicating that some cultivars may be more receptive to the introgression from landraces and other sources of diversity than others (Lopes *et al.*, 2015; Laugerotte, Baumann and Sourdille, 2022). Despite this, landraces still offer a huge benefit to wheat crop improvement strategies – if not always through introgression, but in the identification of beneficial alleles for other kinds of genetic improvement.

A second approach to improving diversity among wheat lines has been to create synthetic hexaploid wheat (AABBDD) lines by recreating the natural crossing of progenitor species: by crossing *A. tauschii* (DD) with modern *T. turgidium* (AABB), researchers have been able to generate synthetic populations with substantially more genetic diversity than among current elite cultivars (Rosyara *et al.*, 2019). Synthetic lines exhibit significant variation beneficial for both biotic and abiotic stresses (Becker *et al.*, 2016; Das *et al.*, 2016; Morgounov *et al.*, 2018) due to the reduced effects of genetic bottlenecking through intense domestication. Selection of specific AABB and DD-donating varieties that show desirable traits or possess beneficial alleles can give rise to hexaploid lines that are particularly stress-tolerant or are pre-adapted to specific environments, making synthetic lines uniquely useful for introgression with elite lines and identification of novel alleles. Screening of synthetic lines revealed considerable variation among yield, but found that populations were largely superior to extant varieties of wheat in agronomic traits, antioxidant activity, and chlorophyll abundance under drought stress conditions (Mokhtari, Majidi and Mirlohi, 2022, 2024; Amini *et al.*, 2023). Other synthetic lines have been found to show enhanced root traits that are beneficial under drought (Liu et al., 2020a). Interestingly, synthetic wheat may also possess drought priming capabilities that exceed that of normal cultivars, with greater yield produced under drought conditions (Amini *et al.*, 2023), suggesting that current elite cultivars may have lost adaptive mechanisms that can help drive tolerance to stresses. The enhanced genetic diversity among synthetic wheat lines marks a promising step in reversing the genetic bottlenecking of elite cultivars but may present several of the same issues as crossing with landraces: time, linkage drag, and lack of genetic resources.

Gene overexpression mechanisms, beneficial alleles, and transgenes can be introduced to elite lines using *Agrobacterium tumefaciens*-mediated transformations, relying on plasmid expression vectors containing T-DNA (the desired region of DNA and a constitutive promoter) and a Vir region (and genes to transpose and integrate the T-DNA with the host) (Gelvin, 2003). This approach has been used to create wheat overexpression (OE) lines that have positively contributed to tolerance under drought: OE lines of *PYL1-1B*, a key ABA receptor, resulted in heightened water use efficiency through greater ABA sensitivity, made possible through the introduction of a MYB recognition site in the gene promoter (Mao *et al.*, 2022), while *Agrobacterium*-mediated overexpression of *DT1-A*, a zinc-finger TF, was found to convey enhanced drought tolerance without growth penalties through escape of repression by *MYC* TFs (Liu et al., 2025b). Meanwhile, introduction of entire transgenes has been used to substantially improve drought tolerance in wheat: transformation with Isopentenyl transferase (*IPT*) genes from *Agrobacterium* itself under the control of a drought-responsive dehydrin promoter was found to substantially increase chlorophyll and water content under drought conditions through the delay of leaf senescence through cytokinin biosynthesis (Ijaz, Bashir and Malik, 2025). While production of overexpression lines or introduction of transgenes can be incredibly useful for validating gene functions and identifying proof-of-concept beneficial alleles, legislation in many countries – including those in the European Union and Japan – restricts the growth and sale of crops containing transgenic modifications introduced through transformation like this, making it less desirable; genetically modified organisms made in this way are grown across the Americas, however (Turnbull, Lillemo and Hvoslef-Eide, 2021).

Ethyl methanesulfonate (EMS)-mediated mutagenesis of plants has been long used to generate mutant lines for diversity and screening experiments. The best example of this in wheat is likely the Germplasm Resources Unit's (John Innes Centre, UK) TILLING (Targeting Induced Local Lesions in Genomes) platforms, which contain over 2,700 mutant lines across both spring and winter wheat backgrounds. EMS-mediated mutations have led to improvements in drought tolerance in wheat, with the *BIG8-1* mutant able to survive severe water-deficit conditions for up to 21 days through slowed degradation of proteins essential for photosynthesis compared to WT plants (le Roux *et al.*, 2021), while EMS-mediated SNPs in drought-associated genes including NAC TFs, cytochrome P450s, and UDP-glucosyltransferases can convey increased drought tolerance through increased proline content and soluble sugars (Zahra *et al.*, 2021). Despite the successful use of EMS mutagenesis in increasing the diversity of homogenous elite varieties in several different commercial crops, it possesses many of the same problems as both landrace- and synthetic variety-based introgression approaches due to the potential introduction of undesirable alleles and the time taken to phenotype and backcross over several generations.

Conversely, gene editing mediated by CRISPR-Cas9 is considered to be more palatable to GMO-sceptic countries, with the United Kingdom passing the Genetic Technology (Precision Breeding) Act 2023, the European Union proposing to classify 'New Genomic Technique' plants (NGTs) and approve them, and Japan enabling faster market entry for genome-edited foods – however the uniting factor here is that all restrict the production and sale to varieties similar to those that have arisen through natural breeding, rather than those with the introduction of transgenes. This has increased the viability of CRISPR-Cas9-mediated plants: mutants containing indels in nucleotidase *Sal1* genes were found to exhibit greater resistance to polyethylene glycol conditions and drought through inherently wider stomata and greater induction of stomatal closure (Abdallah *et al.*, 2025), while deletion of sites in genes encoding receptor-like kinases (*RPK1*) has been found to increase tiller number, grain weight, root length and depth, and yield under drought conditions (Abdul Rahim *et al.*, 2024). CRISPR-Cas9 systems also have the potential to generate methylation variants through specific region targeting to modulate the gene expression (Gahlaut et al., 2020; Papikian et al., 2019). Despite the benefits, few clear examples exist of CRISPR-Cas9-mediated transformation for improving drought or other abiotic stress tolerance in wheat, likely due to challenges of using this approach in complex polyploids with homoeologous genes and repetitive sequences.

DNA methylation may be a useful tool for crop improvement in the future. Differing methylome profiles among genetically similar plants have previously been associated with increased abiotic stress tolerance and greater adaptability at the cost of reduced growth under normal conditions (Bossdorf et al., 2010; Kottler, VanWallendael and Franks, 2018; Zhang et al., 2013; Puy et al., 2018), suggesting that DNA methylation plays a role in plasticity to environmental cues. With a greater understanding of wheat's methylome and its response to stress, methylation plasticity under stress could potentially represent a selectable or editable trait; improvement programmes seeking to improve drought tolerance should consider epialleles and epigenetic diversity in their selection criteria should the wheat methylome prove to play a significant role in the regulation of the response to water deficit conditions (Puy *et al.*, 2018; Gahlaut *et al.*, 2020).

Taken together, these approaches propel the development of elite wheat cultivars: leveraging both natural and synthetic diversity to identify beneficial alleles while carrying out precision editing to introduce them to elite lines should accelerate the development of crops that can better resist environmental stresses and bring benefits to more markets. Further understanding of the regulation of abiotic stress responses however is required to maximise gains from these approaches: holistic multi-omics approaches comprise the most promising pathways to delivering high yielding, stress tolerant wheat crops.

1.8. Delineating between stress responses and stress tolerance

Plants cope with environmental stress in two complementary ways: by effecting immediate and reversible responses, and by holding inherent tolerance traits that enhance their survival across their life cycle. We describe stress responses as the cascade of molecular and physiological changes a plant may undergo in order to ameliorate deleterious biotic and abiotic environmental conditions – essentially how a plant deals with stressors in the moment, normally in a reversible manner (Zhang et al., 2023b; Zhang, Zhao and Zhu, 2020). Conversely, we define stress tolerance as a plant's inherent ability to survive, grow, and achieve reproductive success under disadvantageous environmental conditions through adaptations (Yadav and Bhatt, 2024; Zhang, Zhao and Zhu, 2020; Bashir et al., 2021).

Not all stress conditions affect plants in the same ways, yet many stress responses overlap. Stomatal closure, often associated with reducing water loss under drought conditions, is also a key part of responses to cold stress and pathogens (Agurla et al., 2018; Zhang et al., 2025c; Chen et al., 2022) as plants try to reduce water loss or block microbial entry respectively. Similarly the influx of reactive oxygen species, a hallmark of many stresses, is induced by various biotic and abiotic stresses with the dual role of a critical signalling molecule and a byproduct of disrupted photosynthesis (Bolwell *et al.*, 2002; Sewelam, Kazan and Schenk, 2016). Some stresses demand unique response strategies: heat stress without drought can induce stomatal opening as plants seek to cool down through evaporative cooling (Pankasem *et al.*, 2024), while salt and heavy metal stress can induce efflux of ions into the vacuole to prevent damage to critical components (Yan et al., 2015b; Li et al., 2022c).

We can further distinguish stress responses into two main categories: chronic and acute responses. Acute responses – those that occur as part of the short-term response to a stress – act to ameliorate immediate stress conditions through the activation of molecular effectors, signalling cascades, and temporary upregulation of genes, while chronic responses – those that may build over days and weeks – represent inducible acclimation traits like long-term osmolyte accumulation, transcriptional reprogramming, or epigenetic remodelling that mitigate sustained or recurring stresses (Georgieva and Vassileva, 2023; Zhang et al., 2023b).

In contrast, stress tolerance arises as a cumulative result of inherent traits that enable greater survival or growth under adverse conditions compared to those without those traits. True tolerance mechanisms are distinguished from stress responses by being inherent qualities of that species or variety, not just what a plant does in response to stress. Inherent traits like stay-green phenotypes, thicker waxy cuticles, advantageous plant architecture, basal gene expression or high antioxidant capacity can all be considered tolerance traits as they can exist prior to environmental challenges and do not require induction. Despite this, basal advantages do not mean that a plant is inherently better at *responding* to a stress than a sibling without those basal advantages. The difference between chronic stress responses and inherent tolerance traits is narrow but critical: tolerance mechanisms do not have to be induced by exposure to stress, nor do they require sustained adjustments to cellular or metabolic activity (Saputro et al., 2023; Lichtenthaler, 1998; Takahashi et al., 2020).

The complex interplay between stress responses and tolerance can be difficult to dissect, especially across large and diverse panels of plants, demonstrating the need for a holistic understanding of both to maximise gains from stress-focused crop improvement strategies.

1.9. How does drought stress affect wheat physiology and morphology?

Drought can bring about damage to wheat from germination all the way to the grain-filling stages. Beyond its effects on final grain yields, drought can lead to altered morphology and architecture during vegetative growth and cause enormous cellular, molecular, and physiological damage: almost all aspects of normal cellular function in plants rely on water availability.

Plants sense stress by detecting changes in their environment that disrupt normal cellular function and damage tissues via a network of plasma membrane-bound and cytoplasm-localised proteins alongside cell wall receptor-like kinases (Zhu, 2016; Zhang, Zhao and Zhu, 2020). Plants initially sense drought through the roots: while the exact signals sensed by plants have not been clearly defined, cell turgor pressure and membrane tension changes, affecting the conformation of osmosensor channel proteins like *OSCA1* and catalysing the release of Ca^{2+} into the cytosol (Kaur et al., 2022; Yuan et al., 2014). That influx of calcium ions catalyses the biosynthesis of phytohormones like abscisic acid and activates Ca^{2+}-dependent kinases which transduce the signal through the xylem and to the shoots (Lamers, van der Meer and Testerink, 2020; Wu et al., 2022a), causing changes to leaf physiological traits.

The morphological impacts of water deficit conditions on wheat are broad: drought is deleterious from sowing to threshing, impeding seed germination and seedling growth (Ahmed, Shabbir and Ahmed, 2025; Sharma et al., 2022), reducing growth, mitotic rates, and biomass accumulation during vegetative stages by inhibiting tillering and promoting leaf senescence (Mu et al., 2022; Nyaupane et al., 2024; Javed et al., 2022; Schuppler et al., 1998; Avramova et al., 2015), and ultimately limiting grain size, quality, and quantity (Sehgal et al., 2018; Yang et al., 2023a). Developmental time, a key agronomic trait, can also be affected; wheat frequently employs drought escape mechanisms under water deficit conditions (Nyaupane et al., 2024; Shavrukov et al., 2017), accelerating floral development to achieve reproductive success as fast as possible and to minimise the exposure of critical stages like anthesis and grain-filling to adverse conditions, significantly reducing days-to-heading and quickening grain-filling (Sharma et al., 2022; Yang and Zhang, 2006).

Many physiological changes are in large part due to reduced photosynthetic capacity: photosynthesis as a process heavily relies on water availability, with extended drought resulting in damaged photosynthetic machinery through the buildup of reactive oxygen species (ROS), reduced abundance of Rubisco, and reduced CO_2 intake as transpiration and gaseous exchange slow due to the lack of water and the closure of stomata (Qiao et al., 2024; Kang et al., 2023). Drought causes a decrease in the rate of photosynthesis in wheat through depletion of cellular carbon stores in mesophyll cells and reduced mesophyll conductance, less efficient photosystem II (PSII) activity, degradation of photosynthetic pigments, and increased hydrolysis of starch (Chen et al., 2022; He et al., 2012; Ramachandra Reddy, Chaitanya and Vivekanandan, 2004). The activity of key enzymes like Rubisco has also been found to significantly decrease as a result of up to 60% inhibitor accumulation at its catalytic sites in response to drought (Parry *et al.*, 2002).

Water-deficit-induced production of reactive oxygen species (ROS) can be severely damaging to the cell over long periods of time. As cellular activity shifts towards photorespiration and away from photosynthesis, and as the electron transport chain is deprived of acceptors due to reduced gaseous exchange, the partial reduction of O_2 generates ROS (Tripathy and Oelmüller, 2012; Foyer, 2018). Reactive oxygen species, like $O_2{\bullet}^-$ or H_2O_2, can oxidise and impair photosynthetic apparatus (PSII; thylakoid membranes) and essential cellular molecules (DNA; RNA; proteins), as well as organelles (Cruz de Carvalho, 2008; Foyer, 2018; Nyaupane *et al.*, 2024), leading to cell death and early senescence. At lower concentrations, ROS act as signalling molecules to trigger osmo- and photo-protective pathways and can promote physiological changes like stomatal closure and Ca^{2+} influx, as well as the upregulation of drought-responsive genes (Lee and Park, 2012), but overaccumulation under prolonged drought conditions negatively affects the cell. ROS hyperaccumulation can catalyse production of antioxidant enzymes, scavengers, and amino acids. Superoxide dismutase, glutathione reductase, ascorbate peroxidase, and catalases are all upregulated as ROS levels increase in the cell (Cruz de Carvalho, 2008; Lee and Park, 2012) to reduce free oxygen radicals to H_2O_2 or O_2 and limit cellular damage; sugars and amino acids like trehalose and proline can also act as antioxidants (Eh *et al.*, 2024).

Plants transpire, drawing water in through the roots and carrying it through the xylem through capillary action to leaves where it moves into foliar cells and eventually exits through open stomata. Drought disrupts this key process by limiting the water that can be drawn up through the roots, leaving sessile plants with few options than to limit water usage and bring about enormous molecular and physiological changes (Kulkarni *et al.*, 2017). Stomata, as microscopic pores in the epidermal surface of leaves, control evapotranspiration and atmospheric gaseous exchange in plants: when stomata are open, water vapour and oxygen are released, and simultaneously CO2 is able to enter the leaf mesophyll for fixation by Rubisco and use in photosynthesis (Lawson and Leakey, 2024; Wall et al., 2022). Periods of water deficit, among other stresses, cause stomatal closure through the decrease in the turgidity of guard cells that surround the pore (Agurla *et al.*, 2018). This is catalysed by the influx of abscisic acid (ABA) produced in response to drought conditions, triggering a signalling cascade that causes the efflux of anions and water from the cell (Munemasa *et al.*, 2015), reducing transpirational water loss. Turgor pressure is aided under mild drought conditions by the overproduction of cellular amino acids like proline, which accumulate and act as osmoprotectants against ROS, stabilise proteins, and reduce water loss by maintaining cellular solute concentrations (Szabados and Savouré, 2010; Ramachandra Reddy, Chaitanya and Vivekanandan, 2004). As a result, the stomata and its guard cells play a key role in wheat's physiological drought response; as stomatal conductance decreases, so does the rate of water uptake from the roots as the capillary pressure gradient decreases, conserving groundwater (Abdalla et al., 2022; Carminati et al., 2020).

Drought affects a number of molecular and physiological processes, but plants are also able to respond and limit its deleterious effects by coordinating physiological and cellular changes.

1.10. How do plants combat drought? How is this regulated? What don't we know?

Under drought, plants deploy enormous cellular and physiological changes that are driven by coordinated synergistic and antagonistic alterations to phytohormone pathways.

ABA is arguably the most critical drought response hormone: under water deficit, ABA is generated in a Ca^{2+}-dependent manner from carotenoids, initially accumulating at the leaves (Xiong and Zhu, 2003; Manzi *et al.*, 2015). ABA is shuttled around the plant via the xylem, enabling responses from root to shoot (Manzi *et al.*, 2015; Yang *et al.*, 2024); foliar ABA is an essential regulator of stomatal aperture (Zulfiqar et al., 2024; Bharath, Gahir and Raghavendra, 2021; Munemasa et al., 2015), a mediator of root cell elongation (Trapeznikov, Ivanov and Kudoyarova, 2003; Muhammad Aslam *et al.*, 2022), a facilitator of essential nutrient uptake (Zulfiqar et al., 2024; Kuromori, Seo and Shinozaki, 2018), and plays a key role in drought stress signalling through inhibition of PP2C phosphatases and upregulation of *SnRK2* protein kinases (Fujii et al., 2009; Ma et al., 2009; Zhang et al., 2023c). ABA concentration is known to be negatively correlated with stomatal conductance and aquaporin expression, while positively correlated with water use efficiency (Ding *et al.*, 2016; Li *et al.*, 2017). Many of these responses however cannot occur through ABA alone – stomatal control requires ABA in concert with ROS, jasmonic acid (JA) and its derivatives, salicylic acid (SA), and brassinosteroids, among others (Wei et al., 2021; Daszkowska-Golec and Szarejko, 2013; Savchenko et al., 2014).

Jasmonic acid is a broad activator of biotic and abiotic plant stress responses (Rehman et al., 2023; Wang et al., 2020b). Under drought, levels of JA and its active conjugate JA-isoleucine (JA-Ile) rapidly rise as part of the acute response: JA and *COI1*-mediated degradation of Jasmonate ZIM-domain (JAZ) repressor protein complexes release *MYC2* transcription factors which can bind to the promoter regions of stress response genes (Wang et al., 2021d; Hickman et al., 2017). After the initial rise, JA levels slowly return to baseline levels under prolonged stress (Riemann *et al.*, 2015). JA and its derivatives, like methyl-jasmonate (MeJA), have been implicated in reducing oxidative stress by enhancing antioxidant enzyme activity and inhibiting ROS production (Alam *et al.*, 2014; Zeng *et al.*, 2024) and promoting the accumulation of beneficial osmolytes like proline and spermidine (Bandurska, Stroiński and Kubiś, 2003; Sirhindi et al., 2016; Ma et al., 2024a; Zeng et al., 2024) through interaction between *NAC* transcription factors. Key JA-inducible transcription factors, like *bHLH148*, interact with *COI1* and JAZ repressors to activate drought-responsive DREB TFs to confer drought resistance (Seo *et al.*, 2011). Sustained high levels of JA can also lead to drought survival through senescence, mediated by free *MYC2* TFs which bind to *NAC* and *SAG* gene promoters and induce their expression (Zhang et al., 2020; Qiao et al., 2021) to initiate senescence, sequestering nutrients and restricting water loss (Schippers *et al.*, 2015).

Other phytohormones that play a role in the drought regulome include salicylic acid (SA), gibberellic acid (GA), indole-3-acetic acid (IAA; auxins) and ethylene (ET). In wheat, SA – much like jasmonic acid – is considered to mainly be involved in biotic stress resistance and immune defences (Qi et al., 2019; Maksimov et al., 2014), but is also known to play the role of a signalling molecule under osmotic stress conditions (Li *et al.*, 2013), promoting accumulation of osmolytes and antioxidants (e.g. glutathione, ascorbate), inducing the expression of dehydrins, chaperones, and protein kinases, and repressing ethylene synthesis (Awadalla et al., 2024; Khalvandi et al., 2021; Nazar et al., 2015). SA has been implicated in alleviating negative effects of drought on photosynthetic capacity through a reduction in ROS abundance and an increase in membrane stability among wheat landraces (Khalvandi et al., 2021; Nazar et al., 2015). Gibberellic acid can mitigate osmotic stress-mediated damage through similar mechanisms to SA, enhancing the abundance and activity of ROS scavengers and rescuing photosynthetic capacity (Emamverdian, Ding and Mokhberdoran, 2020; Iqbal and Ashraf, 2013), though this is in addition to its main role as a major positive growth regulator (Al-Huqail et al., 2023; Pfleger et al., 2011). Auxins like IAA, mainly involved in mediating growth, have also been found to mediate drought responses through *ARF* transcription factors, moderating soluble sugar content, chlorophyll concentration, root development and transpiration rate under drought stress conditions (Sadok and Schoppach, 2019; Verma et al., 2022; Mal and Panchal, 2024; Todorova et al., 2022). ET however represents a key restrictor of growth and promoter of senescence, but has also been positively associated with osmotic stress tolerance by supporting photosynthetic capacity (Yan et al., 2023a; Apelbaum and Yang, 1981; Sehar et al., 2021).

These stress-responsive phytohormone pathways are complex and intersect in different ways under different stress conditions; ABA production under drought tends to be antagonistic to the production of several other stress hormones, suppressing the biosynthesis of GA and foliar auxins (Liu and Hou, 2018; Liao et al., 2023; Lorrai et al., 2018) to slow cellular elongation and conserve resource use. High levels of ABA also interfere with SA biosynthesis by inhibiting *ICS1* expression through ABA-dependent *NAC* TFs as the plant shifts towards combatting abiotic rather than biotic stresses (Lorrai et al., 2018; Guo et al., 2016). The relationship between ABA and the closely linked JA and ethylene pathways is somewhat more complex: under drought stress, ABA tends to depress the biosynthesis of JA and ethylene (Anderson et al., 2004; Yang et al., 2019; de Ollas, Arbona and Gómez-Cadenas, 2015), though under some combination stress conditions they have been identified as being co-abundant with synergistic activity, with some studies noting that antagonism likely acts to prevent simultaneous expression of both biotic and abiotic stress responses (Kissoudis et al., 2016; Wan et al., 2025; Hasan et al., 2024; de Ollas, Arbona and Gómez-Cadenas, 2015). Exogenous spraying with ABA and JA precursors is known to induce stomatal closure both together and individually (Savchenko *et al.*, 2014), demonstrating a summative redundant role of different signalling pathways in stomatal dynamics – though this likely also reflects the roles of the different stress pathways, with JA more associated with responding to pathogenic threats (Liu et al., 2017a; Singh and Mukhopadhyay, 2021).

These drought-induced phytohormones can have a substantial impact on the wheat transcriptome. Abundant ABA is known to upregulate the expression of key osmotic stress genes encoding Late Embryogenesis Abundant (*LEA*) proteins which act as chaperones to facilitate the continued function of other proteins, prevent the formation of protein aggregates, and stabilise cellular membranes (Liu *et al.*, 2019; Yu *et al.*, 2019). Of the 179-281 *LEA* genes identified in wheat, 139 are known to be responsive to general abiotic stress, while at least 50 are specifically drought-responsive (Liu *et al.*, 2019; Zan *et al.*, 2020), signifying the family as a major element of the molecular drought response. Enhanced expression of *LEA* proteins like dehydrins are associated with greater drought tolerance (Hanin et al., 2011; Mehrabad Pour-Benab, Fabriki-Ourang and Mehrabi, 2019; Hassan et al., 2015). Phytohormones also play key roles in regulating the expression of other genes, like aquaporins, which play the role of molecular gatekeepers to cellular water (Yang et al., 2022a). These transmembrane channel proteins (*TIP/ PIPs*) are involved in controlling membrane permeability to water and facilitating water mobilisation (Madrid-Espinoza *et al.*, 2018; Ayadi, Brini and Masmoudi, 2019), and increased expression is positively correlated with drought tolerance via enhanced water uptake and retention and reduced ion leakage (Zhou et al., 2012; Ayadi, Brini and Masmoudi, 2019; Yang et al., 2022a).

The induction of stress phytohormones and their mediation of drought stress conditions is not without costs, however: these responses come with trade-offs between survival under water deficit, continued growth, and increased disease susceptibility (Zhang, Zhao and Zhu, 2020; Yang et al., 2019; Caarls et al., 2017a). Under drought, rising ABA and increased antagonism with other phytohormones is associated with increased disease susceptibility (O'Hara, Rest and Franks, 2016; Mohr and Cahill, 2007; Thaler, Owen and Higgins, 2004; Nyaupane et al., 2024), though this may be a tolerable cost under drought due to water deficit conditions often being unfavourable for some wheat pathogens (Maurya et al., 2022; Jeger, 2022). ABA and JA abundance must be balanced with growth-promoting hormones to avoid excessive growth inhibition or premature senescence under drought stress conditions (Janicka et al., 2023; Uyehara et al., 2023; Noir et al., 2013), limiting the efficacy of these drought responses in favour of continued plant function.

These phytohormones however do not exist in a vacuum, nor do they only bring about changes in the stress response: many of them also play roles in normal growth or floral phenology. Floral development, often accelerated under drought conditions as part of a drought escape mechanism, can be catalysed by ABA through GIGANTEA (*GI*) signalling (Riboni *et al.*, 2016), though high ABA can also repress flowering by promoting flowering locus C (*FLC*) expression through *ABI4* and *ABI5* (Shu *et al.*, 2016), highlighting the dual roles of ABA in flowering under stress. *FT*-like genes have previously been implicated in the integration of flowering and abiotic stress pathways under drought and enhancing tolerance in rice (*Oryza sativa*) (Gu *et al.*, 2022).

Despite characterisation of the main stress-responsive phytohormone pathways and their roles under drought, several gaps remain in our understanding. Deconvoluting the complex phytohormone network remains challenging due to the functional redundancy and antagonism that can obscure the key master regulators of the drought response. Better harnessing these pathways and stress mechanisms to fine-tune them and develop more climate-resilient wheat requires a greater understanding of how they work. We must further understand how wheat responds to drought by identifying and characterising master regulators that influence the drought response and give rise to drought tolerance, exploring the role of epigenetic modifications like DNA methylation, histone marks, and chromatin structure in controlling master regulators and downstream genes under drought, and elucidating how these all interact to successfully mediate drought. By addressing these knowledge gaps, we can hopefully guide molecular crop improvement strategies to pinpoint and manipulate the major hubs that control the drought response.

1.11. Epigenetics, DNA methylation, and abiotic stress

Epigenetic mechanisms are involved in almost all aspects of plant development, managing the accessibility of regions of DNA and moderating the ways in which DNA, RNA, and proteins can interact (Tresas et al., 2025; Kong et al., 2020; Zhang, Lang and Zhu, 2018; Liu and Chang, 2021; Lv et al., 2019). Epigenetics is a broad field, encompassing large-scale factors like chromatin structure and histone modifications, and smaller-scale factors like DNA and RNA methylation small RNAs, and long non-coding RNAs.

The structure of chromatin, composed of DNA wrapped around histone octamers (comprised of H2A, H2B, H3, H4 surrounding linker histone H1) (Deal and Henikoff, 2011), is a known regulator of gene expression: densely coiled heterochromatic chromatin limits accessibility of the DNA to RNA polymerases and transcription factors, preventing the transcription of genes and other elements. Chromatin remodelling can enable the expression of genes by 'opening' these dense coils (Bannister and Kouzarides, 2011; Tresas *et al.*, 2025). Histone acetylation, controlled by histone acetyltransferases and deacetylases, is involved in determining chromatin structure; acetylation confers a looser euchromatin structure, increasing gene expression (Kumar, Thakur and Prasad, 2021; Wang *et al.*, 2024). ATP-dependent remodelers can slide nucleosomes – packages of histone complexes – along the DNA, shifting accessibility of regions as they move (Armeev *et al.*, 2021; Reid, Zhong and Mackay, 2024). On a larger scale, chromatin structure can promote *trans*-regulation of genes by folding distant regions of DNA into topologically associating domains that bring enhancers, TFs, and promoters into spatial proximity despite their genomic linear distance (Kumar *et al.*, 2021; Xie and Dean, 2025).

Histone post-transcriptional modifications can also act as epigenetic regulators of gene expression. Histone modifications come in many forms – acetylation, citrullionation, methylation, serotonylation, or phosphorylation – that can affect the overall chromatin structure and subsequently transcription. (Zhou et al., 2020; Pfluger and Wagner, 2007). Methylation of lysine residues on histone H3 is known to be associated with changes in transcription: H3 lysine 4 (H3K4me3) and H3K36me3 trimethylation are strongly associated with active transcription, while H3K27me3 and H3K9me2/3 methylation are associated with gene repression, with the latter notable as a promoter of dense heterochromatinization (Beacon *et al.*, 2021; Haider and Farrona, 2024; Yabe *et al.*, 2024). Other histone modifications, including phosphorylation and acetylation, can be associated with marking sites of DNA damage for later repair.

Non-coding RNAs (ncRNAs) also play a substantial role in epigenetic gene regulation, encompassing small interfering RNAs (siRNAs), microRNAs (miRNAs), and long ncRNAs (lncRNAs). RNAse III Dicer-like (*DCL*) proteins cleave dsRNAs generated by RNA polymerases into significantly smaller transcripts to modulate gene transcription and translation (Kakiyama et al., 2019; Blevins et al., 2006; Voinnet, 2009): *DCL1* predominantly cleaves precursor dsRNAs into 20-21 nt miRNAs which can target complementary messenger RNAs (mRNAs) for degradation by the RISC complex and AGO proteins (Le et al., 2025; Blevins et al., 2006); *DCL2* and *DCL4* process dsRNAs into 22 and 21 nt siRNAs respectively, both of which also target mRNAs for cleavage – both are known to play a role in the plant immune response to viral infection with some redundancy (Nielsen et al., 2024; Liu et al., 2025c; Qin et al., 2017; Parent et al., 2015); *DCL3* is known for generating 24 nt siRNAs which primarily act through the RNA-directed DNA methylation (RdDM) pathway, directing methyltransferases via *AGO* proteins to complementary genes and transposable elements for silencing (Xie and Yu, 2015; Li et al., 2024a). Functional redundancy among the DCLs – for example, *DCL4* mutant lines show compensation by *DCL2/ 3* (Gasciolli *et al.*, 2005), while *DCL1/ DCL3* exhibit overlapping functions in flowering regulation (Schmitz *et al.*, 2007) – can ensure robust gene regulation.

Beyond chromatin and small RNA regulation is methylation of adenosine nucleosides (N6-methyladenosine; m6A), a modification involved in facilitating mRNA splicing, stability, translation, and processing (Liu et al., 2015; Li et al., 2023a; Arribas-Hernández and Brodersen, 2020). m6A is primarily enriched within the final 150 bp, often spanning the stop codon and 3' untranslated region (UTR) of transcripts prior to their polyA tail (Luo et al., 2014; Bodi et al., 2012), and is mediated by writers (RNA methyltransferases), erasers (RNA demethylases), and readers (proteins that bind to m6As) (Yang *et al.*, 2018; Ganguly *et al.*, 2025). m6A is dynamically altered in response to environmental changes, fine-tuning post-transcriptional gene expression and splicing (Song, Cai and Jia, 2024); m6A abundance is negatively associated with gene expression variability (Wang et al., 2022a) but potentially positively associated with gene expression (Wang et al., 2023b; Zhang et al., 2022a), and is known to interact with other epigenetic mechanisms (Yang et al., 2025; Hu, Xu and Kang, 2024; Zhao et al., 2021).

This thesis partly focuses on the role of DNA methylation, where the fifth position of cytosine in DNA is methylated. DNA methylation is perhaps the most pervasive epigenetic mark, interacting with almost all other epigenetic mechanisms and with large implications for organism stability and development. Methylation acts as an regulator of the genome, partly determining chromatin structure, TE silencing, and influencing gene expression (Zhang, Lang and Zhu, 2018). The genomic distribution of DNA methylation in plants is substantially different to that seen in mammalian species: while methylation in mammals primarily occurs at CG sites, organised largely into CG-rich CpG Islands, methylation in plants can occur at CG, CHG, and CHH sites (H = A, C, or T), with heavy methylation of the expansive repetitive regions and transposable elements (TEs); TEs in plant genomes are highly methylated, with their demethylation resulting in substantial developmental abnormalities (Miura et al., 2001; He et al., 2022b). Across plant species there is a considerable amount of variation in DNA methylation patterns, with CG methylation abundance averaging ~80-90% in wheat (Singh et al., 2022a), ~56% in the model monocot *Brachypodium distachyon* (Takuno and Gaut, 2013), and only ~18% in rice varieties (Yan *et al.*, 2025).

Localisation of methylation is particularly important: heavy methylation of promoter, enhancer, transcription start site, and first exon regions have classically been linked to gene silencing or low transcription levels (Mette et al., 2000; Dalakouras and Ganopoulos, 2021; Gallego-Bartolomé, 2020; Brenet et al., 2011), while gene body methylation appears to be associated with sustained and low-noise gene expression (Lee et al., 2023; Horvath et al., 2019). Despite this, some plants appear to broadly lack gene body methylation, creating debate over the importance of its role in plant function (He et al., 2022b; Horvath et al., 2019; Takuno and Gaut, 2013).

De novo DNA methylation – methylation of previously unmethylated sites – is mediated by Domains Rearranged Methyltransferase (DRM) proteins, recruited by the 24 nt-siRNA-binding *AGO4* protein and directed towards sites complementary to the siRNAs (Zhong *et al.*, 2014). Methylation maintenance, however, is mediated by *MET1* (CG sites), *CMT3* (CHG) and *CMT2* (CHG and CHH) methyltransferases (Stroud et al., 2014; Zhang, Lang and Zhu, 2018; Lucibelli, Valoroso and Aceto, 2022; He et al., 2022b) which recognise hemi-methylated DNA (DNA methylated only on the template strand) following DNA replication. Histone methylation also plays a role in the recognition of sites marked for DNA methylation, with *CMT2* and *CMT3* preferentially binding to DNA associated with H3K9me2-marked heterochromatin (Bewick et al., 2017; Du et al., 2012). Conversely, DNA methylation is passively lost across cell cycles due to methyltransferase inactivity or actively mediated by DNA glycosylases known as DEMETER-like (*DML*) or REPRESSOR OF SILENCING (*ROS*) family proteins through the methylation-sensitive base-excision pathway (Zhu, Xue and Qian, 2025; Gahlaut et al., 2022; Kapazoglou et al., 2013).

DNA methylation represents a currently understudied aspect of large polyploid crops, and likely an underexploited mechanism of gene and genomic element regulation in wheat.

1.12. The DNA methylome, stress regulation, and thirsty crops

DNA methylation may play a substantial role in stress regulation, with various plant species displaying substantially different DNA methylation profiles post stress exposure (Song et al., 2016; Verhoeven et al., 2010; Sun et al., 2022a; Talarico et al., 2024).

Abiotic stress has been demonstrated to cause substantial remodelling of the plant methylome. Water deficit conditions in *A. thaliana* are associated with methylation-induced changes in the expression of stomatal control (Wanke and Üner Kolukisaoglu, 2010; Kong *et al.*, 2020), while under extreme drought *Brassica juncea* plants demonstrated significant increases in genome-wide methylation accompanied by demethylation at some known stress-responsive genes (Sharma *et al.*, 2017). High temperature treatment on cotton (*Gossypium hirsutum*) is associated with genome-wide decreases in symmetrical cytosine contexts (CG & CHG) and increases in the asymmetric CHH context (Gong *et al.*, 2024), while drought treatment was associated with increased methylation in all contexts (Lu *et al.*, 2017), however both noted that DNA methylation changes were rarely associated with changes in gene expression, suggesting that changes in methylation may play a broadly subtle, or non-immediate, role in mediating gene expression under stress.

Wheat remains a species with little known about the drought response of the methylome across the genome, with most studies examining genomic fragments. Drought has been observed to induce broad hypermethylation (an increase in methylation) in wheat, with substantial changes to methylation observed in both shoots and roots in a tissue-specific manner (Duan et al., 2020; Kaur, Grewal and Sharma, 2018; Demirel et al., 2025), with most changes observed in the CG context (Naderi *et al.*, 2024), while some studies have identified negatively correlations between promoter region methylation and gene expression of stress-associated genes under drought (Li et al., 2023c; Naderi et al., 2024;

Wang et al., 2021a). Interestingly, drought-tolerant genotypes appear to exhibit a greater abundance of methylation polymorphisms than drought-susceptible genotypes under drought conditions (Kaur, Grewal and Sharma, 2018; Duan *et al.*, 2020; Naderi *et al.*, 2024). Other abiotic stresses, like high salinity, heat, and cold, have also been found to induce methylation reprogramming in the roots (Lan, Yan hao and Jian bo, 2009; Hoseini *et al.*, 2024) and modulation of DNA methylation machinery (Li et al., 2021a; Gahlaut, Samtani and Khurana, 2020). The study of biotic stress likewise remains limited: differential CHH methylation in conjunction with changing histone methylation has been determined to play a significant role in the response to wheat-leaf rust infection (Singh et al., 2022a), while in-silico approaches indicate that methylation machinery is mobilised to counteract *Fusarium graminearum* head blight (Kharbikar *et al.*, 2025).

A common thread behind studies examining the role of DNA methylation in regulating stress responses is their approach: a large proportion use a combination of bisulphite PCR and Methylation-Sensitive Amplification Polymorphism (MSAP), as opposed to genome-wide higher-resolution methods like Whole-Genome Bisulphite Sequencing (WGBS), Reduced Representation Bisulphite Sequencing (RRBS), Nanopore Sequencing, or Methylated DNA Immunoprecipitaton (MeDIP) (Khodadadi et al., 2021; Simpson et al., 2017). This is likely largely due to the expense of whole-genome and long-read approaches to profiling the methylome, especially in species with large genomes (Suzuki et al., 2018; Pereyra et al., 2025; Nyaga et al., 2024). As of writing, at least one study that examines the effects of biotic stress on DNA methylation using genome-wide approaches in wheat has been published (Singh et al., 2022a); the role of DNA methylation in responding to abiotic stress like drought remains a critical gap in our understanding of how the wheat drought response is controlled, with very little understanding of the changes to the methylome in non-genic regions like TEs, at enhancers, or in the vast unannotated intergenic regions that make up much of the wheat genome.

DNA methylation has also been identified as creating stably heritable epigenetic marks: the progeny of plants that have experienced abnormal treatment or adverse conditions may carry similar regions of DNA methylation that are not found in plants that have been grown in control conditions in the same generational timespan, with heritable marks observed in the progeny of plants exposed to high CO2 levels (Panda *et al.*, 2023), drought (Zheng *et al.*, 2013), heat (Guo, Wang and Lisch, 2021), heavy metal stress (Ou *et al.*, 2012), nitrogen-deficiency (Kou *et al.*, 2011), or regenerated from tissue culture (Stelpflug *et al.*, 2014). Despite this, not all species or cultivars appear to exhibit epigenetic heritability (Van Dooren et al., 2020; Suter and Widmer, 2013) – though some suggest it happens to a limited degree (Wibowo *et al.*, 2016; Ganguly *et al.*, 2017) – suggesting that the heritability of DNA methylation may be subject to a combination of inherent priming ability, stimuli, and time. This heritability has been observed in wheat, though largely in response to genome manipulation, as opposed to environmental conditions (Yuan et al., 2020; Gao et al., 2016).

1.13. DNA methylation and stress priming

Heritable epigenetic marks and stable epialleles are potential mechanisms that may give rise to priming responses, comprising a plant's 'stress memory'. Priming a plant involves an initial treatment or stress exposure that can enable a plant to induce stronger responses to that stimuli in the future (Hilker and Schmülling, 2019; Liu, Able and Able, 2022; Zhang et al., 2025d). This priming response can occur within a single generation (*intra*-generational) or across generations (*inter*-generational); the former requires short-term maintenance of DNA methylation marks, while the latter requires active maintenance across recombination.

As sessile organisms, plants are unable to escape unfavourable environmental conditions: priming can offer several adaptive benefits and enhance stress tolerance, enabling faster, stronger, and more efficient activation and deployment of amelioratory stress responses. Improvement programmes for important crops like wheat have delivered just under 1% yield gain per year, well below the yearly 1.5% gain required to meet projected demand in 2050 (Rahman et al., 2021; Ray et al., 2013). Coupled with the expected yield penalties from a more agriculturally hostile climate, novel ways of maintaining and increasing yield under sub-optimal conditions – like priming – are required.

Priming ability has been observed in model crops like *O. sativa* and *A. thaliana*, demonstrating primed responses to both biotic and abiotic stresses like heat, drought, pathogens and fungi, or predation (Clarke et al., 2004; Friedrich et al., 2021; Yao et al., 2022; Westman et al., 2019; Wilkinson et al., 2023; Kushawaha et al., 2021; Choi et al., 2025). Exotic cultivars of wheat, such as landraces and synthetic lines, have also been identified as potentially exhibiting a drought-priming ability by maintaining a drought 'stress memory' (Amini *et al.*, 2023). Despite studies identifying its presence, many studies neglect the mechanistic basis of priming responses, focusing primarily on sustained physiological and agronomic traits, but indications that abiotic stress priming exists are promising.

Studies largely examine the effects of priming but fail to dissect the regulatory transcriptomic and epigenetic mechanisms that may underpin it. Some studies explore the physiological basis of priming-associated tolerance, with salt treatment on *Sorghum bicolor* seedlings conveying increased tolerance to saline conditions through reduced root Na+ uptake and increased photosynthetic capacity compared to unprimed plants (Yan et al., 2015b), while others examine the molecular and transcriptomic basis of priming, with *A. thaliana* being found to recruit demethylases via TFs following heat stress to induce transcription of memory-related genes (Yamaguchi *et al.*, 2021), and mild drought priming in *Brassica napus* activating salicylic acid-mediated modulation of drought-responsive pathways to improve tolerance to subsequent stresses (Muchlas *et al.*, 2023), but the role of the epigenome and DNA methylation in these changes remains understudied.

Priming presents a potential adaptive application for crop improvement strategies and for optimised agricultural seedstocks. Breeders can select for lines that exhibit high beneficial priming abilities and potentially cross these traits back into the elite germplasm (Liu, Able and Able, 2022), while the identification of heritable intergenerational priming could give rise to the availability of 'pre-primed' seed that could better survive harsh stress events.

1.14. Aims and approach

Understanding the underlying regulation of stress responses in wheat is arduous, limited by community genomic resources, cost, genome complexity, and prior study. In this thesis, I aimed to identify potential markers and mechanisms that were associated with the response to and tolerance to drought stress among hexaploid wheat landraces, and to examine whether these were able to convey stress tolerance both within- and across generations. Exploration of the regulatory basis of the response to early drought and inherent drought tolerance was approached using a more holistic approach, consisting of transcriptomic, methylomic, and physiological analyses. I aimed to identify genes whose variation in expression across both landraces and stress conditions was significantly associated with responding to and enhancing wheat's response to stress, but by pairing this with analysis of the changing drought methylome I also aimed to understand the potential higher regulation of these responses.

Examination of the transcriptional and epigenetic changes in response to drought hinged on a core within-plant sampling strategy: leaf tissue was sampled from the same plants before and after drought, rather than from independent control and drought group plants. For each timepoint, extracted nucleic acids were pooled, resulting in a single sample per sampling point per genotype for RNA, and a single sample per sampling point for DNA. This approach was chosen for two main reasons: this before-after sampling strategy was thought to better capture the direct *response* to drought, and would avoid the differences in development and growth that would be seen between plants developmentally stunted by drought and their control counterparts (Dodd and Ryan, 2016; Nyaupane et al., 2024). Secondly, trends observed in other studies suggested that DNA methylation was likely to be inherently noisy, even within the same genotype under the same conditions (Wilkinson et al., 2023; Zastąpiło et al., 2024; Yan et al., 2025); a within-plant approach with pooling would somewhat control for this stochasticity, potentially reducing the impact of plant-specific methylation patterns.

In essence, this approach treated drought + time as a combined factor for all analyses, with interpretation guided by gene functional annotation. This approach obviously has its own caveats: the initial sampling stage would likely result in an identifiable molecular wounding response in the analysis, and there would still be a degree of developmental difference between the two sampling points, though this would be through intentional design rather than an unquantifiable difference between groups.

Alternative approaches to this experimental design would have been to conduct a conventional drought/ control group experiment, or to pool across genotypes with varying levels of drought tolerance to account for different levels of developmental disturbance. Pooling across genotypes would have provided a stronger 'generalised' wheat drought response in **Chapters 2** and **4**, but entirely prevented the tolerance-focused **Chapter 3**, while conventional drought/ control groups could have introduced development as a greater confounding variable for **Chapter 4**.

Ultimately, we hope that the genes, markers, and mechanisms identified in this body of work can be used to inform breeding programmes and propel the production of drought-tolerant wheat that can better survive the burgeoning hostile climate on the horizon.

To meet these aims, this thesis first explores the response to drought stress among a panel of wheat landraces through RNA-Seq and transcriptome profiling, differential expression, and co-expression networks, identifying potential transcriptional master regulators that may govern amelioratory responses to stress in wheat seedlings (**Chapter 2**). Following on from this, the dataset was further examined to identify candidate transcriptional master regulators of drought tolerance in spring wheat landraces identified as tolerant or susceptible to drought conditions (**Chapter 3**). The use of wheat landrace panels in ways like this is somewhat novel, enabling the capture of responses across a wide variety of diverse plants; this approach to using gene networks encompassing landraces and stress conditions also remains uncommon, with current publications largely comprised of output from myself and **Chapter 2**'s co-author, Liam J. Barratt. The drought response was then further explored at the epigenetic level in **Chapter 4**: novel analyses into the drought methylome profile of spring wheat landraces describe the role of DNA methylation in determining gene expression potential and variability, as well as the potential roles of the different methylation contexts. As transposable elements make up almost 90% of wheat's genome, their expression and methylation states are explored, resulting in the identification of novel TE transcript activity that may modulate the expression of genes that display sequence similarity. This exploration of the methylome is completely novel: as of writing, no current examples exist of genome-wide methylation profiling under abiotic stress conditions in wheat – let alone comparisons to gene expression patterns derived from the same samples – making this an exciting first step into understanding the role of the methylome in the wheat drought stress response. Finally, lingering questions remained around the presence of differential methylation that was not clearly associated with changes to gene expression, suggesting that they may play a role in drought priming responses: a subset of wheat landraces used in the other chapters were used to explore the priming potential of landraces using a variety of physiological measures of drought tolerance, both across- and within-generations (**Chapter 5**). Implications of these results, both individually and in combination, will subsequently be discussed in detail alongside the next steps and future directions of this project (**Chapter 6**).

Ultimately, the work in this thesis aims to employ forwards genetics approaches to identifying transcriptional master regulators and regulatory mechanisms that comprise the wheat drought regulome, followed by the use of reverse genetics approaches for validation of some of these markers.

Thank you for taking the time to read this thesis, and I hope it is useful, interesting, and enjoyable.

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

2.1. Abstract

Introduction: Over four billion people around the world rely on bread wheat (Triticum aestivum L.) as a major constituent of their diet. The changing climate, however, threatens the food security of these people, with periods of intense drought stress already causing widespread wheat yield losses. Much of the research into the wheat drought response has centred on the response to drought events later in development, during anthesis or grain filling. But as the timing of periods of drought stress become increasingly unpredictable, a more complete understanding of the response to drought during early development is also needed.

Methods: Here, we utilized the YoGI landrace panel to identify 10,199 genes which were differentially expressed under early drought stress, before weighted gene co-expression network analysis (WGCNA) was used to construct a co-expression network and identify hub genes in modules particularly associated with the early drought response.

Results: Of these hub genes, two stood out as novel candidate master regulators of the early drought response – one as an activator (*TaDHN4-D1*; *TraesCS5D02G379200*) and the other as a repressor (uncharacterised gene; *TraesCS3D02G361500*).

Discussion: As well as appearing to coordinate the transcriptional early drought response, we propose that these hub genes may be able to regulate the physiological early drought response due to potential control over the expression of members of gene families well-known for their involvement in the drought response in many plant species, namely dehydrins and aquaporins, as well as other genes seemingly involved in key processes such as, stomatal opening, stomatal closing, stomatal morphogenesis and stress hormone signalling.

2.2. 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 we examined the effect of drought stress exposure during early development on gene expression in spring habit wheat landrace accessions. 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., 2018b; 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., 2022a), 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, 2022b), 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, 2022a). 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 we can identify candidate master-regulators of these groups of genes (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 can 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). We have 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 (Barratt et al., 2023a), but the present work 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.

2.3. Materials and methods

2.3.1. Selection, Growth, and Sampling of Plants

14 spring habit accessions with a range of drought tolerance levels were used in the present work (**Supplementary Data S2.1**). Genomic tile plots visualising the A, B, and D genomes for each accession in the YoGI landrace panel (Barratt et al., 2023a) 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.

2.3.2. 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. Our experimental design included both technical and biological replication. Prior to sequencing, we pooled RNA from 4 replicate plants per accession, per condition (pre- or post-drought) to help control the effect of the environment on the transcriptome, whilst the different accessions provided biological replication for each treatment.

2.3.3. 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).

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.

2.3.4. 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.

2.3.5. 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, we adopted an approach used previously (Borrill et al., 2019; Andleeb, Knight and Borrill, 2023), 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.

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.

2.3.6. 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 (version 1.72-1; (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.

2.3.7. 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.

2.3.8. 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 accordingly to module size, we would expect each module to contain this proportion of DEGs. To determine whether the observed proportion of DEGs in each module was significantly greater than this predicted proportion, we used a one-proportion Z test. Modules were deemed to be significantly enriched in DEGs if $p < 0.05$.

2.3.9. 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. 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.

2.4. Results

2.4.1. 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 $p < 2.2e-16$) between stressed and control plants (**Figure 2.1A**). Soil Moisture Content (SMC%) was measured over the course of the experiment (**Figure 2.1B**), with the drought stress treatment causing SMC% values of the control and drought groups to differ significantly ($p < 2.2e-16$) 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 2.1A** and **2.1B** are available in **Supplementary Data S2.2**.

2.4.2. 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 2.1C**). 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.

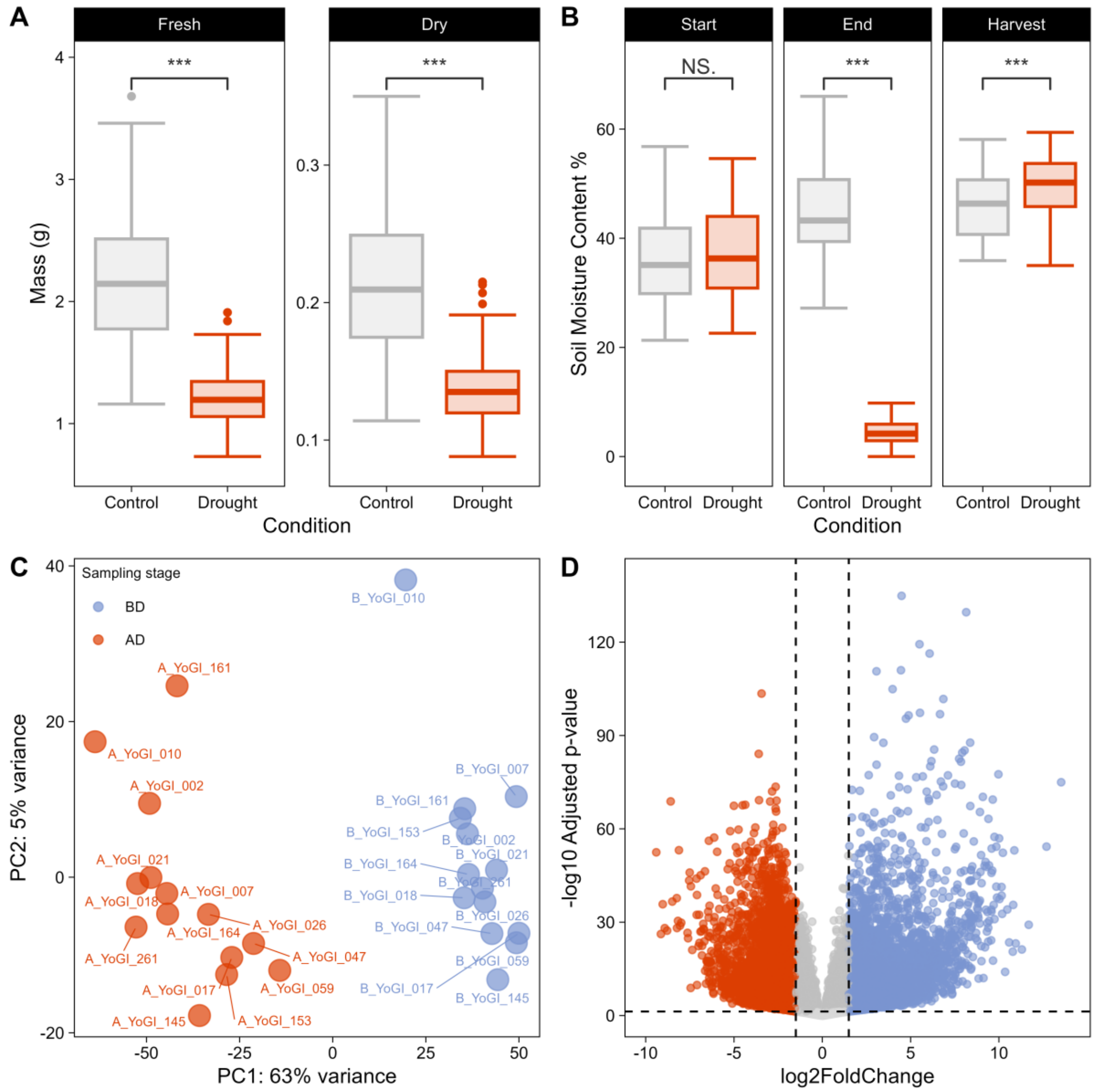

Figure 2.1: Drought treatment resulted in substantial differences across the panel in both phenotypic measurements and transcriptomic profiles. Ten days of drought stress was found to significantly reduce (*t*-test: both $p < 2.2e-16$) average fresh weight (g) by 42.9% and average dry weight (g) by 34.8% **(A)**, while soil moisture content (SMC%) was significantly different at the end of the drought treatment **(B)**. No significant difference was observed between control and drought groups at the start of the drought period. 'Harvest' refers to the end of the 3-day recovery period after the end of the drought period, where all above-ground biomass was harvested per individual. Asterisks **(A, B)** represent significance thresholds; '***' represents p < 0.001. 'N.S' represents no significance. An initial exploration of samples and expression data suggested that samples before and after drought had distinct transcriptional profiles **(C, D)**. Principal component analysis (PCA) of variance-stabilised transcript counts from all 28 samples **(C)** showed clear separation between the samples before drought (BD) and after drought (AD) on PC1, while differential expression analysis identified 10,199 DEGs with differing expression before and after drought, 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. The x-axis represents the log2FC, while the y-axis represents the negative log10 of the p-value for each gene.

2.4.3. 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, we carried out differential expression analysis between samples taken before and after drought stress. Genes were deemed to be differentially expressed (DEGs) when their FDR-adjusted *p*-value < 0.05, and their $\log_2$FoldChange greater/less than 1.5/-1.5.

We identified a total of 10,199 DEGs; 6051 and 4148 with significantly increased and decreased expression, respectively, in response to drought (**Figure 2.1D**). Wide dispersion of the genes in **Figure 2.1D** 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 S2.3** and **S2.4**, respectively.

To investigate gene function among DEGs, we conducted GO enrichment analysis 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 S2.5** and **S2.6**.

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 regulation of gene expression and transcription (‘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 to 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).

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

The co-expression network contained 84,888 genes, housed within 81 modules (**Supplementary Data S2.7**). 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, we conducted GO enrichment analysis on each module, using all the genes included in network construction as background. We expected that modules containing genes involved in regulating the drought response would 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 2.1**), with the black and turquoise modules being enriched in the GO term 'response to water' (FDR = 4.8e-08 and 0.029, respectively).

Table 2.1: 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*)

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, we would expect 12% of the genes in each module to be DEGs. We found that 17 modules contained a significantly higher proportion of DEGs than expected (**Table 2.2**) 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.

Table 2.2: 17 modules were significantly enriched in DEGs. These modules contained a significantly higher proportion of DEGs than expected should the total number have been distributed across modules according to their size (12%). 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 log2FoldChange 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

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 2.1** and **2.2**, therefore, were examined further to determine whether they may be promising candidate master-regulators of the transcriptional early drought response.

2.4.5. Hub Gene Identification

The hub genes within those modules deemed to stress-associated (**Tables 2.1** and **2.2**) 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 2.3**) 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.

Table 2.3: 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.

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., 2012)
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, 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 et al., 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 et al., 2017)

Hub genes in these stress-associated modules (**Table 2.3**) 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 candidates as 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.

2.5. Discussion

2.5.1. 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., 2019; 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 (Barratt et al., 2023a), here we show 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., 2018b; 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., 2022a), but as the climate continues to change, periods of water shortage coinciding with the early growth stages of spring wheat crop growth 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, 2022a, 2022b). 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

2.5.2. Drought Stress Causes Substantial Changes in the Wheat Transcriptome

Our 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., 2020a; 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., 2021a). 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).

Our 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).

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

The black module was significantly enriched in DEGs (**Table 2.2**), as well as the GO term 'response to water' (FDR-adjusted *p*-value = 4.8E-08, **Table 2.1**), 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 subnetwork contained 1544 genes, and 6562 connections between genes (**Figure 2.2A**).

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). We found that expression of the gene was upregulated significantly (log2FC = 5.87) after drought stress (**Figure 2.2B**); consistent with the commonly observed expression responses of dehydrins in response to drought stress (Tiwari and Chakrabarty, 2021; Sun et al., 2021b). *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.

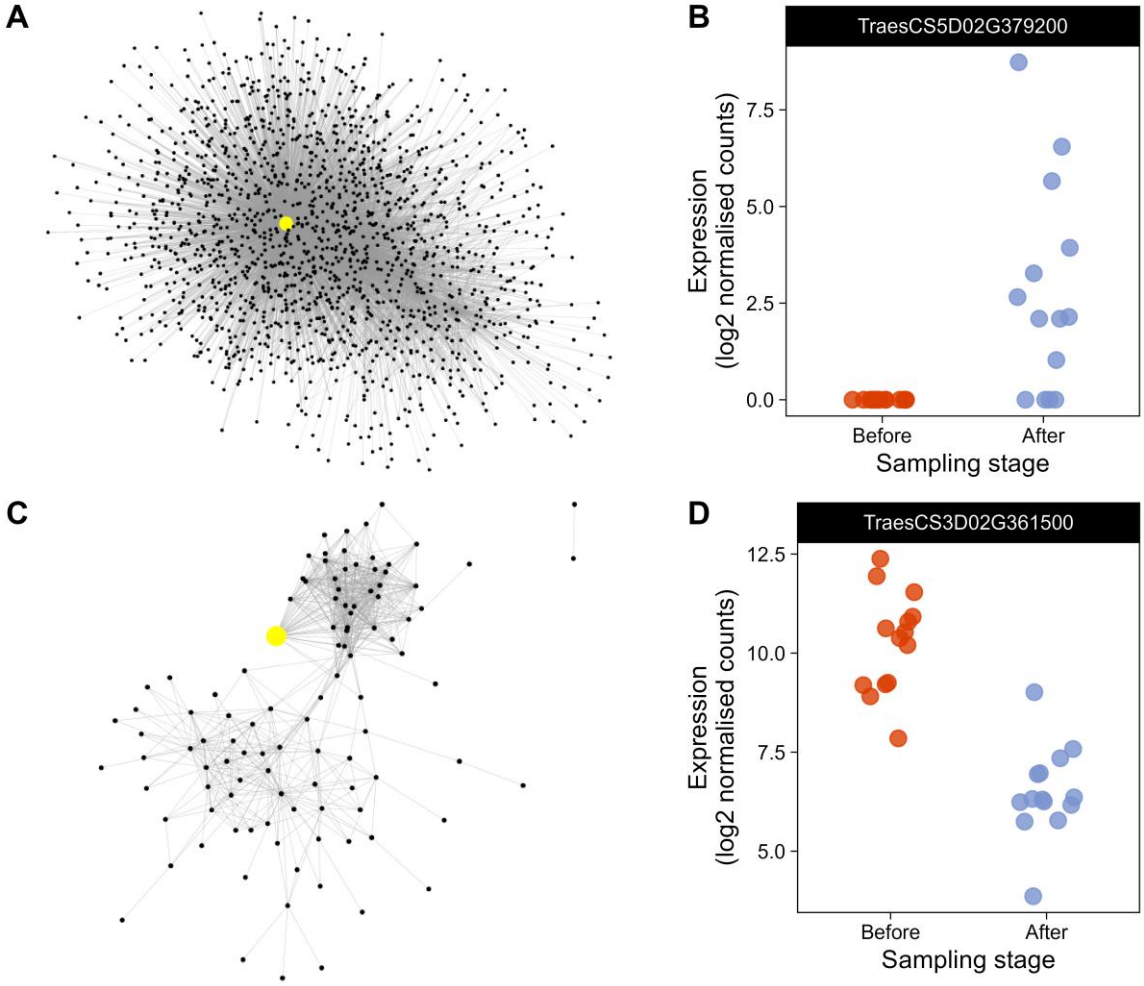

Figure 2.2: Drought-associated modules may house candidate transcriptional master-regulators of the early drought response. To focus on genes likely involved in the drought response within the large black module, a subnetwork was created **(A)**, whereas the ivory module **(C)** was small enough to be 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).

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. We found that 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 *A. thaliana* (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). In the present work, we have 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.

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

The ivory module (**Figure 2.2C**) was identified as drought-associated, as it was significantly enriched in DEGs (**Table 2.2**). 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 2.2D**), 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), *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., 2017b). 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 (identified using Ensembl Plants) 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 we see *TraesCS4B02G282700* expression being 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, we suggest that *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.

Here, we present 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. We identified thousands of genes differentially expressed before and after exposure to drought stress during early development. The use of co-expression network analysis allowed us to identify several hub genes which may act as master-regulators of the transcriptional early drought response. 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.

2.6. Data availability

Raw sequence read data, TPMs, and transcript length data from this article can be found in the SRA data library under accession number GSE225797.

2.7. Author Contributions

LB, IJR and AH conceived and planned the project. LB and IJR performed plant growth experiments and RNA extraction. IJR and SF performed transcriptome data mapping. IJR conducted initial analyses, data visualisation, and differential expression analysis. LB conducted co-expression analysis with input from IJR. LB, IJR and AH wrote the manuscript, and all authors reviewed it. All authors contributed to the article and approved the original submitted version.

2.8. Commentary

Chapter 2 identifies genes that I and the chapter's co-author posit are candidate transcriptional master regulator genes of the drought response, but it should be emphasised that these genes are largely unvalidated, and that our proposed models are only that: hypotheses that must be further validated. This chapter, and WGCNA, cannot explicitly identify causation, but merely explores correlations to identify potential regulatory genes. While dehydrin proteins have been observed to bind to DNA (Tiwari et al., 2019; Hara et al., 2009; Boddington and Graether, 2019) with transcription factor or ROS-protectant activity, the specific hub gene has not been observed to do this, and this cannot be identified from the current RNA-Seq evidence alone. To test the model, ChIP-Seq experiments could identify potential sites that the dehydrins may bind to. More broadly, knockout experiments with TILLING mutants would help to validate the putative positive role in the drought response. Similarly, the hypothetical model proposed for the uncharacterised hub gene could be further explored through identification of its primary cellular localisation through spatial transcriptomics or single cell RNA-Seq experiments, while co-immunoprecipitation experiments may identify potential transcriptional targets.

Identifying which hub genes were strong candidate transcriptional regulators required querying several statistics and traits of the hub gene. Hub genes are defined in WGCNA as genes with high intramodular connectivity – the strength of the correlation between the gene and the module's eigengene – and therefore they are likely to have a high degree score (the number of connections to and from this gene to other genes within the module). Hub genes are not defined by their differential expression status. Modules frequently have multiple hub genes that can be examined collectively or individually, and so we selected interesting hub genes based on several criteria. For example, with *TraesCS5D02G379200,* the dehydrin-encoding gene within the black module, the module had been subset using GO terms to identify a drought-responsive core of genes, the hub genes of which were then curated based on degree score, differential expression status, and molecular function. This multi-step selection process allowed for the identification of biologically relevant genes that were likely responsive to our trait of interest.

The experimental design used in **Chapter 2** (and subsequent chapters in this thesis) comes with caveats: the before and after (BD/ AD) design used for sequencing data inherently confounds the drought treatment and the time between the two sampling points, though this is somewhat mitigated through the use of careful gene functional annotation filtering. Additionally, drought is known to delay development, which I believe additionally mitigates the differences between these plants before and after drought; the plants were also not substantially developmentally different at the end of the drought period, having not substantially increased in tillers or leaf number. The inclusion of plants expressing a broad variety of drought tolerance phenotypes would also go a way towards potentially mitigating this experimental choice. The experimental design was chosen largely due to the paired nature of the methylation experiments in **Chapter 4**, in which I attempted to reduce confounding effects from the highly variable nature of DNA methylation I had observed in other studies even among replicates of the same genotype and conditions.

As a result of these design choices, it should be noted that some of the genes discussed may be differentially expressed as a result of time – however the functional annotation, and co-expressed genes of the identified hub genes, suggest that these genes are primarily changing in expression due to the environmental stimulus of water-deficit conditions, rather than just changing due to the different time points. Key genes may also have been identified due to the latent wounding response brought about by the initial sampling – something again considered in the downstream analysis, but the impact of wounding on hormonal responses (especially jasmonic acid signalling) cannot be discounted.

In an ideal redesign, based solely around this RNA-Seq experiment, samples would be compared between control and drought groups, removing the potential confounding wounding response, and analysis considerations would be made to control for developmental differences that would arise due to the control group having unrestricted growth for the experimental period.

3. Identifying candidate master regulators of early drought tolerance using transcriptomic and co-expression network analyses

3.1. Abstract

Introduction:

Bread wheat (*Triticum aestivum*) is a staple in the diets of over 4 billion people and one of the most widely consumed grains worldwide. The double-pronged challenge of a growing population and climate change threatens the food security of people globally, as increasing demand meets stagnating yields due to droughts increasing in intensity and frequency. Plants can counter drought events by altering phytohormone levels to coordinate physiological changes and moderate water use. Despite the importance of early plant development, few studies have investigated drought tolerance traits during this critical period; as drought becomes increasingly more variable and unpredictable, a stronger understanding of what makes seedlings better tolerate drought is paramount for ensuring food security.

Methods:

Using the YoGI landrace panel, we identified 560 genes differentially expressed between groups with significantly different drought tolerance phenotypes under early drought stress. I further explored this by applying weighted gene co-expression network analysis (WGCNA) to dissect the relationship between these genes and determine hub genes that may potentially regulate transcriptional abundance.

Results:

Our analysis revealed two hub genes with substantial potential as candidate transcriptional master regulators of early drought tolerance; *TraesCS1D02G072700 (TaJOX1),* a jasmonate oxygenase, and *TraesCS5D02G145800 (WRKY19),* a transcription factor potentially involved modulating free JA activity. Both genes appear to interact with the JA signalling pathway, which plays a key role in balancing the growth/ stress trade-off.

Discussion:

These candidate transcriptional regulators likely coordinate some of the nuanced transcriptional changes that govern drought tolerance in seedlings and form a part of distinct responses from tolerant and susceptible genotypes. By modulating JA signalling, these genes may reduce drought-induced JA hypersensitivity and optimise the balance between stress responses and growth, bolstering plant survival and enhancing yields. These findings offer potential targets for optimising stress tolerance in elite wheat and may inform future breeding strategies to enhance yields in adverse climates.

3.2. Introduction

This chapter builds upon **Chapter 2**, which used differential expression and WGCNA-based analyses to identify key transcriptional candidate hub genes involved in wheat's drought response. The focus of this analysis on the drought response however meant that a key trait, phenotypic tolerance to drought, was out of scope; what genes and modules are involved in coordinating inherent tolerance to water-deficit conditions? The approach used in this chapter somewhat mirrors that of **Chapter 2**'s, though additionally leverages phenotypic drought tolerance measures to explore the genetic basis of these traits, furthering our understanding of plant drought strategies and identifying potential targets for enhancing their tolerance. Here, drought tolerance is differentiated from the drought response itself: tolerance is the innate ability of a plant to endure water-deficit conditions and continue functioning, while the response is better regarded as the strategies that the plant employs to cope with the disadvantageous conditions.

Bread wheat (*Triticum aestivum*) is one of the most consequential, nutritious, and consumed cereals in the world; at the start of the decade wheat was the most widely grown food crop, spanning ~545 million acres of arable land across the globe (FAO, 2024; Erenstein, Chamberlin and Sonder, 2021). Wheat plays a vital role in global diets, accounting for over 20% of calories consumed worldwide (Pfeifer *et al.*, 2014). Despite enormous production of wheat grains by global agriculture, suppliers and farmers must obtain higher yields in the coming years to ensure the food security of a growing global population (Kettlewell, Byrne and Jeffery, 2023; Jaggard, Qi and Ober, 2010; Ray et al., 2013) while simultaneously facing the pressures of climate change, which could drive rising demand to outpace yield increases obtained through conventional breeding programmes (Zhang et al., 2022b).

Drought represents just one facet of the increasingly arid conditions wheat is grown under (Senapati, Halford and Semenov, 2021; Leng and Hall, 2019). Water scarcity and drought events are currently responsible for up to 37% reduction in wheat production (Zhang et al., 2024a) and up to 60% reduction in yield (Nyaupane *et al.*, 2024); similarly, up to 60% of land currently used for growing wheat is expected to face substantial water scarcity issues by the turn of the century (Trnka *et al.*, 2019), presenting a monumental threat to agriculture that current elite varieties may not be equipped to deal with. Rainfed agriculture is particularly at risk, facing up to 50% yield reduction as precipitation becomes less frequent and predictable (Ghazouani et al., 2025; Nazari, Mirgol and Salehi, 2021).

Elite varieties, bred intensively to achieve huge advances in yields since the Green Revolution, have been found to display considerable allelic homogeneity and vulnerability to changing environments due to a lack of novel tolerance traits found in their wild relatives (Keneni *et al.*, 2012; Fu, 2015; Salgotra and Chauhan, 2023). These intensive breeding programmes can unintentionally genetically bottleneck varieties and reduce genetic diversity in the pursuit of favourable traits (Tanksley and McCouch, 1997; Keneni *et al.*, 2012), resulting in crops that perform incredibly well under favourable environmental conditions – however they are potentially less able to cope with disease or severe water shortages. Identifying novel sources of drought tolerance outside of that found within these homogenous varieties is imperative to preventing excessive yield losses and meeting the nutritional demand of the future; wheat landraces can be incredibly beneficial for identifying candidate genes for crop improvement. As locally bred domesticates under small-scale crop improvement strategies, and more exposed to natural forces that shape selection, landraces likely capture adaptations to a wide variety of climates and are thought to display greater resistance to adverse conditions compared to elite varieties (Arca et al., 2023; Lopes et al., 2015). Landrace collections like the YoGI panel (Barratt et al., 2023a), contain accessions from across huge geographic ranges and environmental conditions, and so are able to capture a much broader variety of phenotypic and genetic diversity compared to elite varieties (Cheng *et al.*, 2024). Vitally, the lack of genetic bottlenecking from intense breeding programmes means that landraces likely contain beneficial haplotypes and novel alleles absent in elite varieties (Cheng et al., 2024; Lopes et al., 2015). Identification of these beneficial alleles, and their potential for introgression back into elite varieties, provides a valuable approach to increasing the tolerance of wheat production to changing water availability. The identification of these novel tolerance traits is crucial for enhancing future wheat tolerance to drought, something I aim to explore in this chapter.

Plants, as sessile organisms, must adapt to their environment through changes to key phytohormone pathways that control different aspects of development to bring about phenotypic changes. In wheat, key aspects of the drought response include balancing of the abscisic acid (ABA), salicylic acid (SA), and jasmonic acid (JA) pathways (Duvnjak et al., 2023; Nyaupane et al., 2024; Kulkarni et al., 2017; Ilyas et al., 2017). These hormones, among others, act as cornerstones of the drought response, catalysing transcriptional and physiological changes that alter cellular osmotic potential, transpiration rates and water uptake, reduce photosynthetic rate, coordinate stomatal closure, change nutrient accumulation rates, and mediate the buildup of damaging reactive oxygen species to avoid excessive damage to DNA (Camaille *et al.*, 2021; Vijayaraghavareddy *et al.*, 2022; Ghaffar *et al.*, 2023). Jasmonic acid plays a substantial role in this plethora of approaches, but not without compromises: by prioritising survival, resources are directed away from growth, and plants may over-accumulate high levels of phytohormones like JA, ultimately hindering growth, complicating efforts to improve drought tolerance while maintaining yield (Polavarapu B et al., 2022; Heinrich et al., 2013); impaired growth and development can also reduce yield or shift harvest time, potentially resulting in crop abandonment (Zhang et al., 2024a). Despite this, the stress response-growth trade-off managed by JA presents an opportunity for crop improvement – by understanding the nuanced adjustments in hormone signalling that enhance drought tolerance in phenotypically tolerant landraces, we may identify opportunities to optimise the balance between survival and productivity, enabling more ‘stay-green’ phenotypes that produce greater and more consistent yields (Christopher *et al.*, 2016; Abdelrahman *et al.*, 2017).

This study focuses on genes associated with phenotypic tolerance among plants responding to stress in the early vegetative stage – attention has largely focused on the impact of drought events at later stages of development, like anthesis or grain filling (Verbeke et al., 2022; Qaseem, Qureshi and Shaheen, 2019; Farooq, Hussain and Siddique, 2014; Javed et al., 2022), though increasingly studies are exploring drought during the vegetative stages; drought stress at tillering can have substantial negative effects on yield through reduction in the number of productive tillers (Khan et al., 2023; Sarwar et al., 2023; Nyaupane et al., 2024). Identifying mitigating factors at this important stage of development could help to future proof wheat production.

Detecting genes involved in abiotic stress tolerance can be challenging due to the sheer number of genes that change in expression in response to stress; inherent differences between genotypes can further obfuscate these responses by contributing ‘signal noise’ at potentially responsive regulatory genes. Weighted Gene Co-expression Network Analysis (Langfelder and Horvath, 2008, 2012) can be used to dissect the nuanced relationships of transcriptionally regulatory genes involved in both stress tolerance and responses, and successfully detect genes with large effects in the network. Network approaches like this have been recently employed to identify key transcriptional regulators of osmotic stresses in wheat (Chen et al., 2024a) and other crops (Tang et al., 2023; Ju et al., 2023; Ren et al., 2022). Leveraging transcriptomics and WGCNA, this study examines the differences between susceptible and tolerant landraces before and after a harsh drought event, and aims to identify key transcriptional regulators involved in early drought tolerance in spring bread wheat. Building on prior work identifying candidate transcriptional master regulators of the overall response to early drought stress in landraces (Barratt et al., 2023b), this study groups the same lines by their drought phenotypes, enabling interrogation of the genetic basis of drought tolerance and sustained growth.

3.3. Materials & methods

3.3.1. Phenotype screening, selection of plants, and growth

The YoGI panel, a diverse collection of ~350 hexaploid *Triticum aestivum* landraces (Barratt et al., 2023a) was used to identify accessions with high and low drought tolerance phenotypes. Twenty accessions from this drought panel with a variety of drought-tolerance phenotypes and a spring growth habit were taken forward for further analysis. Accessions were compared using tile plots to screen for large scale homoeologous exchanges, structural rearrangements, and hexaploidy, as described in Barratt et. al, 2023. Accessions with significant dominance or deletion were removed. Seeds were sown in a mix of Levington Advance Seed & Modular F2S compost and Aggregate Industries Garside Sands 16/30 sand (80:20 ratio) to promote drainage, treated with CaLypso insecticide (Bayer CropScience Ltd., 0.083ml in 100ml water per litre of compost) and grown in long day (16/8h, 20°C/14°C) glasshouse conditions.

Each accession had four replicates per treatment. Normal watering was applied until individual plants reached Zadoks' GS13 (Zadoks, Chang and Konzak, 1974), after which stress was applied by withholding water for a ten-day period, followed by three days of normal watering to allow for recovery. 6cm length of leaf tissue was collected from wheat seedlings upon reaching GS13 and at the end of the drought period. At each sampling stage, as well as after drought recovery, soil moisture content (SMC%) was recorded via an ML3 Thetaprobe Soil Moisture Sensor and HH2 Moisture Meter (Delta-T Devices, Cambridge, United Kingdom) to ensure individuals were receiving similar levels of watering or water deficit. Tissue was collected individually for each accession and replicate and immediately immersed in liquid nitrogen to prevent nucleic acid degradation, then stored at -80°C.

Post-recovery (13 days from treatment onset), all above-ground biomass was harvested and dried for two days at 70°C and weighed on a scale. Here normalised dry biomass (nDW) was used as a measure of drought tolerance ($nDW = \frac{DW_{drought}}{DW_{control}}$) across lines, where $DW_{drought}$ represents an individual plant's dry biomass under drought conditions and $DW_{control}$ represents the mean dry biomass for that line under control conditions. nDW is expressed as a proportion. Plants with a mean nDW in the top quartile (>0.75) were designated drought tolerant (DT) while plants with low mean nDW (<0.6) were designated drought susceptible (DS). Normalised dry biomass, or similar measures, has previously been used to measure plant tolerance to various stresses and across different genotypes (de Jong and Lin, 2017; Sherstneva et al., 2024; Li et al., 2022a; Henderson et al., 2020).

3.3.2. RNA Isolation and sequencing

RNA was isolated from plant tissue for sequencing. Total RNA was extracted from <100 mg of leaf tissue samples using the E.Z.N.A Plant RNA Kit (Omega Bio-Tek, GA, USA) and treated with DNase according to the manufacturer's protocol. RNA concentration was quantified by running 1 µl of each sample on both the NanoDrop ND-1000 Spectrophotometer (Thermo-Fisher Scientific, MA, USA) and the Qubit 4 Fluorometer (Life Technologies, CA, USA), while RNA quality was assessed by running 2 µl of sample on an Agilent Technology 2100 Bioanalyzer (Agilent Technologies, CA, USA). Samples with RNA Integrity Number (RIN) values >7 were sequenced. Biological replicates were pooled into 1 sample per accession per group at equimolar proportions. Samples were sequenced at Novogene (Cambridge, United Kingdom) using the Novaseq 6000 platform (Illumina, CA, USA) with an 150bp paired-end sequencing strategy.

3.3.3. Data processing and QC

Quality control was conducted to ensure accurate downstream analyses. QC was carried out using FastQC (Andrews, 2010). Raw reads underwent trimming of low quality sequences (average Phred score < 15), short length reads (<36bp), and Illumina adapters using Trimmomatic v0.39 (Bolger, Lohse and Usadel, 2014). IWGSC RefSeq v1.1 was used as the reference and gene model annotation, and is available from the International Wheat Genome Sequencing Consortium (https://www.wheatgenome.org).

Salmon's (Patro *et al.*, 2017) mapping-based mode was used to index the reference transcriptome, and quantification of the trimmed reads. Salmon output files were processed for input to DESeq2 using the R package TxImport (Soneson, Love and Robinson, 2015) to generate a table containing transcript abundance, counts, and length.

3.3.4. Exploration of gene expression data and differential expression analysis

Initial exploration of transcriptome data was conducted via PCA and boxplots in R to check for global trends. DESeq2 (Love, Huber and Anders, 2014) was used for differential expression analysis. The data was initially filtered to remove genes with <10 counts across the dataset. In DESeq2 an additive model was used to identify differentially expressed genes (DEGs) between before (BD) and after drought (AD) samples, as well as between the DS and DT groups. Log2FoldChange values for all comparisons were shrunk using the R package Ashr (Stephens, 2017).

Genes were considered differentially expressed if they fulfilled an adjusted p-value (FDR) < 0.05 and an absolute log2FoldChange ≥ 1 for drought tolerance DEGs (DS-DT comparison) or an absolute log2FC ≥ 1.5 for drought response DEGs (BD-AD comparison). The DS group was used as the reference level for the DS-DT comparison; positive log2FC values correspond with genes that are more highly expressed in the DT group, while negative values are associated with genes that are more highly expressed in the DS group. BD was used as the reference level for the BD-AD comparison.

3.3.5. Functional annotation & GO enrichment of differentially expressed genes

The agriGO V2 Singular Enrichment Analysis tool (Tian *et al.*, 2017) was used to identify gene ontology (GO) terms significantly enriched in each list of differentially expressed genes. Up- and down-regulated genes were submitted separately for analysis. The analysis used all genes present in the differential expression analysis post-count filtering as the background, with a Benjamini Hochberg multiple-test adjustment (FDR). GO terms were considered significantly enriched with an FDR-adjusted p- value ≤ 0.05. GO terms with fewer than 5 mapped genes were discarded.

3.3.6. Network Construction and Module Detection

A network was constructed using WGCNA to dissect the relationships between tolerance-associated genes. TPM abundance datasets generated using Salmon from the RNA sequencing were used as input for WGCNA (Langfelder and Horvath, 2008, 2012) in order to construct a co-expression network. All samples were used for network construction. Genes were filtered out if they contained too many zero values across samples; genes with many zero values are considered to be low-variance, cannot be well-correlated with other genes, may instead represent technical artefacts like low read depth or confounding homoeologues, and can invalidate the scale-free assumptions of WGCNA. blockwiseModules() was used for blockwise network construction and module detection using 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 0.8), minimum module size = 30, merge cut height = 0.25. The exportNetworkToCytoscape() function was used following module detection to create edge and node files for module analysis and visualization. A threshold of 0.1 on edge weights was used to filter out weak connections (edges) between genes (nodes) (**Supplementary Data S3.1-S3.2**).

3.3.7. Module GO Term Enrichment Analysis

Each module in the network was submitted for GO enrichment analysis (as above) to aid identification of interesting modules. For a given module, the node list produced by exportNetworkToCytoscape() was used as the gene list, analysed against a background of all genes included in the network.

3.3.8. DEG Enrichment Analysis

Enrichment analysis was conducted to identify modules that contained a higher-than-average proportion of DEGs. If DEGs were evenly distributed across all modules proportionally to their size, one would expect each module in the network to contain a similar proportion of differentially expressed genes. I.e., if 10% of genes in the network were DEGs, each module might be expected to contain 10% DEGs. A one-proportion Z test in R, using the function prop.test() with the argument *alternative = “greater”* was used to test whether the observed proportion of DEGs for a given module was significantly higher than expected with an even distribution. A given module was found to be enriched in DEGs if p ≤ 0.05.

3.3.9. Network Visualization and Hub Identification

Networks were visualised and hub genes were identified to further explore the genetic basis of tolerance traits in the wheat landraces. Identification of hub genes was done using custom scripts in R, counting the number of connections (edges) to and from each gene in the threshold-filtered WGCNA edge file produced by the function exportNetworkToCytoscape(). The number of total connections was referred to as a 'degree score' and used to initially rank genes in the module. These scripts are available on GitHub (https://github.com/andreaharper/HarperLabScripts). Well-connected genes were screened according to their putative function and expression profiles to ensure that they were consistently expressed, well-expressed, and showed differences between our groups of interest. Genes within a module with the highest degree score were identified as the central hub genes; in highly connected modules, where many of the genes shared the same degree score, or in modules where none of the well-connected genes were differentially expressed between the groups of interest, a subnetwork was created from the initial module edge file using the differentially expressed genes as 'guide genes'. This involved filtering the module to only include genes that shared an edge with any differentially expressed gene, removing the noise of genes that did not significantly change in expression. The highest scoring (most well connected) tolerance DEG was identified as a module's tolerance hub gene, as this is considered a reliable metric for the identification of transcriptionally regulatory hubs within a given module (Zhu et al., 2023; Tang et al., 2023).

Visualisation of modules was done using Cosmograph in Python (Rokotyan, N., Stukova, O., Kolmakova D. & Ovsyannikov, D., 2022). The edge tables were supplied in .csv format for Cosmograph.

3.3.10. Validation of Hub Gene Function

TILLING mutants were used in an attempt to validate gene function. TILLING mutant lines were obtained from SeedStor (https://seedstor.ac.uk/). Identification of TILLING lines with mutations in for *WRKY19* (*TraesCS5D02G145800*) was carried out using the gene's EnsemblPlants variant table (https://plants.ensembl.org/Triticum_aestivum). A variant (Cadenza1792) with the spring Cadenza background was selected based on the presence of a nonsense gain-of-stop mutation which was predicted to disrupt gene function. These lines were generated using EMS mutagenesis (Krasileva *et al.*, 2017) but current seed stocks are likely the result of several generations of self-pollination. The presence of TILLING mutations was confirmed using PCR-based genotyping (see **Supplementary Data S3.10** for primers and sequence targets)

TILLING mutants were confirmed using polymerase chain reaction (PCR)-based genotyping and sequencing. Polyploids often contain multiple homoeologues of a given gene across its genomes; homoeologue-specific primers are needed to amplify only the region of interest on the gene of interest. Primers were designed using Primer3

(Untergasser *et al.*, 2012) and screened for GC content, appropriate melting temperature, and the likelihood of self-dimerisation or hairpin formation. Primers were also tested for specificity against the homoeologues using NCBI's primer-BLAST tool: non-homoeologue specific primers were noted and the nucleotide at the site of interest was checked to ensure that homoeologues could be differentiated when examining the sequencing trace at the genotyping stage. Primers were reconstituted as per the manufacturer's instructions, and a working aliquot was made at 10 µM concentration.

PCR was carried out on DNA extracted from leaf tissue (harvested, immersed in liquid nitrogen, and stored at -80°c) using an Edwards DNA Extraction buffer protocol. GoTaq Green Master Mix (MM) (Promega, WI, USA) was used for PCR amplification. Each PCR reaction contained 10 µL GoTaq MM, 1 µL of DNA, 1 µL Forward Primer, 1 µL Reverse Primer, and 7 µL H_2O to a total volume of 20 µL. PCRs were carried out using the same cycle quantity and length, though primers may differ by annealing temperature due to differing GC proportions. PCR products were purified prior to sequencing using the Wizard SV Gel and PCR Cleanup Kit (Promega, WI, USA) as per the manufacturer's protocols. 2.5 µL of purified PCR product was added to 5 µL of nuclease-free H_2O with 2.5 µL of Forward Primer to a total volume of 10 µL and was sent for sequencing using Eurofins' LightRun Tube Sanger Sequencing service. Presence of mutation at the desired site was determined and plants were designated WRKY19^{WT} (no point mutation) or WRKY19^{MUT} (point mutation present).

Confirmed mutants were subsequently backcrossed onto wild-type Cadenza plants. Mutant lines were used as male pollen donors on emasculated Cadenza plants. Backcrossing was carried out to reduce mutation load of the progeny while maintaining the mutation of interest. Experimental plants were backcrossed once and self-pollinated to obtain homozygous mutants. BC_1S_1 (once backcrossed, once selfed) plants were used for drought screening. Plants were genotyped at each stage to ensure that the lines maintained the mutation of interest.

Drought treatment was carried out as described previously and measured using an Aligned Rank Transformed ANOVA on both raw and normalised dry weight. The ARTool (ver. 0.11.2) (Kay *et al.*, 2025) package was used followed by post-hoc Tukey testing via the ARTool art.con() function. Data were assessed for homogeneity of variance and normality of residuals using the shapiro_test() and levene_test() functions from the rstatix (ver. 0.7.2) (Kassambara, 2023) package in R.

24 BC_1S_1 plants were sown and genotyped. 8 Cadenza cultivar plants were grown alongside as a comparison.

3.4. Results

3.4.1. Assessing drought tolerance phenotypes

As identified in a previous study involving these landraces (Barratt et al., 2023b), drought treatment had a significant (p = 3.25e-14) impact upon both fresh and dry weight between the drought and control groups by two-way ANOVA. The dry weight of droughted plants was normalised (nDW) as a proportion of its control counterparts to identify drought tolerance phenotypes across the panel. The accession with the highest mean nDW (YoGI_047) saw an average reduction in biomass of only 20% compared to its control group, while the most drought-susceptible accession (YoGI_010) was reduced by 51%, indicating broad variation in drought phenotypes.

I sought to identify whether these accessions expressed different drought tolerance phenotypes. From an initial panel of 20 accessions, seven ‘drought-tolerant’ (DT) and seven ‘drought-susceptible’ (DS) accessions were identified, providing two groups with significantly different average drought tolerance (ANOVA: p = 7.31e-07) (**Figure 3.1**). To ensure that the resultant normalised dry weight was not simply a result of differing biomass under control conditions, I compared the relationship between dry biomass under control conditions and normalised dry biomass under drought conditions for each line across the panel using linear regression in R. A significant correlation was identified between control dry biomass and normalised dry biomass (p = 2.1e-4), though this was accompanied by an R^2 value of 0.226, suggesting that the vast majority of variation in the normalised dry biomass across the panel was not explained by biomass under control conditions, and therefore I was satisfied that normalised dry biomass was a sufficient measure of early drought tolerance for these plants.

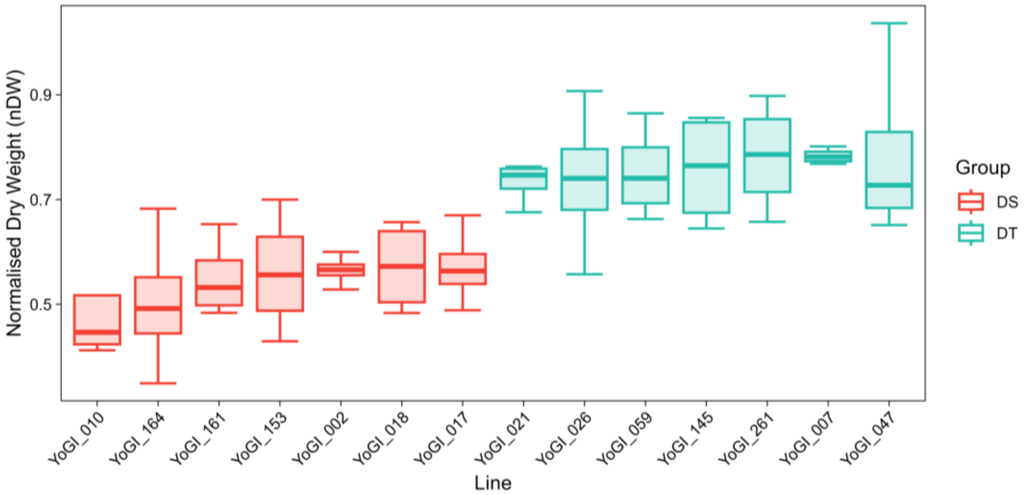

Figure 3.1: The YoGI panel contains lines with a variety of drought tolerance phenotypes. Two groups (7 lines each) displaying significantly different drought tolerance phenotypes (ANOVA: p = 7.31e-07) were identified using Normalised Dry Weight (nDW). nDW for each line was used to compare drought tolerance across the panel. 14 accessions were selected from a larger panel of 20 and designated drought tolerant (DT) or drought susceptible (DS).

Tolerant accessions were considered to be those that displayed significantly less drought-related growth reduction than the susceptible accessions. Six intermediate remaining accessions (not pictured) were removed from further analysis and were not sequenced.

3.4.2. Transcriptome sequencing, quantification, and overview

Sequencing data used was detailed in Barratt et. al (2023); in brief, 921.6 Gb of raw data was generated across 28 RNA-seq samples (samples taken before and after drought stress for 14 accessions), which were individually pooled from 4 biological replicates. After filtering, data was deemed to be of sufficient quality and mapped to the transcriptome reference using Salmon, resulting in an average unique mapping rate across samples of 61%. Raw sequence read data is available in NCBI's Gene Expression Omnibus (GSE225797).

Initial exploration of the dataset was conducted to identify batch effects, quantify treatment and phenotype effect sizes, and view trends among differentially expressed genes. The PCA (**Figure 3.2A**) identified that a substantial amount of the variance was explained by the samples before and after drought, as expected due to drought's massive transcriptional reprogramming. However, none of the first 10 principal components were able to clearly explain variance between the tolerance phenotypes, indicating that differences in tolerance are perhaps brought about by a small core of genes, or by small and nuanced changes in expression across a wide variety of genes. PC1 and PC2 together explained 68% of the variance across the dataset, with PC2 only representing 5% of the total variance; differences across the two tolerance phenotype groups were likely to be small in magnitude, so this was not entirely unexpected. There was little or no clustering that appeared to be due to geographical origin of accessions.

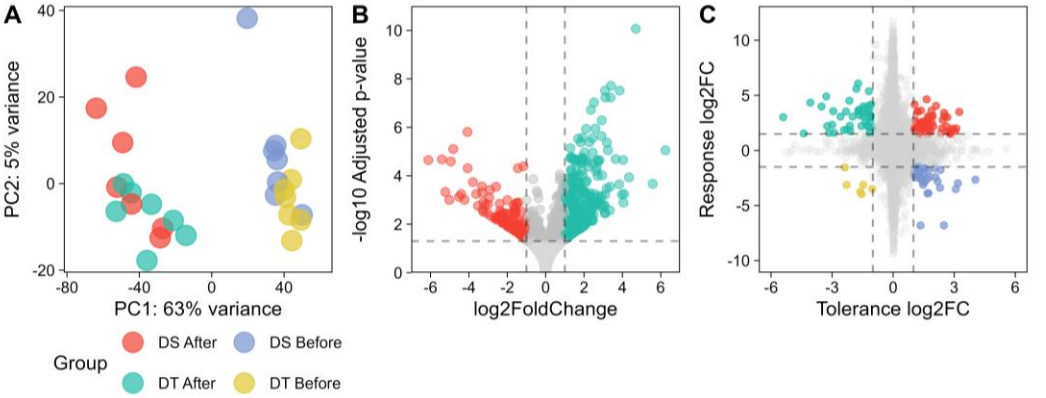

Figure 3.2: Transcriptome analysis identified wheat transcriptome shifts across drought exposure and potentially tolerance phenotypes. Samples (7 per group) and gene groups are coloured according to their treatment/ phenotype and differential expression groups respectively. **(A)** Principal component analysis identified clear differences between samples taken before and after drought (PC1), while no single PC was able to fully separate the samples by their phenotypic groups. **(B)** Differential expression analysis identified 560 genes that were significantly differentially expressed between phenotypic tolerance groups. Vertical lines represent a log2FC threshold of ±1, while the horizontal line represents a p-value threshold of 0.05. DEGs outside of those thresholds are coloured in grey. **(C)** The relationship between tolerance and response DEGs was also examined, identifying that a proportion of drought responsive DEGs were also tolerance DEGs.

3.5. Tolerance-associated DEGs are dominated by phytohormone stress response functions

560 DEGs were identified using DESeq2 between the drought susceptible (DS) and drought tolerant (DT) groups; a greater proportion appeared upregulated, with 367 genes with higher expression in DT samples (65.6% of observed DEGs) and 193 genes with lower expression (34.4%). Only 0.7% of genes tested overall were deemed tolerance DEGs. 10,199 genes were identified as being differentially expressed between the before drought (BD) and after drought (AD) groups at the log2FoldChange threshold of ±1.5.

GO enrichment analysis was conducted to further understand the biological functions of these DEGs. GO terms associated with phytohormone signalling pathways were identified – especially the jasmonic acid pathway – as well as specific stress response functions that were enriched in genes with increased expression in tolerant samples. The GO terms '*jasmonic acid metabolic process'* (GO:0009694; FDR = 1.2E-26), followed by '*response to wounding'* (GO:0009611; FDR = 1.2E-26) and '*response to jasmonic acid'* (GO:0009753; FDR = 1.30E-21) were found amongst the most enriched. Other stress-related enriched GO terms included '*response to water deprivation'* (GO:0009414; FDR = 7.20E-11) and '*hyperosmotic salinity response'* (GO:0042538; FDR = 2.60E-11). Broad stress terms like '*response to stress'* (GO:0006950; FDR = 9.00E-06) and other stress-response mechanisms like '*salicylic acid mediated signalling pathway'* (GO:0009863; FDR = 3.20E-06) were also enriched. The most enriched Molecular Function GO term was '*transcription corepressor activity'* (GO:0003714; FDR = 1.90E-17), while no Cellular Component GO terms were enriched.

GO enrichment analysis of genes with higher expression in susceptible samples (or with lower expression in tolerant samples) primarily obtained several enriched terms related to biotic stress responses – the most enriched term was identified as '*defense response to fungus'* (GO:0050832; FDR = 3.70E-12) – while other enriched stress terms included '*chitin catabolic process'* (GO:0006032; FDR = 1.00E-11) and '*response to bacterium'* (GO:0009617; FDR = 7.50E-06). Some abiotic stress related GO terms were also identified, with '*response to osmotic stress'* (GO:0006970; FDR = 0.0067) and '*response to salt stress'* (GO:0009651; FDR = 0.0041) enriched. The most significantly enriched Molecular Function GO term was '*chitinase activity'* (GO:0004568; FDR = 1.40E-11), while the most significantly enriched Cellular Component GO term was '*anchored component of membrane'* (GO:0031225; FDR = 4.30E-06). The term *'cell wall'* (GO:0005618; FDR = 0.00011) was also enriched.

Analysis of DEGs shared between the response (comparing expression between before drought (BD) and after drought (AD) groups) and tolerance (comparing expression between DS and DT) (**Figure 3.2C; Supplementary Data S3.6-S3.9**) identified 152 genes significantly differentially expressed in both contrasts. The largest group (63 genes) of these 'crossover DEGs' included genes with increased expression both after drought and in tolerant accessions. These genes were enriched in GO terms including '*regulation of jasmonic acid mediated signalling pathway'* (GO:2000022; FDR = 2.8e-14), '*response to jasmonic acid'* (GO:0009753; FDR = 1e-06), '*regulation of signalling'* (GO:0023051; FDR = 1.7e-05), '*transcription factor activity, DNA binding'* (GO:0000989; FDR = 5.9e-08), and '*abscisic acid-activated signalling pathway*' (GO:0009738; FDR = 0.042) . The second largest overlap was of genes with increased expression in tolerant accessions but decreased expression across the panel in response to drought – of these 37 genes, '*root hair cell development'* (GO:0080147; FDR = 0.017) was the most enriched GO term. Other GO terms included '*jasmonic acid mediated signaling pathway'* (GO:0009867; FDR = 0.022), '*response to wounding'* (GO:0009611; FDR = 0.047) and '*integral component of organelle membrane'* (GO:0031300; FDR = 0.00025). The last functionally enriched crossover group, which consisted of 44 genes with increased expression among susceptible accessions but increased expression after drought, was enriched in the GO terms '*cell killing'* (GO:0001906; FDR = 1.5e-10), '*defense response to fungus'* (GO:0009817; FDR = 1.5e-10), '*defense response'* (GO:0006952; FDR = 0.00054), '*response to salt stress'* (GO:0009651; FDR = 0.012), and '*apoplast'* (GO:0048046; FDR = 0.044).

Expression data, DEGs, and GO enrichment results for all comparisons can be found in **Supplementary Data S3.3-S3.9.**

3.5.1. Co-expression network construction & identification of tolerance-associated modules

The co-expression network was constructed using WGCNA to identify and explore any interesting hub genes. The network consisted of 84,888 genes across 81 modules after filtering; mean average module size was 1,048, while the median module size was 165. Module size varied significantly, with the smallest identified module housing 30 genes, and the largest housing 19,380 genes. 18,952 genes were placed in the unstructured grey module, indicating that they had no strong or meaningful co-expression relationships with the eigengene of any module. Strong variation in module size was to be expected with a strong driver of gene expression variation in this dataset, with larger modules likely containing broad cellular processes and stress responses, and smaller modules likely containing tightly regulated processes controlled by small cores of genes.

To identify modules associated with tolerance phenotypes, as opposed to the wider drought response, WGCNA's trait association functions were used to correlate module eigengenes (representatives of the overall expression pattern across samples within that module, expressed as the first principal component of that module) with the experimental groups, identifying 18 modules significantly positively associated with tolerant samples after drought treatment, and 5 modules significantly positively associated with tolerant samples before drought treatment (**Supplemental Figure S3.1**). Modules positively associated with tolerant samples were expected to house genes with a higher expression in those samples, either at the basal level or after drought, and may contain hubs with beneficial regulatory functions under stress conditions.

To further refine module selection and identify modules particularly associated with drought tolerance among these samples, DEG and GO enrichment analyses were used. 12 modules containing significantly more (greater than 0.7%) tolerance DEGs were identified (**Table 3.1),** suggesting they could be involved in determining an accession's inherent level of drought tolerance. GO enrichment was used to further refine module selection, with 8 DEG-enriched modules also found to be enriched in stress-associated GO terms. Modules that contained genes involved in drought mechanisms were thought likely to be enriched in GO terms like '*response to osmotic stress'*, or to broader phytohormone GO terms like '*response to jasmonic acid'*.

To further ensure the focus on differences related to drought tolerance, as opposed to differences in inherent growth between phenotypes, any modules significantly enriched in growth-related GO terms (GO:0040008: '*regulation of growth*', GO:0001558: '*regulation of cell growth*'; GO:0040009: '*regulation of growth rate'*) were filtered from further analysis.

Table 3.1: All modules enriched in tolerance DEGs are listed, with the average log2FC of DEGs within the module for both tolerance and response contrasts, along with stress-associated enriched GO terms for that module. Log2FCs are only given when the module contained significant ($p < 0.05$) DEGs of that comparison. Only the log2FC of DEGs was used when calculating mean LFCs. For each module, the most enriched GO term is listed first in bold, followed up to three enriched stress-associated GO terms possessed by the module. FDR-adjusted *p* values are provided in brackets. *P* values for tolerance DEG enrichment are provided for each one-proportion Z test used.

Module	Module size	Mean tolerance LFC	Mean response LFC	Enriched GO terms	DEG Enrichment
coral1	65	14.09	2.59	-	p ≤ 0.001
palevioletred3	96	2.41	-	-	p ≤ 0.001
salmon4	98	2.01	-	**Jasmonic acid biosynthetic process (p = 1.07e-08)** Response to water deprivation (p = 2.46e-02)	p ≤ 0.001
darkred	868	1.9	2.47	**Regulation of jasmonic acid mediated signalling pathway (p = 7.54e-18)** Regulation of plant-type hypersensitive response (p = 2.70e-08) Response to salicylic acid (p = 4.34e-02) Regulation of transcription (1.61e-02)	p ≤ 0.001
darkgreen	786	1.84	-2.54	**Cellular response to toxic substance (p = 8.01e-06)** Calcium-mediated signalling (p = 3.85e-04) Jasmonic acid mediated signalling pathway (p = 3.72e-03) Response to wounding (p = 4.63e-04)	p ≤ 0.001
pink	1781	1.8	1.45	**Protein phosphorylation (p = 2.84e-05)** Plant-type hypersensitive response (p = 3.58e-05)	p ≤ 0.001
yellow	2709	1.62	2.53	**Response to chitin (p = 2.09e-72)** Regulation of transcription (p = 3.35e-12) Response to water deprivation (p = 6.45e-16) Response to salt stress (p = 3.62e-12)	p ≤ 0.001
ivory	136	1.28	-2.76	**Jasmonic acid biosynthetic process (p = 2.13e-11)**	p ≤ 0.001
blue	4314	-1.9	-1.14	**Protein phosphorylation (p = 1.78e-88)** Negative regulation of defense response (p = 1.03e-53) Response to oxidative stress (9.83e-19)	p ≤ 0.001
darkorange2	122	-1.63	2.67	-	p = 0.0154
salmon	1433	-1.98	3.26	**Negative regulation of endopeptidase activity (4.82e-14)** Response to oxidative stress (p = 2.78e-02)	p = 0.0028
skyblue3	194	-1.22	23.92	-	p = 0.0018

3.5.2. Identification of stress-associated hub genes

Further refinement of the network was required to identify the top candidate hub genes from the modules in **Table 3.1**. Hub genes obtained from candidate tolerance modules were identified using NCBI BLASTX (Camacho *et al.*, 2009) to compare their gene model sequences to proteins across the *Poaceae* and other related families. Several of these hub genes shared substantial sequence identity with genes that were likely to have stress-responsive or transcriptionally regulatory functions (**Table 3.2**). Many of these genes may act as part of a pathway with other genes within the module, and or attenuate aspects of the drought response by mediating stress hormone signalling in the cell.

Identification of the tolerance-associated hub genes within modules was sometimes aided by the construction of subnetworks from large modules (e.g. *darkgreen*) using the DEGs as guide genes, where only edges to or from a tolerance-associated gene are preserved, to observe their specific role within a module. This has been used previously to dissect genes of interest from large modules (Barratt et al., 2023a, 2023b)

Very small modules with few tolerance DEGs (e.g. coral1) or no enriched GO terms were removed from further analysis despite their DEG enrichment and average log2FoldChange.

The darkred module (**Figure 3.3A**) was significantly enriched in tolerance DEGs, housing 868 genes, 37 of which were differentially expressed between the DS and DT groups (4.26% of genes in the module; *p* = 8.08e-33). The module was drought responsive, with 10% of its genes identified as response DEGs. Tolerance DEGs (average LFC = 1.90) showed higher expression among tolerant accessions, while drought-response DEGs (average LFC = 2.47) were transcriptionally upregulated, suggesting that tolerance DEGs may also be involved in directly and positively responding to drought. The module was also enriched in several stress associated GO terms, such as '*jasmonic acid mediated signalling pathway', 'regulation of plant-type hypersensitive response', 'defense response', 'response to salicylic acid',* and '*regulation of cell death',* indicating a strong likelihood that its genes were involved in the tolerance of and response to drought.

The darkgreen module (**Figure 3.3C**) was significantly enriched in tolerance DEGs, housing 786 genes, 52 of them being differentially expressed (*p* = 6.67e-80), and was also enriched in the GO terms '*jasmonic acid metabolic process'* (GO:0009694, *p* = 7.11e-04), '*response to jasmonic acid'* (GO:0009753; *p* = 1.6e-02), '*response to wounding'* (GO:0009611; *p* = 2.2e-03), '*calcium-mediated signalling'* (GO:0019722, *p* = 7.1e-04), and '*terpene synthase activity'* (GO:0010333, *p* = 9.2e-03). The module was also found to be significantly associated with tolerant samples prior to drought. Many of the genes in the module possessed the same number of connections (454), confounding hub gene selection – as a result, a subnetwork was created from the module using the tolerance DEGs as guide genes. The hub gene of this subnetwork was found to be *TraesCS1D02G072700*, a DEG (tolerance LFC = 2.3) connected to 428 genes within the module, including all other DEGs present. Despite the gene's higher expression in tolerant accessions across both conditions, the gene appeared transcriptionally downregulated in response to drought (response LFC = -3.39). Across the module, all differentially expressed genes saw transcriptional downregulation after drought, indicating that the module was negatively associated with the drought response.

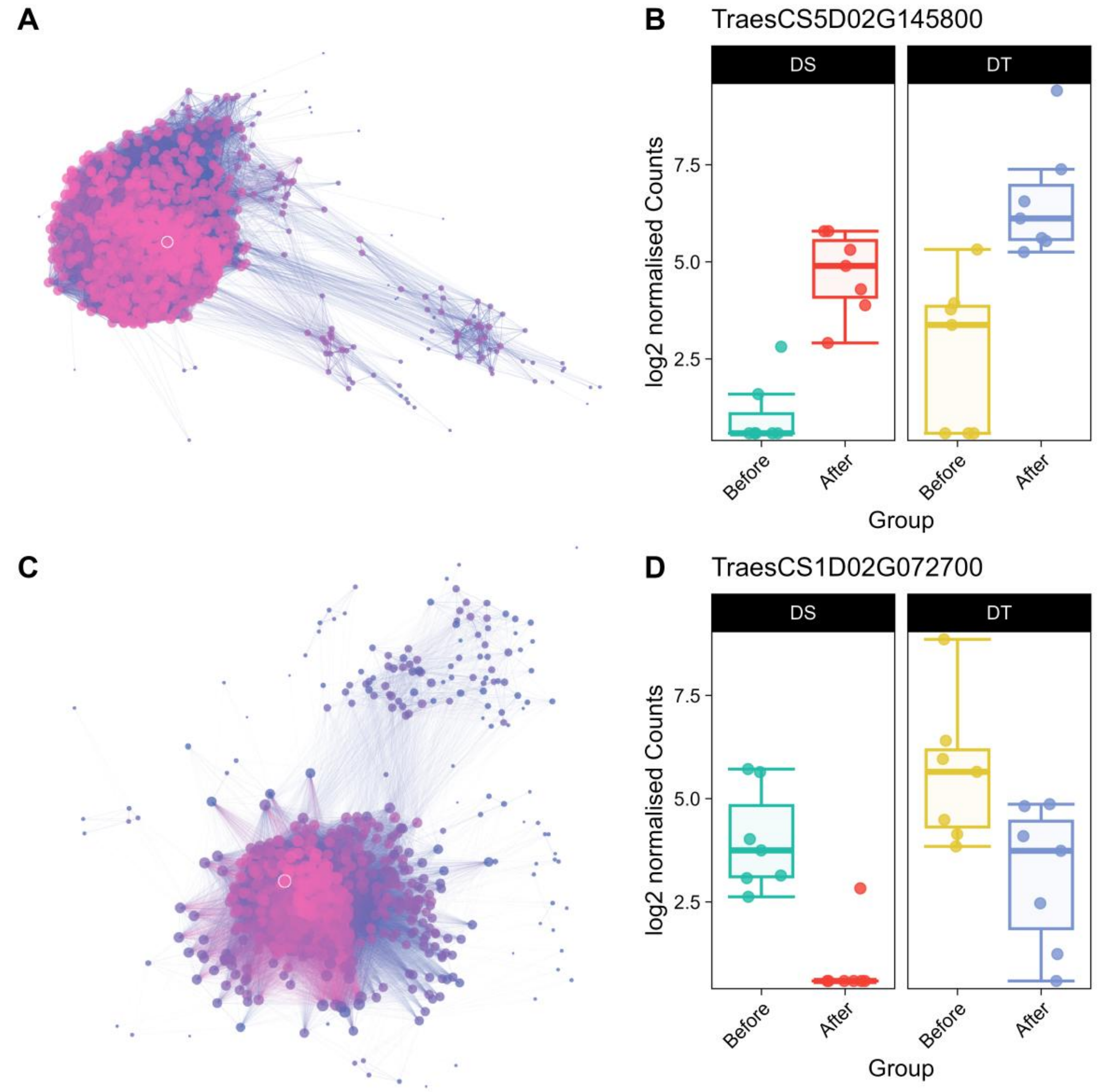

Figure 3.3: **darkred and darkgreen modules were visualised, and their main hub genes were plotted to show module composition and gene trends**. In module visualisations **(A, C)**, the size and colour of the nodes relate to the number of connections to or from that node. DS and DT refer to 'drought susceptible' and 'drought tolerant' phenotype groups respectively. **(A)** The darkred module was another mid-size module enriched in tolerance DEGs**.** The hub gene, *TraesCS5D02G145800*, has been highlighted with a white circle. **(B)** log2 normalised expression of *TraesCS5D02G145800* between before and after and between drought sensitive and drought tolerant lines (7 samples per group); the gene was significantly upregulated in response to drought, as well as showing higher expression among tolerant lines. **(C)** The darkgreen module was a mid-size module enriched in tolerance DEGs**.** The hub gene, *TraesCS1D02G072700*, has been highlighted with a white circle. **(D)** log2 normalised expression of *TraesCS1D02G072700* across both drought response (before and after) and between drought sensitive and drought tolerant lines (7 samples per group); the gene was significantly downregulated under drought, though showed higher expression among tolerant lines both before and after drought.

Table 3.2: Eight hub genes were identified as being potentially involved in mediating drought tolerance based on their high number of connections to other genes within the module (degree score), changing expression across samples, putative function, and DEG enrichment of the module. Degree score is given for each putative hub in brackets alongside the gene ID, while module size is given for each module in brackets alongside the module name. Degree scores also note where a DEG subnetwork was used to interrogate the genes connected to the hub.

Hub Gene ID	Module	Tolerance LFC	Response LFC	Top BLASTX Hit	Putative Function
TraesCS1A02G218600 (24)	coral1 *(65)*	1.36	3.62	Phospholipase A1-Ibeta2, chloroplastic-like [Triticum aestivum]	JA biosynthetic pathway & fatty acid metabolism (H. Zhang *et al.*, 2021)
TraesCS5D02G219300 (66)	salmon4 *(98)*	3.38	-	Protein TIFY 10c-like [Triticum aestivum]	Repressor of jasmonic acid, response to drought stress (X. Wang *et al.*, 2023)
TraesCS5D02G145800 (630)	darkred *(868)*	2.11	4.04	Transcription factor WRKY19-like [Triticum aestivum]	Transcription factor activity; promote response gene expression (Niu *et al.*, 2012)
TraesCS1D02G072700 (428 – DEG subnetwork)	darkgreen *(786)*	2.3	-3.39	Jasmonate-induced oxygenase 1-like [Triticum aestivum]	Hydroxylation of jasmonic acid to 12OH-JA; repression of plant immune responses (Caarls *et al.*, 2017)
TraesCS2B02G034600 (1069 – DEG subnetwork)	pink *(1781)*	1.66	-	Putative receptor-like protein kinase At4g00960 [Triticum aestivum]	Receptor protein kinase activity; regulator of immune responses (Wrzaczek *et al.*, 2010)
TraesCS4D02G325200 (1527)	yellow *(2709)*	-	1.65	Serine/ threonine protein kinase BSK1-2 [Aegilops tauschii subsp. Strangulata]	Regulator of plant immunity; positive regulator of brassinosteroid signalling (Tang *et al.*, 2008)
TraesCS5D02G194500 (3091)	blue *(4314)*	-	2.04	Senescence-induced receptor-like serine/threonine-protein kinase, partial [Aegilops tauschii subsp. strangulata]	Senescence-related membrane receptor kinase activity
TraesCS2D02G224200 (790)	salmon *(1433)*	-	10.4	Isocitrate lyase [Triticum aestivum]	Osmotic stress response via glyoxylate cycle (Yuenyong *et al.*, 2019)

3.5.3. Hub Gene Validation

The *WRKY19* hub gene was tested using TILLING mutants. Both treatment (Aligned Rank Transformed (ART) ANOVA: p = 1.22e-08) and genotype (ART ANOVA: p = 5.86e-04) were found to have a significant effect on dry weight (g) (**Figure 3.4A**). Post-hoc Tukey tests identified that Cadenza was significantly bigger than both WRKY19^{WT} (+59.5%, p = 0.0154) and WRKY19^{MUT} (+97.9%, p = 4.21e-04) plants across both treatment groups, though WRKY19^{WT} and WRKY19^{MUT} plants did not show significantly different dry weight (p = 0.131), suggesting the target mutation itself did not have a large impact.

Normalised dry weight was used to compare tolerance across the three genotypes. Plants from each group were normalised against the average dry weight of their control counterparts. Genotype was found to have a non-significant effect on normalised dry weight (ART ANOVA: p = 0.0807); post-hoc Tukey tests found that no groups were significantly different, despite average nDW being higher among WRKY19^{MUT} plants (0.422) than WRKY19^{WT} plants (0.372) and CAD (0.322), though this is likely due to low sample sizes.

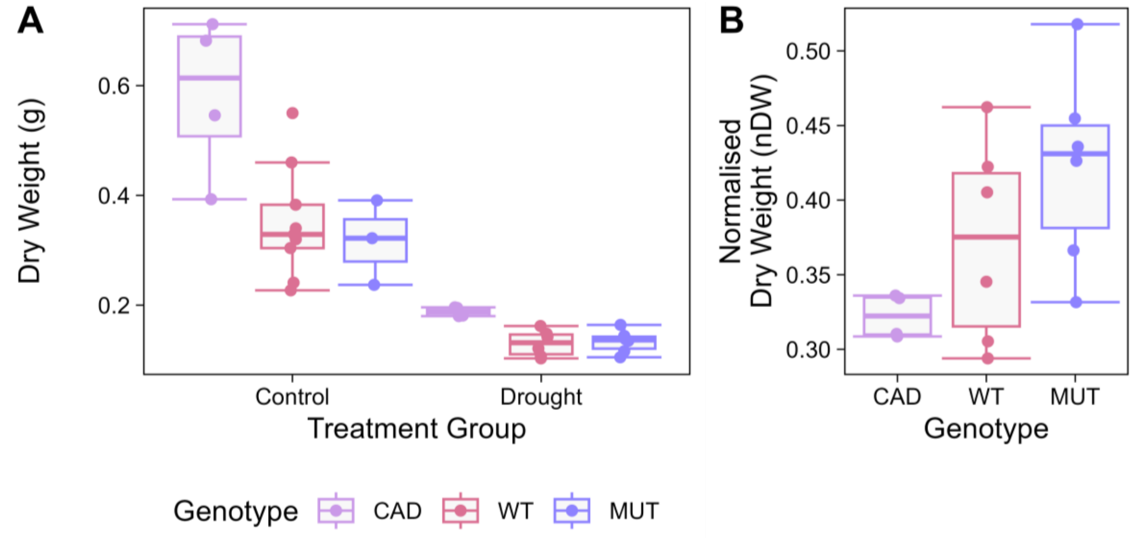

Figure 3.4: ***TraesCS5D02G145800's* proposed function was unable to be validated using TILLING mutants**. TILLING mutants for the WRKY hub gene (*WRKY19^{MUT}*) were compared to plants from the same background without the stop-gain mutation (*WRKY19^{WT}*) and plants from the Cadenza background (CAD), identifying a significant effect of treatment and genotype on nDW. **(A)** Raw dry biomass (g) was compared between the three groups of plants before and after drought. **(B)** Normalised dry weight (nDW) was compared between the three genotypes; *WRKY19^{WT}* and *WRKY19^{MUT}* plants exhibited substantially higher nDW than Cadenza. Dots represent individual data points. There were fewer MUT plants (n=3) than the WT (n=7) and CAD (n=4) genotypes under control conditions, and fewer MUT (n=4) plants than the WT (n=6) and CAD (n=4) genotypes under drought conditions. Low group size of mutant lines may have limited analysis.

3.6. Discussion

Landraces are an essential pool of genetic and phenotypic diversity that can be leveraged to identify and reintroduce beneficial alleles to elite crop varieties, ensuring that they are better able to survive in a variety of climate conditions. Resources involving wheat landrace panels have grown in in recent years, providing a stronger framework to support wheat breeding efforts for improving abiotic stress tolerance (Cheng et al., 2024; Korpetis et al., 2023; Farooq, 2023).

Genetic sources of drought tolerance – basal differences in the expression of genes, even under control conditions – can change the fitness of plants under stress. Identifying plant phenotypes with more vigour under stress conditions is particularly beneficial; this analysis has used above-ground dry weight biomass as a marker of drought tolerance, with the assumption that plants able to continue accumulating biomass and developing under stress are more tolerant to drought stress than those that cannot. Some landraces are inherently more resistant to senescent processes and degreening, beneficial traits under stress conditions which can allow for maintained photosynthesis and a longer grain filling period (Riache et al., 2023; Ochieng et al., 2021). Stress hormone signalling is involved in the senescent response in leaves in response to drought stress; JA can promote both stress response genes and degreening (Zhu et al., 2015; Ono et al., 2019; Zhao et al., 2016), suggesting that attenuating its responses could allow for plants to better balance the relationship between responding to stress and growth. The YoGI panel has previously been used identify potential key transcriptional regulators of the wheat drought response (Barratt et al., 2023b), thermotolerance (Barratt et al., 2023a), and the heat stress response (Barratt, Franco Ortega and Harper, 2023). Here I have aimed to identify nuanced changes to the wheat transcriptome between drought-tolerant and drought-susceptible wheat landrace seedlings under harsh water deficit. Understanding the drought response is critical – finding ways to fine-tune that response is also deeply important for crop improvement strategies.

As observed in the differential expression GO enrichment, jasmonic acid-related GO terms dominated the DEGs shared by the two differential expression contrasts; among the 152 shared DEGs, representing over a quarter of tolerance DEGs, 36 of these were associated with JA, and largely transcriptionally upregulated post-drought and more highly expressed among tolerant lines. JA, as a well-established regulator of both biotic and abiotic stresses, is likely to be involved in promoting drought tolerance in tolerant lines by modulating growth and stress responses (Rehman *et al.*, 2023), and so the gene regulatory network surrounding it is a potential source of drought tolerance.

Genes transcriptionally downregulated under drought conditions but with increased expression in tolerant lines included genes annotated with the highly enriched GO term 'root hair cell development', which could suggest that tolerant lines can establish more substantial or more efficient root systems to maximise water uptake under growth-restricted conditions; root system architecture and root surface area are associated with drought tolerance levels (Zhang et al., 2025e; Kulkarni et al., 2017; Li et al., 2021c), and so plants with greater expression of genes involved in root hair development may have enhanced water use efficiency: of these genes, a triad of homoeologous hexokinase-3 (*HXK3*) genes (*TraesCS3A02G480900, TraesCS3D02G475600,* and *TraesCS3D02G475600*) stood out. HXKs have previously been implicated in promoting root growth, drought tolerance, and improving water use efficiency when overexpressed (Kelly et al., 2019; Pérez-Díaz et al., 2021; Park and Lazarovits, 2014), suggesting that their increased expression in tolerant lines may benefit inherent tolerance.

Genes transcriptionally upregulated after drought but with lower expression in tolerant lines were also interestingly enriched in fungal defence GO terms, indicating that susceptible and tolerant lines may respond to drought in slightly different ways. Though induction of pathogenic/ fungal response genes could contribute to a plant's ability to tolerate drought stress and continue to grow under adverse conditions via overlapping responses between biotic and abiotic stresses (Su *et al.*, 2021), drought tolerance and disease resistance are conventionally negatively correlated due to suppression of the SA pathways by ABA (Mohr and Cahill, 2007; Audenaert, De Meyer and Höfte, 2002), a mechanism potentially seen here. Some genes in this group, like those encoding pathogenesis-related protein 5 (*PR5*)*,* have known dual pathogen-resistance and osmoprotectant roles (Weber et al., 2014); others, including several genes encoding 1,3-beta glucanases, sarcosine oxidases*, RGA1,* and *GTL1*, are thought to be negatively associated with drought tolerance – either their knockdowns improve drought tolerance, or their overexpression is deleterious to drought tolerance – suggesting that these tolerant lines may have a more optimised relationship between the drought and disease response pathways (Baek et al., 2019; Lee et al., 2008; Yoo et al., 2010; Ferrero-Serrano and Assmann, 2016). Tolerant lines exhibiting lower expression of these pathogen-associated genes, despite their transcriptional upregulation in response to drought, may suggest that these plants have more fine-tuned crosstalk between the abiotic and biotic stress pathways, potentially mediated by a greater degree of ABA present in the cell, as implied by the enrichment of ABA-associated GO terms among genes transcriptionally upregulated after drought and in tolerant lines. Greater suppression of energetically costly SA-mediated pathogenic responses under drought would better allow limited resources to be targeted towards drought-mitigation and better conserve water through ABA-associated stomatal control at the cost of pathogen resistance (Choudhary and Senthil-Kumar, 2022; Cao, Yoshioka and Desveaux, 2011).

Further exploration of the genes with differential expression between drought - susceptible and drought-tolerant groups relied on analysis of the weighted gene co-expression network; this analysis has identified candidate transcriptional regulator hub genes which may potentially mediate aspects of the drought response and contribute to a plant's inherent drought tolerance. By focusing on modules enriched in stress- and tolerance-associated genes, this analysis aims to provide robust insight into the regulatory network of early drought tolerance in wheat. Understanding the potential roles of these hubs requires examination of the gene's changes in expression across all contexts; exploration of modules that contain an overrepresentation of stress- and tolerance-associated genes allows focusing the scope to the strongest signals and elucidate genes involved in conveying stress tolerance in spring wheat seedlings.

This study discusses two of the promising candidate transcriptional master regulators of early drought tolerance in spring wheat landraces, prioritised based on their expression profiles, putative functions, connected genes, and characterisation of the modules that house them. The remaining hub genes (**Table 3.2**) may also present potential targets for breeders to optimise early drought tolerance in wheat crops. From this exploration of the network, we have identified that the most promising hubs appear to have an almost singular focus: mediating the jasmonic acid phytohormone pathways.

3.6.1. WRKY19 transcription factors may convey long-term drought tolerance through weak bioactive jasmonates and repression of JA

The darkred module's hub gene, *TraesCS5D02G145800* (tolerance LFC = 2.11; response LFC = 4.04) (**Figure 3.3B**), was identified as *TaWRKY19-like*, a WRKY transcription factor that has previously been suggested to convey broad spectrum tolerance to osmotic stresses when overexpressed in *A. thaliana*. (Niu *et al.*, 2012). While both susceptible and tolerant groups appear to increase transcription of this gene in response to drought, tolerant lines exhibited a higher basal expression, suggesting that it may be an adaptation inherent to these specific landraces that promotes stress tolerance. WRKY genes in wheat have previously been identified as strong targets for understanding abiotic stress responses and may play a role in conveying tolerance phenotypes (Gao et al., 2018; Ye et al., 2021b). WRKY transcription factors affect expression of their target genes by binding to a W-box element in the target gene's promoter region (Wang et al., 2023a). Both the hub and the genes connected to it had higher transcript abundance both in tolerant accessions and after drought, suggesting that here it is involved in both promoting tolerance and responding to drought. The hub gene also showed substantial similarity to *WRKY22-like* from *Triticum dicoccoides* and *TaWRKY47-like*. The present work indicates that the WRKY hub may be a coordinator of drought tolerance and response through mediation of jasmonic acid signalling.

The hub gene was connected to several tolerance-associated drought-responsive genes involved in jasmonic acid metabolism and signalling. Two of these, *TraesCS5D02G137000* (response LFC = 3.45) and *TraesCS5B02G128100* (response LFC = 3.41; tolerance LFC = 2.49), were found to be *cytochrome P450 94C1-like*, a cytochrome involved in jasmonyl-isoleucine (JA-Ile) turnover. *CYP94C1* comprises part of the pathway that hydroxylates JA-Ile into the weakly bioactive 12OH-JA-Ile, with its expression associated with decreased JA-mediated pathogenic defence (Heitz et al., 2012; Yang et al., 2023c), while elevated expression of an orthologue in *O. sativa* has been shown to convey an improved salt stress response (Kurotani *et al.*, 2015), and similarly overexpression in *A. thaliana* has been implicated in alleviating early senescence (Cao *et al.*, 2025). 12OH-JA-Ile has previously been implicated in the modulation of longer-term JA-Ile activated processes after an initial pathogen response (Jimenez-Aleman *et al.*, 2019); if similar mechanisms exist for abiotic stress, long-term weak activation of JA-responsive genes may convey drought tolerance phenotypes, but 12OH-JA-Ile's role in abiotic stress responses is relatively under-explored. Here, higher CYP94C1 transcriptional abundance in tolerant lines implies that JA-Ile may be actively attenuated under drought, shifting signalling towards weaker, but longer-term, jasmonate responses.

WRKY transcription factors have previously been implicated in the regulation of CYPs: *AtWRKY33* is a known positive upstream regulator of high salinity tolerance and *CYP94B1* in *A. thaliana* (Krishnamurthy et al., 2020; Birkenbihl, Diezel and Somssich, 2012), which similarly is involved in the catalysis of JA-Ile, raising the possibility that *TaWRKY19* could act similarly to promote osmotic stress tolerance through CYPs. A second pair of CYPs was also well connected to the hub gene: *TraesCS1B02G253600* (response LFC = 5.91) and *TraesCS1A02G242000* (response LFC = 3.49) were genes found to be *T. aestivum CYP71A1-like* genes. *CYP71A1*s have previously been found to be downregulated by heat stress exposure (Pandian *et al.*, 2020), and are likely involved in production of indole alkaloid secologanin (Chakraborty *et al.*, 2023), but have also been found to positively regulate drought tolerance in dicots like alfalfa (*Medicago sativa*). Unlike the first pair of CYPs, *CYP71A1* has no clear relationship to JA, but WRKY TFs have previously been implicated in the regulation of *CYP71* genes in *A. thaliana* (Birkenbihl *et al.*, 2017) and could potentially be transcriptionally influenced by the hub gene to promote drought tolerance.

Several members of the jasmonic acid biosynthesis pathway were also connected to the hub: *TraesCS1D02G220300* (response LFC = 1.75; tolerance LFC = 3.04) and *TraesCS1A02G266000* (tolerance LFC = 2.04), were identified as phospholipase A1 proteins involved in the production of the JA-precursor alpha-linolenic acid (Zhang et al., 2021a; Ruan et al., 2019), *TraesCS4D02G195600* (response LFC = 1.95; tolerance LFC = 1.29), found to be an omega-3 fatty acid desaturase also involved in the production of linolenic acid (Singh *et al.*, 2011), and *TraesCS4B02G037700* (tolerance LFC = 1.13), a *linoleate 9S-lipoxygenase 1-like*, another vital JA biosynthesis enzyme (Singh et al., 2022b), as well as *TraesCS6A02G224200* (response LFC = 2.18), a *T. aestivum MYB30*-like transcription factor annotated with the GO terms '*jasmonic acid biosynthetic process*' and '*response to water deprivation*'. The co-expression of the hub gene and multiple JA biosynthesis genes would suggest that WRKY19 may influence both JA production and jasmonate-responsive transcriptional targets under drought, supporting a model in which it contributes to moderating the jasmonate signalling pathway rather than controlling a conventional JA stress response.

Several homoeologues and duplicates of the *TIFY11e* jasmonic acid co-repressor were identified as being connected to the hub, suggesting that it may act to suppress general jasmonic acid signalling. The connected *TIFY11e* copies broadly split into two groups: 1. drought responsive only (mean response LFC = 3.00) and 2. drought responsive and with higher expression in tolerant lines (mean response LFC = 1.89; mean tolerance LFC = 2.22) *TIFY11e*, or *TaJAZ14* (Xie et al., 2019a), acts as a transcriptional regulator of jasmonic acid signalling. Members of the orthologous *Oryza sativa* TIFY11 family were found to promote plant growth when overexpressed (Singh and Mukhopadhyay, 2021; Hakata et al., 2017), suggesting that high expression of these TIFY proteins may desensitise plants to JA and reduce JA-associated growth inhibition; it is possible that the WRKY hub here is involved in repressing jasmonate-associated gene expression to promote growth over traditional stress responses under harsh drought stress conditions. This would be consistent with a model in which the hub gene suppresses stronger JA responses in favour of long-term weak responses.

WRKY genes have been implicated in the positive and negative regulation of abiotic stress responses; here I hypothesise that increased expression of *TaWRKY19* may enhance growth under water-deficit conditions by preventing a JA-hypersensitive response through co-expression with JA biosynthesis genes, JA-Ile catabolic enzymes, and JAZ repressors. This model would suggest that the hub gene mediates, rather than suppresses, JA-responsive gene expression; it would attenuate a strong conventional JA-based stress response, while still allowing for low-level expression of JA-responsive genes. High-level JA regulators have previously been found to be co-expressed with both positive and negative regulators of downstream JA responses (Chen et al., 2024b), suggesting that genes like the hub may be involved in balancing stress responses to maximise fitness under stressful conditions.

3.6.2. TaJOX1 may be involved in reducing JA hypersensitivity, promoting growth under drought

The darkgreen module's central hub gene was identified as *T. aestivum* jasmonate-induced oxygenase 1-like (*TaJOX1*), a JA-induced oxygenase involved in inhibiting plant immune responses through the inactivation of JA. JOXs, or JAOs, have previously been characterised in *Arabidopsis thaliana* as a missing step in JA metabolism responsible for balancing growth and defence processes under biotic stress conditions through the hydroxylation of JA to 12OH-JA (Caarls *et al.*, 2017a; Smirnova *et al.*, 2017), a non-bioactive jasmonate. Under drought stress conditions, *A. thaliana* and *Nicotiana tabacum* JOX mutants have been shown to display greater drought survival rates compared to wild type (Marquis et al., 2022; Zhang et al., 2024b). JOX knockouts in *A. thaliana* consistently display stunted growth and hyperaccumulation of JA (Caarls et al., 2017a; Zhang et al., 2021b), suggesting that higher expression of JOX genes would allow for less restricted growth. JOX genes have previously been found to promote growth of wheat and *A. thaliana* seedlings in warm conditions through repression of JA and an increase in the abundance of *JAZ* proteins (Zhu et al., 2021b), further supporting the potential relationship between *JOX1*, our hub gene, and increased growth under stress conditions.

Interestingly, in the present work, *TaJOX1*, though transcriptionally downregulated under drought conditions, saw elevated baseline expression in tolerant lines and was transcriptionally downregulated to a lesser extent than in susceptible lines (**Figure 3.3D**) – this is congruent with the idea that reduced JOX expression is part of the drought response, therefore reducing the amount of JA converted to 12OH-JA – however the increased basal expression in tolerant accessions and less severe transcriptional downregulation under drought may contribute to preventing JA hypersensitivity without substantially limiting JA's role in the drought response, allowing for greater growth compared to susceptible accessions, which express very low levels of *JOX1* under drought, and therefore potentially accumulate more JA, and experience greater JA-mediated growth restrictions. The hub gene may form part of a model in which tolerant lines partially attenuate JA responses under drought via JA hydroxylation, preventing excessive JA accumulation and therefore limiting growth inhibition.

The hub gene was also connected to one of its homoeologues, *TaJOX1-B* (*TraesCS1B02G08860*: response LFC = -3.91; tolerance LFC = 1.68), and one of its paralogues, *TaJOX2* (*TraesCS4A02G109200*: response LFC = -1.68; tolerance LFC = 1.52), both of which followed a similar pattern of expression to *JOX1*, suggesting it may work in tandem with its closely related genes; these genes are all 'crossover' genes, as noted above, suggesting that the nuanced mediation of phytohormone responses is important for managing drought tolerance and response strategies. Co-expression of JOX1 and its closely related genes may suggest that tolerant lines may possess a broader capacity for JA hydroxylation compared to more susceptible lines, reinforcing this JA attenuation.

Supporting the hub's putative role in repressing the JA response through the removal of bioactive JA, several connected tolerance DEGs were also annotated with jasmonate signalling-related GO terms. *TraesCS4B02G364800* (response LFC = -1.82; tolerance LFC = 1.72) showed substantial similarity (94.87% identity) to *bHLH6-like* in *Triticum uratu*, a basic helix-loop-helix transcription factor; *OsbHLH6*, its orthologue in rice, has previously been implicated in the positive mediation of jasmonate signalling (Chen et al., 2024b). *bHLH* TFs are tightly linked to jasmonate signalling due to their roles in promoting or repressing JA responses; the presence or absence of a canonical activation domain has been implicated in determining the exact role of bHLHs in relation to JA signalling. *MYC* bHLHs lacking the domain, known as *JAM* proteins, are unable to recruit RNA polymerase II to activate gene expression (Sasaki-Sekimoto *et al.*, 2014; Goossens, Mertens and Goossens, 2017). *bHLH* genes like *MYC* transcription factors often act as transcriptional activators under stress conditions, and higher expression of these would result in greater expression of JA-responsive genes. InterProScan domain analysis (Jones *et al.*, 2014) identified the presence of an *AIB/MYC*-like domain, further suggesting involvement of the TF in JA signalling as well as potentially ABA signalling (Ortigosa *et al.*, 2020), as well as an *ACT*-like domain commonly found on plant transcription factors. InterProScan was unable to predict presence of an activation domain – though this may be present in the N-terminal disordered region – indicating that *TabHLH6* may be a negative transcriptional regulator of JA signalling, unlike its orthologue. *TraesCS4B02G364900* (response LFC = -2.81; tolerance LFC = 2.18) was identified as the transcription factor *bHLH168*-like, an uncharacterised TF annotated with the GO terms 'defence response to fungus' and 'protein dimerization activity'. Another stress hormone-related TF was *TraesCS1A02G220000* (response LFC = -2.03; tolerance LFC = 1.19), which was identified as *ERF039-like*, an ethylene-responsive transcription factor likely involved in regulating cell growth. The ethylene and jasmonic acid hormonal pathways have a considerable amount of crosstalk (Yang *et al.*, 2019; Ma *et al.*, 2020), with some ERFs being induced by JA (Cai *et al.*, 2014; Ye *et al.*, 2020). *ERF039-like*'s increased expression in tolerant lines may suggest that it could be involved in mediating growth under stressful conditions, further enabling the tolerant phenotype, though members of the ERF superfamily have previously been characterised as both positive (Zhang et al., 2024c) and negative regulators (Xie et al., 2019b) of growth under stress. Together, the presence of both positive and negative regulators of JA-responsive transcription here supports a model in which JOX1 is involved in the fine-tuning of JA responses, rather than broad scale suppression or promotion under drought.

Members of the sesquiterpene biosynthesis pathway, which produces bitter terpenes involved in preventing herbivory (Tomlin *et al.*, 2000), were also connected to the hub. *TraesCS2B02G445800* (tolerance LFC = 1.81) was identified as acyclic sesquiterpene synthase-like*,* while *TraesCS5D02G024200* (response LFC = -3.12; tolerance LFC = 1.47) and *TraesCS5A02G016700* (tolerance LFC = 3.11) were identified as beta-carophyllene synthases. Although the expression of these genes decreased in response to drought, likely due to the depreciation of non-essential pathways, they were expressed more highly in tolerant lines; terpenoids have previously been implicated in weakly conferring drought tolerance in other species (Zhang et al., 2025a; Munné-Bosch et al., 2001) and are known to be influenced by jasmonates (Xu et al., 2016; Taniguchi et al., 2014). Their higher expression in tolerant lines here may therefore reflect moderated JA signalling, as opposed to fully repressed JA signalling, consistent with a model in which JOX1 contributes to the coordination of JA attenuation.

I hypothesise that the darkgreen module's hub gene, *JOX1*, potentially attenuates jasmonic acid signalling through the conversion of JA to 12OH-JA, reducing the expression of other JA-responsive genes, and limiting JA-mediated growth inhibition while still enabling the JA-dependent drought response. Under drought conditions, the hub's expression was substantially transcriptionally downregulated, perhaps relieving some of JA-responsive gene repression. The higher expression in tolerant accessions may potentially act to moderate the levels of free bioactive JA in the cell, potentially attenuating the expression of JA-responsive genes under drought conditions to reduce JA-associated growth penalties; this is supported by the expression patterns of its connected paralogues, transcription factors, and stress-response genes. Under drought conditions, *JOX1*'s higher expression in tolerant lines may contribute to a greater attenuation of JA responses when compared to the susceptible lines, while its overall transcriptional downregulation could still enable JA-responsive gene expression; I posit that this hub may promote a stronger growth phenotype under drought stress conditions through reducing JA sensitivity.

3.6.3. WRKY Hub Gene Validation

I hypothesised that the WRKY hub was a positive regulator of drought tolerance, with a role in the drought response, though I was unable to confirm this using TILLING lines carrying mutations in the gene. Validation was attempted using *Cadenza1792, a* TILLING line containing a stop-gain nonsense mutation that should prevent the production of functional transcripts. Both WRKY19^{WT} (BC_1S_1 plants without the mutant allele; out-segregants) and WRKY19^{MUT} (BC_1S_1 plants containing the stop-gain point mutation) plants exhibited substantially higher average normalised dry weight compared to their Cadenza background, in part likely due to the significantly higher raw dry weight of Cadenza under control conditions, though WRKY19^{WT} and WRKY19^{MUT} plants were not significantly different.

Homozygous mutants for the nonsense mutation exhibited slightly higher average nDW than both Cadenza and the wild-type plants with the same mutation background – though this was not significant – tentatively suggesting that *WRKY19* may not function in the expected way based on its expression profile and the roles of its co-expressed genes. If *WRKY19* was a positive regulator of drought tolerance, its knockout might have been expected to have a negative impact on drought tolerance – instead here we see that knocking out *WRKY19* may instead slightly enhance drought tolerance.

Our observed trends come with substantial caveats: EMS mutagenesis introduces many mutations in addition to the mutation of interest, which could give rise to beneficial or deleterious effects on biomass independently of changes to *WRKY19*; homoeologous compensation and changes to genome dosage of the *WRKY19* homoeologues could mitigate the negative impacts of the stop-gain mutation to buffer the loss of function (He et al., 2022a), obscuring the effect of a single knockout; group sizes were different due to unequal segregation of WRKY19^{MUT} and WRKY19^{WT} plants – despite the self-pollination generation most likely generating progeny where half carried the mutant allele and the other half did not, only 3 confirmed mutants were present among the WRKY19^{MUT} plants under control conditions, compared to 8 confirmed wild-type plants, which may have led to a higher average nDW among mutant plants if those control individuals were biased towards high or low biomass.

As a result, the prediction of *WRKY19*'s positive function in drought tolerance could not be confirmed, and as a result further experiments – ideally with further knockouts of its homoeologues and larger sample sizes – should be carried out to determine the role of *WRKY19* in conferring drought tolerance.

3.7. Conclusions

The YoGI panel continues to be an invaluable resource for the investigation of transcriptional regulation of how wheat seedlings respond to drought, providing potential genes for breeders to develop more drought tolerant wheat varieties. This analysis has identified several hundred genes with differing expression between phenotypically drought-susceptible and drought-tolerant plants, with a substantial number of these also being implicated in the wider response to drought stress.

Through WGCNA I have identified several hub genes with strong co-expression to other genes and posit that they may act as transcriptional master regulators of drought tolerance through negative mediation of the jasmonic acid signalling pathways, acting to reduce the amount of free JA available to induce transcription of JA-responsive genes, reducing JA-hypersensitive responses, and to potentially promote chronic responses to drought through weakly bioactive jasmonates. The candidate hubs and their co-expressed genes may elucidate some of the relationship between jasmonate signalling, the drought response, and drought tolerance: JA mobilisation is clearly an important part of the drought response, but tolerance under drought appears to involve mechanisms that fine-tune JA signalling to balance stress responses and growth penalties. This may also point to a dual role of JA under drought, in which JA activation contributes to the immediate drought response, but moderated activity is more associated with longer-term drought tolerance. Here, both the JOX1 and WRKY19 hub genes point towards a model in which tolerant plants have controlled, rather than unfettered, jasmonate activity under drought. I was unable to confirm *WRKY19*'s function as a positive drought regulator.

These findings may be of interest to wheat breeders, as the candidate genes highlighted could be used for marker-assisted selection for crop improvement strategies and contribute to ongoing efforts to improve the resilience of global wheat crops in the face of climate change. By further understanding the role of JA signalling in the drought stress response and the genes that control it, selecting genotypes for breeding to obtain a more optimal balance in JA signalling to prevent JA hypersensitivity-induced growth suppression could help to develop more drought tolerant varieties. Promoting plant productivity under water deficit conditions will only become more vital as we progress through the 21st century.

3.8. Author Contributions

LB, IJR and AH conceived and planned the project (see **Chapter 2**). IJR repeated network construction to ensure validity, conducted transcriptomic and network analysis, and created all figures. IJR carried out validation experiments and analysis using TILLING lines. IR wrote the chapter in its entirety, though some methods are adapted from those in **Chapter 2**.

3.9. Acknowledgements

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4. Dissecting the drought regulome: epigenomic mechanisms of the early drought response in bread wheat

4.1. Abstract

Introduction: Half the global population relies on bread wheat (*Triticum aestivum*) for a major part of their diets; increasingly harsh climates threaten food security, as periods of drought occur more frequently during seedling stages of staple crops like wheat. Wheat seedlings experience global transcriptional reprogramming under drought, but DNA methylation's role in this response is poorly understood. Understanding wheat's drought methylome may enable us to develop more drought-tolerant varieties, futureproofing our food.

Methods: WGBS was conducted on pools of genomic DNA from wheat landraces before and after a drought event, and paired with transcriptome data from the same tissue. DSS with smoothing was used to compare global methylation, with DMRs analysed against genome annotations and gene expression.

Results: DNA methylation and gene expression appeared highly correlated, with methylation positively correlated with expression but negatively with stress responsiveness. 120,000+ DMRs were observed across all C contexts. DMRs overlapping with gene bodies revealed differential methylation targeting stress-associated genes. DMRs mostly mapped to intergenic regions and transposable elements. Some TE families were enriched in DMRs in all C contexts, and also showed substantial sequence similarity to drought-responsive genes. ROS1a likely plays a substantial demethylation role in wheat's drought response.

Discussion: Wheat's drought methylome primarily undergoes CG hypomethylation and non-CG hypermethylation. Some genes were drought responsive across both the transcriptome and methylome, though most differentially methylated genes exhibited stable expression, suggesting that the methylome may be a subtle regulator of expression or act as a priming mechanism. *RLX_famc9*, an LTR retrotransposon, was enriched among DMRs and displayed significant homology to many drought-responsive genes. *RLX_famc9* may play a role in generating regulatory siRNAs in control conditions, which are repressed under drought conditions via hypermethylation to allow for the expression of downstream genes.

4.2. Introduction

Climate change, a growing population, resource scarcity, and increasing aridity of arable land threaten global agriculture's ability to meet rising nutritional demand. Crops are continually exposed to a variety of stresses in the environment: mitigation is paramount for obtaining sufficient yield and ensuring food security. Environmental challenges like intense heat, salinity, and drought trigger dynamic molecular changes in plants as they adapt to survive and reproduce. Environmental cues prompt plants to undergo transcriptional reprogramming steered by regulatory genes and various epigenetic mechanisms (Himanen and Sistonen, 2019; Sun et al., 2022a; Talarico et al., 2024; Rajkumar et al., 2020). Epigenetic changes – like DNA methylation – are known to cause shifts in gene expression (Gupta and Salgotra, 2022; Wang and Yamaguchi, 2024), though the extent and effects of these shifts remains a subject of debate. Malleable plant epigenomes may give rise to greater phenotypic plasticity, enabling plants to adopt more beneficial phenotypes under stress and ultimately attain greater survival and yield in adverse conditions (Dar et al., 2022; Liu and He, 2020).

Bread wheat (*Triticum aestivum*), as previously described, is a staple grain crop relied upon by billions globally as a major source of calories, protein, and fibre (Erenstein et al., 2022; Shiferaw et al., 2013); further crop development to ensure its yields among increasingly harsh climates is fundamental to achieving and maintaining food security across the world. Among the most critical threats to wheat production is climate-change-driven drought, with water deficits shown found to reduce grain and grain protein yields by almost half (Qaseem, Qureshi and Shaheen, 2019; Wan et al., 2022a). Early drought stress continues to be an area of increasingly intense research, with a number of studies in recent years exploring the impacts of early drought on wheat and stressing the importance of identifying tolerant varieties (Nardino et al., 2022; Sharma et al., 2022; Guo et al., 2017; Sallam et al., 2018; Barratt et al., 2023b), but the higher regulation of wheat's drought response remains underexplored. Periods of water shortage are becoming increasingly common throughout the year (Chen *et al.*, 2025), overlapping with the early stages of spring wheat growth: following current trends, this will continue to intensify as climate change progresses, and so understanding the mechanisms underlying plant responses to drought at the genomic level will enable us to better exploit their potential and bolster their survival.

DNA methylation is one mechanism known to play a role in the regulation of abiotic stress responses; in plants this is mediated by the RNA-directed DNA methylation (RdDM) and demethylation pathways through interactions between small RNAs, protein complexes, and DNA methyltransferases or glycosylases. Cytosine-based DNA methylation can occur at three cytosine contexts: CG, CHG, or CHH (where H represents any base other than G). DNA methylation likely has different impacts on transcription depending on its localisation; methylation markers in the gene body may be positively associated with expression (Muyle *et al.*, 2022), while methylation at the promoter regions and beyond is typically associated with gene and transposable element silencing (Zhang, Lang and Zhu, 2018).

Investigation into wheat's methylome and its role in stress responses is limited, with current research largely relying on techniques like methylation sensitive amplification polymorphism (MSAP) to profile the methylome and identify changes at broad regional levels (Duan *et al.*, 2020; Naderi *et al.*, 2024), targeting specific genes (Li et al., 2023c), or using lower-resolution approaches like meDIP-Seq (Wang et al., 2016a). Genome-wide approaches have been used in studying wheat's biotic stress response (Singh et al., 2022a; Xu et al., 2022), but whole-genome bisulphite sequencing (WGBS) or long-read sequencing remains rare in wheat, in part due to the extensive cost and time of sequencing and analysis for the large genomes of complex polyploids. These approaches have been used to investigate the drought response methylome of crops like rice (Ding *et al.*, 2022) and maize (Wang et al., 2021b), identifying correlations with gene expression and differentially methylated genes that may be involved in the drought response, but drought in wheat remains an underexplored frontier.

Despite making up a large proportion of plant genomes and being associated with a greater proportion of differential methylation, the regulatory potential of transposable elements (TEs) remains underexplored. Most TEs in plant genomes are highly and stably methylated, but some have been identified as being involved in stress responses through *cis*- or *trans*-regulation: the *Helitron* TE family *ATREP2* is likely integral for induced resistance to herbivory in *Arabidopsis thaliana* (Wilkinson *et al.*, 2023), while non-coding RNAs derived from TEs have been identified as important for abiotic stresses in Moso bamboo (Ding *et al.*, 2024) and maize (Lv *et al.*, 2019), suggesting that they may be a significant target for DNA methylation studies.

This analysis follows on from the exploration of wheat's drought-response and drought-tolerance transcriptome in **Chapters 2** and **3**, in which many questions remained about the regulation of drought strategies in wheat landraces. The analysis in these chapters identified massive transcriptional reprogramming in response to drought, and small but substantial differences in landraces with different phenotypic drought responses, and enabled the generation of hypotheses regarding gene clusters, but is fundamentally unable to identify mechanisms that regulate the hub genes themselves. The DNA methylome could identify key high-level regulatory processes that coordinate key drought response genes: drought-responsive methylation at upstream regulatory regions, like promoters, or exapted transposable elements, could drive expression changes in the key hub genes previously identified. Conducting an integrated analysis of the transcriptome and the methylome could disentangle homoeologous redundancy, help identify the regulatory basis of changing gene expression, and identify potential transcriptional impacts of methylation-controlled non-coding regulatory elements in the wheat genome.

The present work represents a novel integration of transcriptomics and whole genome bisulphite sequencing data to explore the thus-far understudied role of epigenetic regulation in wheat's drought response, with a focus on changes at genes and transposable elements. By examining changes in methylation dynamics across the genome and identifying correlations with transcriptional shifts, I aimed to elucidate this regulatory layer of abiotic stress responses and identify potential avenues for crop improvement under challenging environmental conditions.

4.3. Materials and Methods

4.3.1. Plant growth

The details of the plant growth conditions have reported previously in Barratt & Reynolds et. al (2023), though only half of the landraces (7 lines) from that study were selected for further investigation in this study. YoGI landrace lines deemed to exhibit a drought tolerant phenotype (YoGI_007, YoGI_021, YoGI_026, YoGI_047, YoGI_059, YoGI_145, and YoGI_261), defined as maintaining >70% biomass by weight compared to their control group, were selected from the wider panel of 20 as they were hypothesised to more likely exhibit beneficial drought-response mechanisms than susceptible lines. Four biological replicates were grown per line.

In summary, plants were sown in an 80:20 Levington's F2/ sand mix treated with CaLypso insecticide (Bayer CropScience Ltd., 0.083ml in 100ml water, applied per litre of compost), grown in long-day conditions (16/8h) and commenced drought conditions upon reaching the growth stage GS13 (Zadok's growth scale; (Zadoks, Chang and Konzak, 1974)). Drought treatment consisted of withholding water for 10 days, followed by 3 days of recovery with regular watering. Plants were sampled for RNA and DNA analyses at onset of drought (day 0) and end of drought (day 10). Dry biomass of above-ground shoots from droughted samples was compared to the above-ground biomass from control samples. Soil moisture content was recorded using an ML3 Thetaprobe Soil Moisture Sensor with an HH2 Moisture Meter (Delta-T Devices, Cambridge, United Kingdom) at onset and end of treatment, as well as at the end of recovery.

4.3.2. TILLING Mutant acquisition, backcrossing, and drought screening

TILLING mutants were used to investigate the role of TaROS1a DNA demethylation enzymes in wheat's drought response. TILLING mutant lines for *TaROS1a* homoeologues were obtained from SeedStor (https://seedstor.ac.uk/). Identification of individual lines with mutations in the genes of interest (*ROS1a-5A*: *TraesCS5A02G169000*; *ROS1a-5B: TraesCS5B02G165800*; *ROS1a-5D: TraesCS5D02G173300*) was carried out using the EnsemblPlants variant tables (https://plants.ensembl.org/Triticum_aestivum) available for each gene. These genes have previously been identified as members of the *TaROS1a* family (Gahlaut *et al.*, 2022) but have also been referred to as *DME* orthologues. Variants with a Cadenza background were selected based on the presence of a predicted nonsense mutation (gain of stop codon) or a missense mutation predicted to disrupt protein function (SIFT = 0). If multiple nonsense mutant lines were identified, the one with the mutation earlier in the sequence was selected to reduce the chance of partially functional but truncated transcripts. Nonsense mutations were selected preferentially to missense mutations.

Cadenza0002, Cadenza1580, and *Cadenza1622* were selected due to the presence of nonsense mutations for *ROS1a-5A, -5B*, and *-5D* respectively. These lines were originally generated using EMS mutagenesis in the mid-2010s (Krasileva *et al.*, 2017) but current seed stocks may be a result of several generations of self-pollination. TILLING mutants were confirmed using polymerase chain reaction (PCR)-based genotyping (**Supplemental Data S4.1**) and sequencing. Polyploids often contain multiple homoeologues of a given gene across its genomes; homoeologue-specific primers are needed to amplify only the region of interest on the gene of interest. Primers were designed using Primer3 (Untergasser *et al.*, 2012) and screened for GC content, appropriate melting temperature, and the likelihood of self-dimerisation or hairpin formation. Primers were also tested for specificity against the homoeologues using NCBI's primer-BLAST tool: non-homoeologue specific primers were noted and the nucleotide at the site of interest was checked to ensure that homoeologues could be differentiated when examining the sequencing trace at the genotyping stage. Primers were reconstituted as per the manufacturer's instructions, and a working aliquot was made at 10 µM concentration.

PCR was carried out on DNA extracted from leaf tissue (harvested, immersed in liquid nitrogen, and stored at -80°c) using an Edwards DNA Extraction buffer. GoTaq Green Master Mix (MM) (Promega, WI, USA) was used for PCR amplification. Each PCR reaction contained 10 µL GoTaq MM, 1 µL of DNA, 1 µL Forward Primer, 1 µL Reverse Primer, and 7 µL H_2O to a total volume of 20 µL. PCRs were carried out using the same cycle quantity and length, though primers may differ by annealing temperature due to differing GC proportions (see **Supplemental Data S4.1**). PCR products were purified prior to sequencing using the Wizard SV Gel and PCR Cleanup Kit (Promega, WI, USA) as per the manufacturer's protocols. 2.5 µL of purified PCR product was added to 5 µL of nuclease-free H_2O with 2.5 µL of Forward Primer to a total volume of 10 µL and was sent for sequencing using Eurofins' LightRun Tube Sanger Sequencing service. Presence of mutation at the desired site was determined and plants were designated ROS1a-5X^{WT} (out-segregants; no point mutation) or ROS1a-5X^{MUT} (point mutation present).

Confirmed mutants were backcrossed onto wild-type Cadenza plants. Mutant lines were used as pollen donors. Backcrossing was carried out to reduce the mutation load of the progeny while retaining the mutation of interest. Experimental plants were backcrossed once, and the progeny was self-pollinated to obtain homozygous mutants for each line. BC_1S_1 (once backcrossed, once selfed) plants were used for drought screening. Plants were genotyped at each stage to ensure that the lines maintained the mutation of interest as described previously.

BC_1S_1 and WT Cadenza plants were grown and screened for drought phenotypes as previously described. Cadenza was used to identify a baseline WT drought phenotype prior to EMS mutagenesis and backcrossing. Mutant BC_1S_1 lines were compared to their WT-allele siblings and WT Cadenza using normalised dry weight (nDW) as a measure of drought tolerance across the different experimental lines. nDW was calculated as

$nDW = \frac{DW_{drought}}{DW_{control}}$, where $DW_{drought}$ refers to an individual plant's dry weight under drought conditions and $DW_{control}$ represents the mean dry weight for that line under control conditions. nDW is expressed as a proportion.

Statistical testing was carried out compare traits between the ROS1a-5X^{WT}, ROS1a-5X^{MUT}, and WT Cadenza lines. Data were assessed for homogeneity of variance and normality of residuals using the shapiro_test() and levene_test() functions from the rstatix (ver. 0.7.2) (Kassambara, 2023) package in R. If assumptions were fulfilled, a two-way Analysis of Variance (ANOVA) test was used in R followed by the TukeyHSD() function; if assumptions were violated, the non-parametric Aligned Rank Transformed ANOVA (ver. 0.11.2) (ART-ANOVA) from the ARTool (Kay *et al.*, 2025) package was used, followed by post-hoc Tukey testing via the ARTool art.con() function.

4.3.3. Nucleic acid extraction and sequencing

Following plant growth experiments and tissue collection, nucleic acids were extracted and sequenced for transcriptome and methylome analysis. The details of the RNA extraction protocols have been reported previously in (Barratt et al., 2023b). In summary, RNA was extracted from ~100 mg of individual leaf tissue before and after drought and sequenced on the Illumina Novaseq 6000 platform (Illumina, CA, USA).

Genomic DNA was extracted using the Wizard HMW DNA Extraction Kit (Promega, WI, USA) according to the manufacturer's protocol, including an RNAse treatment. Genomic DNA concentration was quantified using a Qubit dsDNA Broad Range Assay Kit and Qubit 4 Fluorometer (Life Technologies, CA, USA), with DNA quality and integrity quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo-Fisher Scientific, MA, USA) and an Agilent Technology Tapestation (Agilent Technologies, CA, USA); samples were only included where their DNA integrity score (DIN) was above 7. DNA samples were pooled together in equimolar proportions into two pools, before drought (BD) and after drought (AD). Samples were then shipped on dry ice to Novogene (Cambridge, United Kingdom) for further QC, bisulphite treatment, library preparation, and sequencing on the Illumina Novaseq 6000 platform (150 bp paired end strategy) at their China facility.

4.3.4. Genomic data processing, mapping, and QC

Post-sequencing quality control was carried out to assess and remove low-quality reads. FastQC (version 0.11.9; www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used for both RNA and genomic DNA reads. Raw reads were filtered using Trim Galore (Krueger *et al.*, 2023) (version 0.6.10) by trimming low quality sequences (average Phred score < 20), trimming short length reads (<20bp) and clipping Illumina adapters. Low Phred-scoring reads were removed due to the higher likelihood of inaccurate base calling, which could affect downstream mapping efficiency and lead to inaccurate methylation calls. For DNA, 8 bp from the 5' end of each read was trimmed to avoid sequencing/ methylation biases. FastQC was also run post trimming to verify adapter and poor-quality sequence removal and estimate final GC content of reads. GC% is a useful metric, especially in this DNA methylation sequencing context, which can allow for the identification of PCR amplification sequencing bias, library preparation issues, or coverage issues.

Further RNA processing was previously detailed in (Barratt et al., 2023b); in summary, reads were aligned using Salmon (Patro *et al.*, 2017) to the IWGSC *Triticum aestivum* v1.1 transcriptome reference.

Genomic DNA sequence processing, including alignment and methylation calling, was carried out using Bismark (Krueger and Andrews, 2011; Langmead and Salzberg, 2012). The genome assembly was pre-processed using the *bismark_genome_preparation* script at default parameters. Paired-end Bismark alignment (bowtie2) was run at mostly default settings (*parameters: '--score_min L,0,-0.4'*) with slightly relaxed penalties to account for differences between the landraces and Chinese Spring. Reads were mapped to the IWGSC *Triticum aestivum* v1.0 assembly (GCA Accession: GCA_900519105.1); the IWGSC *Triticum aestivum* v1.1 annotation was used for annotating features. Trimmed sequences were aligned using end-to-end alignment mode. Following alignment, the Bismark script *deduplicate_bismark* was used for deduplication of reads, after which *bismark2bedGraph* (*parameters: '--CX', '--ample_memory'*) was used for methylation extraction for all three methylation contexts for both samples.

Coverage and sequencing depth of genomic reads was assessed using PanDepth (Yu *et al.*, 2024) following alignment. Coverage and depth were calculated to ensure that downstream analysis was accurate and robust, identifying regions that were effectively sequenced, and giving statistical testing adequate power from which to draw conclusions, and to allow for the identification and removal of low-depth sites.

4.3.5. BS-Seq, initial visualisation, and differential methylation analysis

Following DNA read alignment, Bismark was used to generate Bismark Coverage format files for use in downstream analysis, which contained methylation percentage, unmethylated reads, and total reads for each sequenced site per chromosome in each sample.

Methylation extraction for all four contexts was run using default parameters apart from '*--ample_memory*' and '*--cutoff 3*'. A cutoff of 3 was used to filter out very-low coverage sites.

Methylation data across gene bodies, TE bodies, and their flanking regions was conducted to identify methylation trends across different genomic elements and explore the association between gene body methylation and gene expression. This was processed using the *MethOverRegion* function from the ViewBS package (Huang *et al.*, 2018), and then refined and visualised using R. Custom scripts were used to visualise and analyse methylation traces across gene bodies per percentile of gene expression and absolute $\log_2$Fold Change.

Differential methylation analysis (DMA) was performed using the R package DSS (Feng and Wu, 2019). DSS input was filtered to remove loci that with coverage less than 3 in one or both samples. The DMA was carried out using the DMLtest() function with smoothing at default parameters. As nearby cytosine methylation sites are assumed to be spatially correlated (Huh et al., 2019; Feng and Wu, 2019), smoothing was used to improve DML and DMR detection.

DMLs and DMLRs obtained using DSS were filtered using thresholds for each sequence context. For all comparisons, a p-value threshold of 0.05 was used. For CG and CHG methylation, DMLs were considered significant with absolute Δ methylation ≥ 20%, while the threshold for CHH DMLs was absolute Δ methylation ≥ 10%. Different thresholds were used due to the different frequency of each type of site and average methylation of each context across the genome; CHH sites are more numerous than CHG or CG sites but are methylated at significantly reduced levels. Thresholds for DMR filtering were identical, though DSS's *areaStat* statistic was used for ad-hoc DMR-ranking instead of p-values as DSS does not provide p values for DMRs. AreaStat is a function of test statistics incorporating width (number of DMLs in the region) and height (Δ methylation). Very C-poor DMRs (less than 5% DMLs) were removed from downstream analysis. Genes overlapping with DMRs by at least 10bp within the gene body or 1.5 kb flanking intergenic regions were defined as differentially methylated genes (DMGs). Transposable elements that overlapped with DMRs by at least 10bp were defined as differentially methylated transposable elements (DMTEs). Overlapping elements were identified using genomation (Akalin *et al.*, 2015) and valr (Riemondy *et al.*, 2017) in R.

Genomic features, DEG, and DMR results were visualised as a Circos plot using Circlize (Gu et al., 2014) in R. Centromeric regions (Zhao et al., 2023) were expanded by 1.5x in each direction to make their position clearer. TE, Gene, DEG, and DMR density was calculated per 20 Mb bins using valr (Riemondy et al., 2017) and then Z-scored per chromosome.

4.3.6. Transcriptome analysis

Transcriptome analysis was conducted on the paired RNA samples to probe trends in gene expression and transcriptome-methylome associations. The differential expression analysis protocol was previously detailed in (Barratt et al., 2023b); count data from Salmon was analysed using DESeq2 (Love, Huber and Anders, 2014) after basic filtering of data. Differential expression analysis was carried out using BD samples as the reference level, and resultant $\log_2$Fold Change values were shrunk using the R package Ashr (Stephens, 2017). Thresholds of absolute $\log_2$Fold Change ≥ 1 and FDR-adjusted p-value ≤ 0.05 were used to screen for differentially expressed genes (DEGs).

4.3.7. Gene ontology and transcription factor binding motif enrichment analysis

Following identification of DEGs and DMGs, Gene Ontology enrichment analysis (GO) was conducted to identify gene functions overrepresented in each dataset. GO enrichment was carried out using clusterProfiler (v 4.12.0) (Wu *et al.*, 2021) against the relevant background for each data set (DEGs against all genes remaining post filtering; DMGs against all wheat genes with GO terms and coverage). GO terms with an FDR-adjusted *p* value ≤ 0.05 by hypergeometric testing were considered significantly enriched.

In addition to GO enrichment analysis, enrichment of other gene regulatory elements was probed. Transcription Factor Binding Motif enrichment analysis was conducted using the findMotifsGenome.pl script from HOMER2 (Heinz *et al.*, 2010). BED files for promoter and downstream DMRs, as well as the genome reference, were supplied as input. Parameters included '*-modelBg'* and '*-mset' plants*. For the -size argument, the average length of the flanking DMRs was given for each context (CG: '*-size -278,278'*; CHG: '*-size -262,262'*; CHH: '*-size -171,171'*) to include motifs both nearby and overlapped by the DMRs; methylation levels surrounding TF binding sites is thought to be associated with TF binding affinity (Morgan, DeMeo and Glass, 2024; Rimoldi *et al.*, 2024).

4.3.8. Transposable enrichment and homology analysis

After identifying enrichment of drought-responsive genes and gene-proximal regulatory regions, I next queried whether methylation at more distal transposable elements were changing in response to drought, and if they could too be involved in stress gene regulation. Enrichment analysis of DMRs at transposable element families was carried out using the TEENA pipeline (Li et al., 2024e), using all DMRs obtained per context from the differential methylation analysis. All DMRs were used to identify TE families selected for any sort of differential methylation activity, as TE families were unlikely to be differentially methylated in one direction only. The TE annotation was summarised to the family level, grouping together all subfamilies due to their high homology. TE families were considered significantly enriched if they had an adjusted p-value ≤ 0.05 and a fold enrichment ≥ 2.

TEs can have *trans*-regulatory roles through the generation of small RNA transcripts, so I queried whether these TE families showed sequence similarity to gene and promoter sequences. BLAST+ (Camacho *et al.*, 2009) (v2.14.1) was then used to identify TE families with high homology to the 1.5 kb promoter regions and gene bodies of DMGs and DEGs. Genomic sequences of the TEs were also queried against a random gene set of equal size as a control. Each gene set was converted into two BLAST *nucl* database (one for promoter regions, one for gene bodies) using the function *makeblastdb* (parameters: '-parse_seqids', '-dbtype nucl'). TE sequences were obtained from the Wheat RefSeq v1.1 annotation using BEDTools (Quinlan and Hall, 2010) and queried against the individual gene set databases using BLAST+ blastn (parameters '*-word_size 8', '-evalue 0.0005')* for both gene bodies and promoter regions. Results were filtered to remove alignments below 19 nt and above 320 nt, as well as removing genes/ promoter regions that overlapped with the queried transposable element family. Alignments were visualised using histograms in ggplot2 alongside the number of unique genes per histogram bin. TEs that showed significantly more alignment to the target genes than the random genes were identified using the function *ks.test*() to carry out a Kolmogorov-Smirnov test in R.

Orthologous TEs were found in the TREP database to the TEs of interest from the IWGSC V1.1 reference annotation using BLAST+. RNA-seq reads were then mapped to the TRansposable Elements Platform (TREP) Complete nucleotide sequence dataset (Schlagenhauf and Wicker, 2019) with gene CDS models supplied as decoys for alignment to ensure that reads preferentially mapped to TEs rather than genes. The TREP database contains unique and consensus transposable element sequences from across the *Triticeae*. Salmon was run with default parameters. Quantified expression was processed as previously described for gene expression data and analysed using DESeq2 (Love, Huber and Anders, 2014).

Furthermore, exploring potentially transcriptionally active regions of the TE sequences required identifying the positional alignment of transcript reads. A multiple sequence alignment of RNA reads and a representative *RLX_famc9* reference sequence was carried out using MAFFT (Katoh and Standley, 2013) with the parameters '*mafft --reorder --keeplength --anysymbol --maxambiguous 0.05 --kimura 1 --addfragments fragments --auto*'. The *RLX_famc9* sequence was selected through all-vs-all comparison of the *RLX_famc9* family sequences using BLAST. After self-hit removal, bitscore was calculated for each sequence. The final sequence was selected by identifying the sequence closest to the mean sequence length within the 95th or above percentile. The final sequence used is available in **Supplemental Data S4.6**.

4.3.9. Confocal microscopy and high pressure spraying of dsRNAs

Following in-silico hypothesis generation, preliminary validation of this proposed TE-derived siRNA mechanism was attempted by synthesising and applying a T1 dsRNA transcript to wheat plants using a high-pressure 'spray-induced gene silencing' (SIGS) method, thus referred to as HPS-dsRNA treatment.

HPS-dsRNA treatment was carried out using a SAC0607S Low Noise Air Compressor (6l) and AB932 Air Brush Kit without Propellant from Sealey (Suffolk, UK). Leaves were lightly pressed into a tray of 400 mesh silicon carbide (Sigma Aldrich, MA, USA) to induce abrasions and increase leaf permeability. Leaves were not rubbed or dragged through the abrasive. Treatments were applied using between 6-8 bar (87-116 PSI) air pressure. Treatments were loaded into modified 2 ml Eppendorf tube with a drilled hole in the lid to allow for the Air Brush tube. Treatments were sprayed on the abaxial side of the leaf blade from 3 cm away; where possible, treatment was delivered along the entire leaf.

The ability of the HPS-dsRNA method to introduce exogenous molecules into the cytoplasm was assessed using fluorescent fluorescein isothiocyanate-dextran (FITC-Dextran; 10,000 MW) from Sigma Aldrich suspended in deionised water at a concentration of 12.5 mg/ mL. Leaves treated with the HPS-delivered FITC-dextran and silicon carbide were imaged using Confocal Microscopy with a LSM980 MP AiryScan 2 (Carl Zeiss, Germany) and analysed in Fiji (Schindelin *et al.*, 2012). Chloroplast autofluorescence was visualised in red to differentiate from FITC-dextran fluorescence in green. Guard cells were primarily observed due to their closeness to the leaf surface and therefore clear signal. FITC-dextran introduction was deemed successful if cells were live (with visible cytoplasmic streaming), fluorescing with FITC-dextran signal, and without significant lesions or damage.

Desired RLX_famc9 transcripts were generated from RNA sequenced in this study. YoGI_145 (BD) was identified as a sample with high expression of RLX_famc9-associated transcripts and was used for cDNA synthesis. The Improm II Reverse Transcriptase Kit from Promega (WI, USA) was used for to generate cDNA from the RNA sample. Primer pairs targeting the RLX_famc9 T1 sequence were designed (**Supplemental Data S4.1**): each forward and reverse primer had a second copy that contained a T7 promoter at the start. Template DNA of the T1 sequence was then synthesised using these primers in two separate PCR reactions (one using the standard forward primer and the reverse primer+T7 promoter, and a second using the forward primer+T7 promoter and the standard reverse primer) to increase the dsRNA yield when carrying out in-vitro transcription. dsRNA template DNA was purified using the Wizard SV Gel and PCR Clean-Up System from Promega (WI, USA). Amplification of template strands was confirmed using a NanoDrop and gel electrophoresis.

The T7 RiboMAX Express Large Scale RNA Production System (Promega, WI, USA) was then used to synthesise RNA from the template DNA. Synthesised RNA was treated with DNase as per the manufacturer's protocol to remove remaining DNA. RNA obtained from both sets of primers was annealed post-transcription by incubating at 75°c for 5 minutes and left to anneal as the samples returned to room temperature, obtaining a greater quantity of dsRNA. dsRNA quantity and purity were assessed using the NanoDrop as previously described.

The HPS-dsRNA treatment method was further assessed using RT-qPCR. I selected a well-used stably expressed housekeeping gene (β-actin) as a knockdown target for HPS-dsRNA treatment and used another (β-tubulin) as the reference gene for relative quantification of expression. 24 plants were used across 4 timepoints (0, 6, 24, and 48 hours); there were 3 biological replicates per timepoint per treatment group. At 10 days post germination the newest leaf of each individual plant was pressed into silicon carbide (as previously described). Plants in the treatment group were sprayed with 600μL β-actin-derived dsRNAs dissolved in nuclease-free water (35 ng/ μL), while plants in the control group were sprayed with an equivalent volume of nuclease-free water, both from a 3 cm distance.

Tissue samples were collected at each timepoint; plants were only sampled once to reduce any effect of wounding on gene expression. RNA was extracted from leaf tissue samples as previously described, quantified using a NanoDrop, and equimolar proportions of RNA per sample were taken forward for cDNA synthesis as previously described. iTaq Universal SYBR Green Supermix (Catalog #1725121) (Bio-Rad Laboratories, CA, US) was used as the qPCR master mix to a final reaction mix of 20µL. Samples were run in triplicate across two plates on an Applied Biosystems QuantStudio 7 Pro (Life Technologies, CA, US) using a Fast Mode qPCR with Melt Curve profile at default settings (20 seconds at 95°c, followed by 40 cycles of denaturing for 1 second @ 95°c and annealing for 35 s @ 60°c, with fluorescence detection at the end of each cycle). The melt curve was run to check for the amplification of a single desired product. Gene expression was determined using relative quantification and the ΔΔCt method, using *β-tubulin* as the endogenous control, and analysed in R.

The effect of HPS-dsRNA application on plants was assessed. 16 plants were treated 10 days post germination; 8 plants were treated with 600µL of RLX_famc9 T1-derived dsRNAs (35 ng/µL) while 8 were treated with 600µL of nuclease free water. Of each treatment, 4 were droughted as previously described, while 4 were kept under a well-watered regime. 1 recovery day was given between the HPS-dsRNA application and drought treatment to allow for the plant to recover from HPS-mediated damage and allow for dsRNA to be processed by cellular machinery. Plants entered recovery 10 days post drought treatment and returned to a well-watered regime. Above-ground biomass was collected after 2 days of recovery and was dried and analysed as previously described.

Primers used for dsRNA template amplification, in-vitro transcription, and qPCR are available in **Supplemental Data S4.5**.

4.3.10. Data availability

The datasets used in this study can be found in online repositories. Raw and processed data is publicly available at NCBI (RNA-seq transcriptome data, GSE225797; WGBS methylome data, GSE311296).

The Cadenza TILLING lines (*Cadenza0002*, *Cadenza1622*, and *Cadenza1580*) are available from the Germplasm Resources Unit (GRU) (Norwich, United Kingdom; www.seedstor.ac.uk).

4.4. Results

4.4.1. Phenotype

Phenotype data involving the plants used in this study was previously reported (Barratt et al., 2023b); drought treatment was found significantly negatively impact both fresh (*p* = 1.01e-09) and dry weight (*p* = 7.75e-06) when comparing the treatment and control groups (one-way ANOVA). Accessions with high mean biomass retention (>70%) in the drought group compared to their controls were used for WGBS. Samples were taken from plants at treatment onset (BD; Zadok's GS13) and 10 days post treatment (AD).

4.4.2. Transcriptional reprogramming in spring wheat seedlings under drought

This present study used 328.1 Gb of raw data generated using the Illumina paired-end Novaseq 6000 platform as per *Barratt & Reynolds et. al 2023*. 7.264 x 10^8 raw reads were generated across 14 samples used with an average of 97.3% and 92.6% bases having a Phred score of ≥20 and ≥30 respectively, alongside an error probability of 0.03. Mean GC content of these reads ranged from 53-57%. Raw reads were assessed using FastQC and then pre-processed using Trim-Galore and quantified using Salmon and were considered suitable for further analysis.

Counts from the 14 samples (7 per group) were transformed using the vst() function of DESeq2 for exploration using principal component analysis (**Figure 4.1A**), in which PC1 (64% variance) clearly distinguished between samples taken before (BD) and after drought (AD).

Differential expression analysis was carried out to identify genes that responded to drought at the seedling stage, identifying 13,932 DEGs. 58% were transcriptionally upregulated after drought. GO terms related to the stress response were enriched in transcriptionally upregulated genes (**Figure 4.1B**) with 'response to water deprivation' (FDR-adjusted *p* value = 1.77e-66) the most significantly enriched term, accompanied by 'leaf senescence' and 'stomatal movement'. GO terms associated with processes like 'photosynthesis, light reaction' (*p* = 2.69e-56) and 'water transport' (*p* = 2.16e-08) were enriched in transcriptionally downregulated genes. Complete lists of all enriched GO terms for both transcriptionally upregulated and downregulated gene sets are available in **Supplemental Data S4.2**.

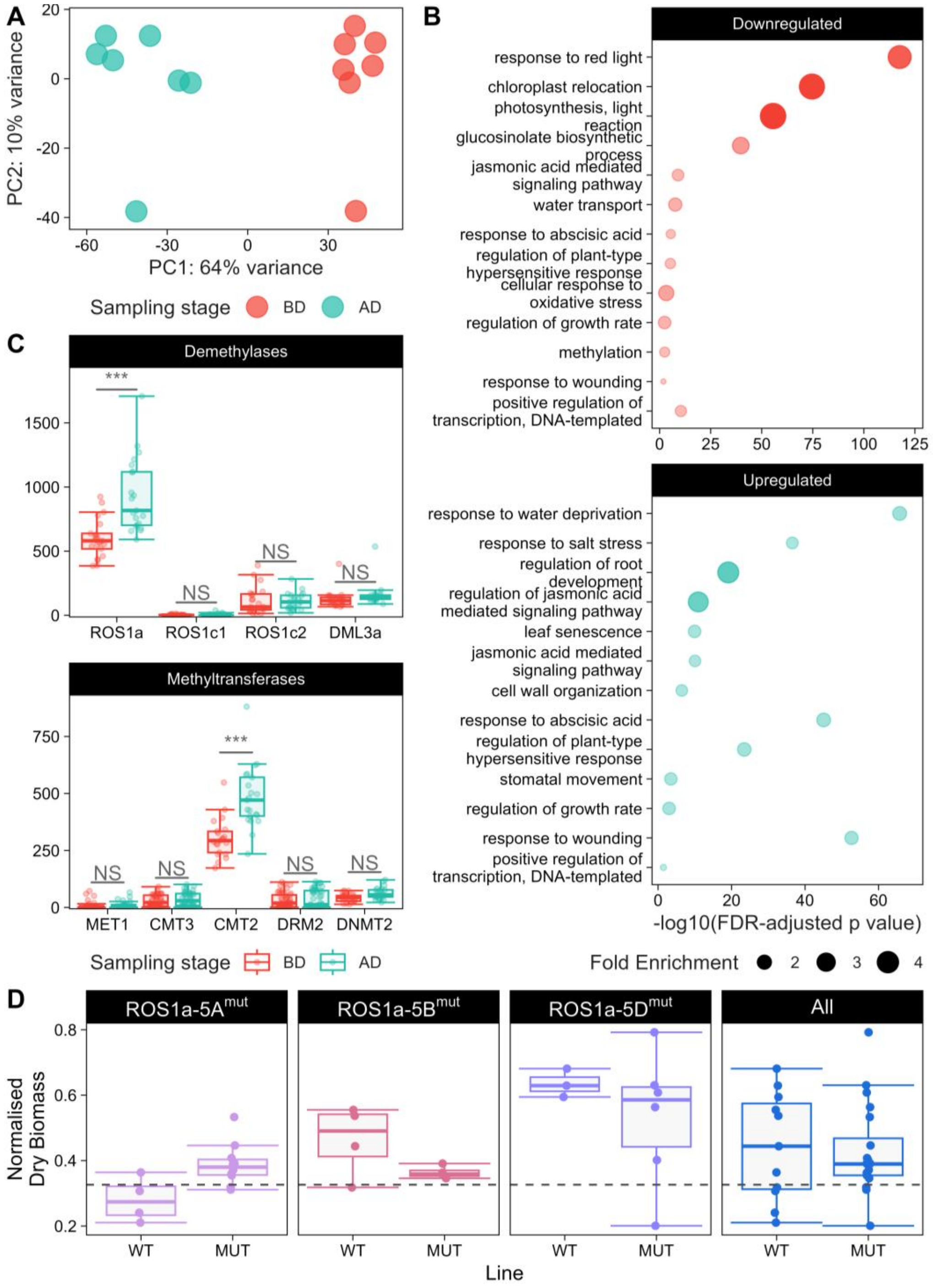

Figure 4.1: **Drought treatment resulted in global transcriptional reprogramming, though only some gene families involved in methylation signalling were affected. ROS1a TILLING mutant lines appear to have diverging effects on drought tolerance**. **(A)** PCA of global gene expression patterns identified that PC1 was the main driver in the dataset and strongly distinguished between BD (before drought) and AD (after drought) samples. **(B)** GO enrichment was performed on both upregulated and downregulated gene sets using ClusterProfiler. A selection of stress-associated GO terms is provided. Complete GO enrichment results for differentially expressed genes can be found in **Supplemental Data S3. (C)** Normalised gene counts of major wheat methyltransferase and DNA glycosylase families were compared before and after drought. *** represents $p < 0.001$; NS represents no significant difference. **(D)** ROS1a TILLING lines were used to examine the role of ROS1a-mediated demethylation in determining drought tolerance. WT and MUT refer to wild-type and mutant BC_1S_1 lines. Dashed line represents the average nDW of Cadenza under the same conditions for comparison.

DNA methyltransferase and glycosylase gene family expression was compared at the family level before and after drought (**Figure 4.1C**). CMT2 family expression was significantly higher (p = 1.81e-05; *Wilcoxon ranked-sum test*) after drought treatment, while all other families showed slightly increased but non-significant changes. ROS1a expression was also significantly higher following drought (p = 2.99e-04; *Wilcoxon ranked-sum test*); though the three other major DNA glycosylase families fluctuated in expression, none were significantly different.

4.4.3. ROS1a knockouts may have homoeologues-specific effects on drought responses

Mutant lines were used to explore the role of ROS1a in the drought response (**Figure 4.1D**). Each line used contained a mutation within one of three ROS1a homoeologues, resulting in three knockout lines: ROS1a-5A^{mut}, ROS1a-5B^{mut}, and ROS1a-5D^{mut}. ROS1a^{mut} lines displayed contrasting trends when compared to both wild-type Cadenza and homozygous wild-type lines with the same mutation load. Lines were also analysed together to examine the broader effects of ROS1a knockouts.

Almost all lines exhibited higher mean nDW than Cadenza under drought conditions. Compared to wild-type plants, mutant lines trended towards a reduction in nDW, with the exception of *ROS1a-5A* mutants. In ROS1a-5A^{mut} plants, genotype (ROS1a-5A^{WT} / ROS1a-5A^{mut} / Cadenza) was identified as having a significant effect on normalised dry weight (ANOVA: p = 0.0139). A post-hoc Tukey test identified that while neither ROS1a-5A^{WT} nor ROS1a-5A^{mut} plants were significantly different to Cadenza, ROS1a-5A^{mut} displayed significantly higher nDW than ROS1a-5A^{WT} (+9.1%, p = 0.0129), suggesting that differences were small but significant.

Similarly, genotype had a significant effect on normalised dry weight among ROS1a-5B^{mut} plants (ANOVA: p = 0.0118); ROS1a-5B^{WT} nDW was significantly higher than Cadenza using a post-hoc Tukey test (p = 0.0124), while ROS1a-5B^{mut} was not significantly different (p = 0.661) to Cadenza or ROS1a-5B^{WT} (p = 0.0572). Though they were not significantly different, ROS1a-5B^{WT} plants displayed a 32.4% higher mean nDW compared to ROS1a-5B^{mut} plants and 49.7% higher than Cadenza. Similarly, genotype had a significant effect on nDW in ROS1a-5D^{mut} plants (ANOVA: p = 0.0442), with post-hoc Tukey testing identifying that while ROS1a-5D^{WT} plants were significantly different to Cadenza (p = 0.0462), ROS1a-5D^{mut} plants were not (p = 0.115), nor were they significantly different to ROS1a-5D^{WT} plants (p = 0.602). ROS1a-5D^{WT} plants displayed a mean 10.2% increase in nDW compared to ROS1a-5D^{mut} plants. Both 5D and 5B plants therefore exhibited a wide spread in differences to the other groups, but were non-significant, suggesting that they may not be responsible for a large change in nDW.

Examination of all lines together identified that there was a significant effect of genotype on nDW overall (Aligned Rank Transformed (ART) ANOVA: p = 0.000196), with the interaction term (*genotype x homoeologue*) also found to be significant (ART ANOVA: p = 0.000226). Post-hoc Tukey tests identified that Cadenza was significantly different to wild-type (p = 0.000650) and mutant plants (p = 0.000665) overall, though wild-type and mutant plants were not significantly different (p = 0.889). Wild-type plants across all three ROS1a knockouts exhibited slightly higher nDW (mean increase = 1.7%) compared to their mutant siblings, but this increase was small and suggests that ROS1a mutants may have broad or contradictory impacts on drought responses.

4.4.4. BS-Seq and differential methylation of the wheat genome under drought

Both the before drought treatment (BD) and after drought treatment (AD) samples were sequenced across 5 lanes. 799.8 GB of raw data was generated, consisting of ~5.3x10^9 reads; an average of 94.7% and 87.4% of bases had a q-value of >20 and >30 respectively, with an error probability of 0.03. GC content of reads ranged between 26.03% - 26.64%, which was expected due to the bisulphite treatment reducing unmethylated cytosine content.

Average unique mapping rates to the RefSeq IWGSC v1.1 assembly (available: International Wheat Genome Sequencing Consortium, http://wheatgenome.org) were 72.9% (BD) and 72.0% (AD), suggesting consistent and adequate mapping efficiency. Remaining reads were discarded due to alignment score or multi-mapping. Average coverage after alignment was 13.6X (BD) and 14.5X (AD), which was deemed high enough to make accurate methylation calls and support downstream analysis. In BD, the proportions of methylated cytosines for each sequence context (CG, CHG, and CHH) were 87.0%, 53.8%, and 1.6% respectively, and in AD were 87.1%, 54.8%, and 1.7% respectively, indicating global methylation was relatively stable. Methylation calls were removed from further analysis if that site had fewer than 3 reads.

The three C contexts (CG, CHG, or CHH) were distinct in average methylation level. Small but statistically significant differences were observed across CHG sites, with the context displaying the largest difference between the BD and AD samples (+1.2%, Tukey: p = 3.20e-07) using an ANOVA and Tukey post-hoc testing (**Figure 4.2A**). Little preference was observed among the methylation state of each C context in its various triplet base contexts; for example, in the CG context, average methylation at each triplet base (e.g CGT, CGG, CGC etc.) ranged between 86.3 - 90.1%. The lack of strong triplet base preference indicates low sequence and methyltransferase bias.

Gene bodies (from start to end of gene) displayed methylation depletion surrounding the transcription start or termination sites, a consistent increase towards the centre of the gene, and increased methylation further away from the gene body in the flanking regions (**Figure 4.2B**). For transposable elements, proximity to genes was a substantial factor in determining the methylation profile of transposable elements across all three contexts, with proximal and gene-overlapping TEs displaying substantially lower methylation levels in flanking regions compared to distal TEs.

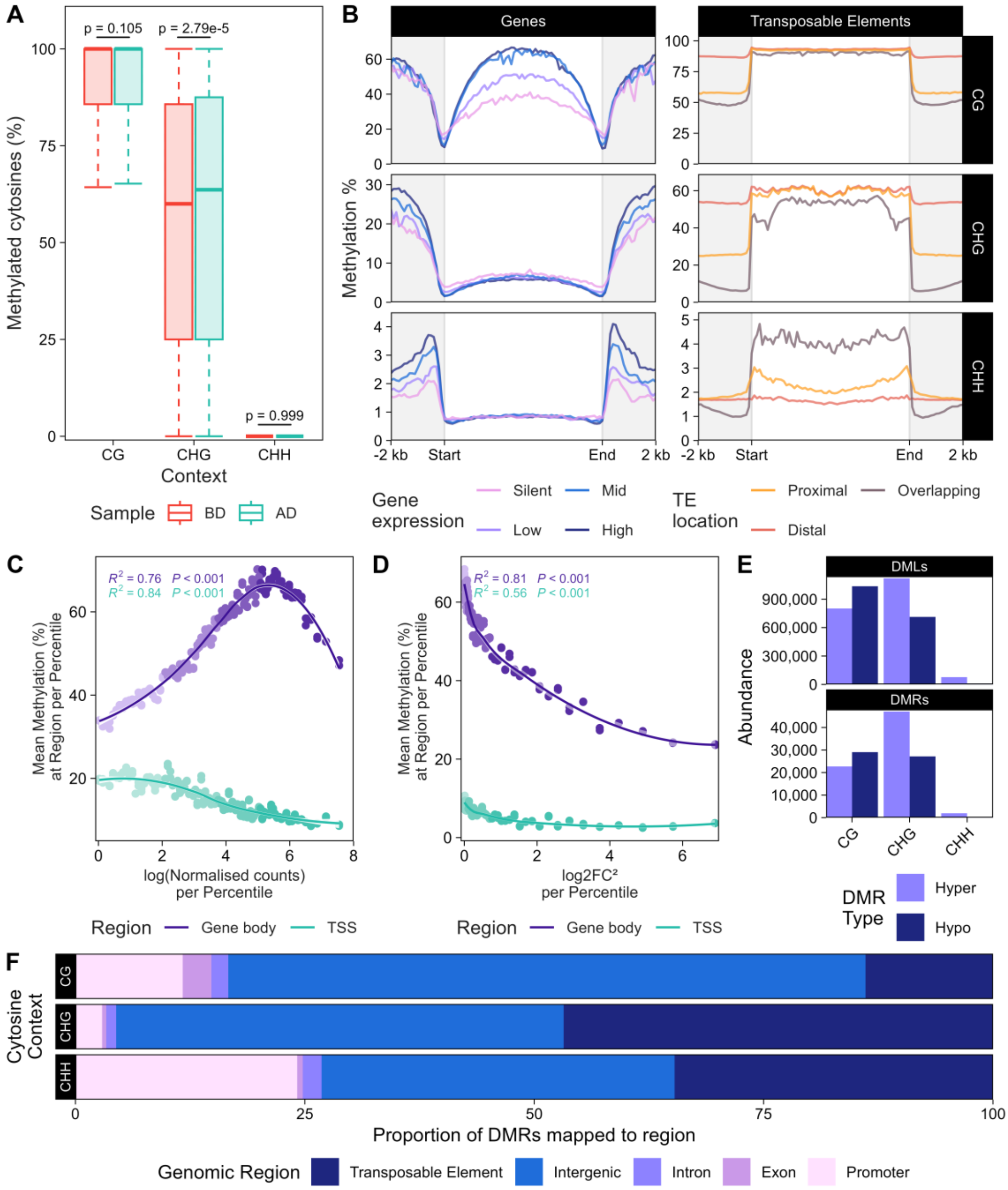

Figure 4.2: Drought treatment may cause small but context-specific changes to DNA methylation, while gene-body methylation appears to correlate with gene expression and variability under drought. (A) A sample of ~5% of methylation calls were sampled from each file and visualised using boxplots. Methylation (%) at each context ranged from 0-100%. **(B)** Mean methylation (%) across genes and transposable elements was calculated per 100 bins (20 bins per 2 kb flanking region, 60 bins per body). Genes were grouped into four expression quartiles and transposable elements were grouped by gene proximity ("overlapping genes"; <10 kb: "proximal"; >10 kb: "distal"). **(C)** Genes were grouped into percentiles by mean expression. Mean log(normalised counts) percentiles were plotted against maximum CG gene body methylation (%) (above) as well as average methylation at the TSS (below). **(D)** Genes were grouped into percentiles using absolute log$_2$FC (BD vs AD); the mean absolute log$_2$FC was plotted against the maximum mean CG gene body methylation (above) as well as average methylation at the TSS (below). **(E)** Differentially methylated loci (DMLs) and regions (DMRs) were summarised per context. **(F)** DMRs were mapped to genomic and genic features using the R package genomation as proportions of all identified DMRs.

Genes, grouped into percentiles by expression (**Figure 4.2C**) and absolute $\log_2$Fold Change **(Figure 4.2D)**, were found to be significantly associated with the peak of CG methylation in the gene body. Regression analysis identified significant correlations between the average expression of percentile groups and their maximum average gene body methylation level ($p < 0.001$; $R^2 = 0.76$), alongside the average methylation level at the bin containing the transcription start site (TSS) ($p < 0.001$; $R^2 = 0.84$). Gene expression variability under drought by percentile was also significantly correlated with DNA methylation: average absolute $\log_2$Fold Change of percentiles and their maximum average methylation level in the gene body ($p < 0.001$; $R^2 = 0.81$) and average methylation at the TSS ($p < 0.001$; $R^2 = 0.56$) both showed significant correlations. DNA methylation of the gene body was positively correlated with expression, while methylation at the TSS was negatively correlated. High methylation at the TSS and gene body was weakly and strongly negatively correlated respectively with $\log_2$Fold Change under drought. Only very small changes were observed between samples, though this is likely an artifact of the quantile approach with expression and binning across genes; this will have obfuscated changes in some gene-specific methylation profiles. Individual gene traces were inherently noisy for several reasons: bins contained both exonic and intronic regions, which are known to usually have different methylation traits (Feng et al., 2010), some genes will have exhibited low sequencing depth, and genes can greatly vary in length; aggregation of these traces was appropriate for identifying broad expression-grouped trends, but would have smoothed over any biologically-meaningful gene-specific methylation changes.

Differential methylation identified both differentially methylated loci (DMLs) and regions (DMRs) between BD and AD. Initial differential methylation results for all three methylation contexts are summarised in **Figure 4.2E.** CG DMRs (Δ Methylation threshold = 20%) displayed preferential hypomethylation (56.1% of 51,775 CG DMRs). Non-CG contexts yielded the most DMRs, with a stronger bias towards hypermethylation; 74,185 CHG DMRs were identified (Δ Methylation threshold = 20%), with 62.9% being hypermethylated. CHH (Δ Methylation threshold = 10%) yielded the fewest DMRs (2,281), with 85.5% of these being hypermethylated. Contexts displayed distinct characteristics; CHG and CHH DMRs were on average 29.0% and 35.9% shorter than CG DMRs, while CHH DMRs were substantially more C-rich, containing over twice as many cytosines on average. DMR counts were used to quantify the abundance of regions that differed in methylation profiles between the two samples in a context-specific manner and identify downstream targets for analysis. CHH DMRs were likely more C-rich due to their genomic localisation at repetitive sequences and transposable elements.

DMRs were mapped to genomic features (**Figure 4.2F**). Over 69%, 48%, and 38% of CG, CHG, and CHH DMRs respectively were localised to intergenic regions. Gene-associated CHG DMRs most frequently localised to the gene body, while CG and CHH DMRs were primarily localised to the gene-flanking promoter and downstream regions. Gene promoter regions were the dominant localisation of genic CHH DMRs (~24% overall), and the second-most overlapped feature for genic CG (~12%) and CHG (~3%) DMRs. Transposable elements were the largest annotated region type for all three contexts, indicating a high degree of differential methylation at non-genic but active loci.

Visualisation of Z-scored gene, TE, DEG, and DMR density per 20 Mb bin as a Circos plot (**Supplementary Figure 4.1**) identified expected trends like TE-rich regions displaying gene depletion, and vice-versa (second track), with linear regression identifying a significant negative association ($R^2 = 0.571$, $p = 1.09e\text{-}132$). Similarly, bins spanning the estimated chromosome centromere regions (outermost track) displayed below-average gene density and average or above-average TE density. DNA methylation (fourth track) also appeared to peak near the centromere in CG and CHG contexts across many chromosomes, consistent with heterochromatin. DMR density (innermost track), however, was significantly positively associated with gene density per bin ($R^2 = 0.672$, $p = 2.89e\text{-}174$) and negatively with TE density ($R^2 = 0.695$, $p = 1.38e\text{-}185$), indicating that high DMR density was associated with gene-richness across the 20 Mb windows, even if most DMRs did not span genes and were instead intergenic, as per **Figure 4.2**. This indicates that intergenic DMRs may preferentially arise in more gene-rich chromosomal regions, rather than TE-dense pericentromeric heterochromatic regions.

4.4.5. Genic DNA methylation may play an influential but secondary role in the drought stress response

9.78% DMRs overall were associated with promoter, exon, or intronic regions (**Figure 4.3A**). CG and CHG DMRs mapped in similar proportions to different genic regions, while CHH DMRs were dominant in promoter regions. Differentially methylated genes (DMGs) were defined as genes that overlapped with a DMR in the gene body or across its flanking regions. Mapping suggested that DMRs primarily occurred in non-genic regions.

GO enrichment was performed using DMGs against all wheat genes annotated with GO terms (**Figure 4.3B**). Stress-associated GO terms were enriched in hypomethylated CG gene body DMGs, including terms such as '*borate transport*' (GO:0046713) '*reactive oxygen species metabolic process*', '*plant-type hypersensitive response*' (GO:0009626), '*hormone-mediated signalling pathway*' (GO:0009755), '*protein kinase activity*' (GO:0004672), '*defense response*' (GO:0006952), '*response to salicylic acid*' (GO:0009751), and '*regulation of stomatal closure*' (GO:0090333). 104 GO terms were enriched in hypermethylated CG gene body DMGs, including '*positive regulation of brassinosteroid mediated signaling pathway*' (GO:1900459), '*calmodulin-dependent protein kinase activity*' (GO:0004683)', '*positive regulation of abscisic acid-activated signalling pathway*' (GO:0009789), '*response to salicylic acid*' (GO:0009751), '*response to cold*' (GO:0009409), and '*response to wounding*' (GO:0009611). Both were enriched in '*protein phosphorylation*' (GO:0006468) and '*signal transduction*' (GO:0007165). These

GO terms suggest that hypomethylated CG gene body DMRs may act to modulate part of the drought response.

Enriched GO terms were also obtained among CHG DMGs: hypermethylated gene body DMGs were enriched in the terms '*potassium ion transmembrane transport*' (GO:0071805) and '*abscisic acid-activated signalling pathway*' (GO:0009738), while hypomethylated gene body DMGs were enriched in similar terms to the CG hypomethylated DMGs, including terms such as '*signal transduction*' (GO:0007165, FDR = 0.00748) and '*plant-type hypersensitive response*' (GO:0009626, FDR = 0.00849). Similarly to the CG gene body DMRs, they may act to modulate the drought response, but in a less targeted way.

No enriched terms were observed among CHH DMGs with gene body-associated DMRs. No enriched GO terms were observed among DMGs with DMRs in the flanking regions for any cytosine context.

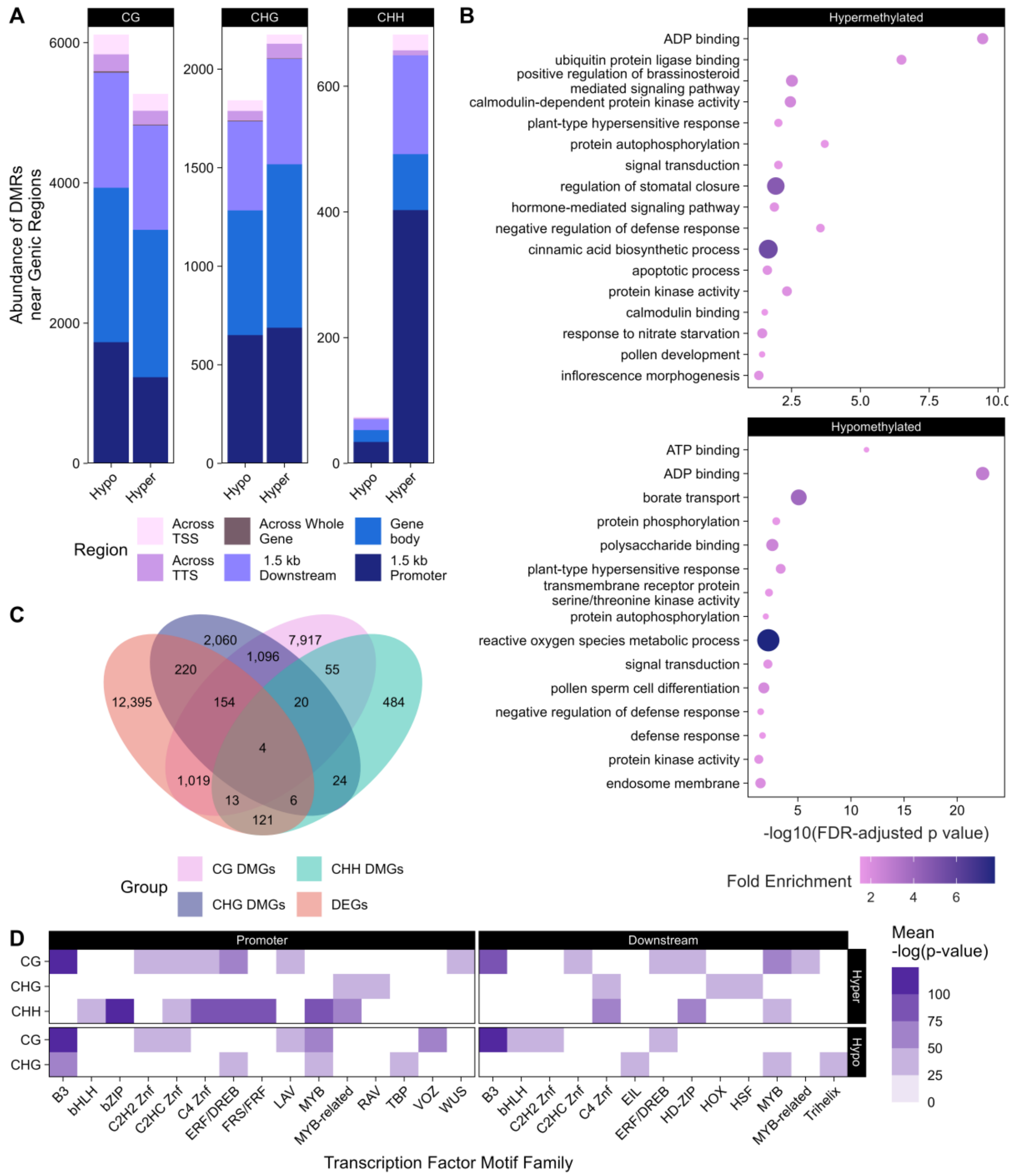

Figure 4.3: Gene bodies and gene-flanking regions appear to be significant targets of differential methylation under drought conditions. **(A)** DMRs mapped to genes and their flanking regions were broken down into locales based on their start and end coordinates relative to the gene annotation. **(B)** GO enrichment analysis was conducted on DMGs associated with gene body DMRs per type against a background of all genes with annotated GO terms using ClusterProfiler. Size and colour represent fold enrichment. **(C)** DEGs and DMGs for all contexts were compared using a Venn diagram. **(D)** Statistical motif enrichment of hypo- and hypermethylated DMR sequences in gene-flanking regions was carried out using HOMER. Results were summarised to the predicted family level. Darker cells indicate higher significance.

4.4.6. DEGs, DMGs, and DMRs are partially shared across contexts

There was substantial overlap between DMGs of each context and DEGs (**Figure 4.3C**). Four genes overlapped all groups: *TraesCS1D02G012000* ($\log_2$FC = 5.65, FDR = 2.11e-10), a defensin-like AMP protein, *TraesCS2A02G313000* ($\log_2$FC = -1.28, FDR = 1.1e-3), a cupin superfamily protein, *TraesCS5D02G317000* ($\log_2$FC = -1.26, FDR = 0.038), a CRT/DRE binding factor protein, and *TraesCS7A02G566200* ($\log_2$FC = 3.40, FDR = 4.1e-4), an ABC transporter G protein. DMG/ DEG overlaps displayed weak relationships between the expected effect of DMRs and $\log_2$Fold Change (e.g. promoter hypermethylation repressing expression, or gene body hypermethylation enhancing it). Only 49.5% CG DMGs associated with promoter or gene body DMRs displayed the expected change in expression, a relationship that remained weak with CHG (48.6%) and CHH (58.7%) DMRs, suggesting that methylation may not always impact gene expression in this way.

DMRs close (within 100bp) to other DMRs, including across contexts, were often positively correlated: of 10,108 CG-CHG proximal DMRs, 99.7% displayed similar methylation profiles, with 55.8% of these being hypomethylated. 88.48% of proximal CG-CHG DMRs were mapped to intergenic regions. Other comparisons yielded fewer proximal DMRs: only 57 CHG-CHH and 70 CG-CHH DMRs were observed, of which 79% and 9% respectively displayed similar methylation profiles, indicating that asymmetric and symmetric DMRs generally target different regions.

4.4.7. Gene-flanking DMRs are enriched in transcription factor binding motifs

Overrepresented transcription factor binding motifs (TFBMs) were identified within DMRs localised to the 1.5 kb flanking regions of genes for all three contexts. The 1.5 kb flanking regions were analysed and enriched motifs were summarised to the family level. 43 unique significantly enriched motif families (*p* value ≤ 1e-10 & average log enrichment ≥ 2) were identified across all three contexts (**Figure 4.3D**). Several TFBMs were associated with families commonly associated with stress responses, including members of the *MYB, MADS, bHLH, ERF/DREB, bZIP*, and different zinc-finger-containing families. The most significantly enriched motif family was the *MADS*-box transcription factors (mean p-value = *1e-28*), driven by presence of *VRN1* and *REM* motifs in gene-flanking DMRs. Some families were enriched in both upstream and downstream flanking regions as well as enriched in both hypo- and hypermethylated DMRs. Due to a low number of hypomethylated CHH DMRs in flanking regions, no significantly enriched families were identified. TFBMs are likely targeted by CG and CHG DMRs which are substantially targeted to genic regions.

4.4.8. Differential methylation in non-genic regions

DMRs were largely localised to non-genic regions, with a large proportion attributed to unannotated regions, but over 40% across all contexts mapped to transposable elements. 7,992 CG DMR-associated transposable elements (DMTEs) were identified, tending towards hypomethylation (54.6%). The RLG (Ty3-like, formerly known as Gypsy) superfamily was most abundant for CG among both methylation states, followed by RLC (Copia), and DTC (CACTA). Substantially more CHG DMRs (46.8%) were associated with transposable elements than CG DMRs (13.9%); of 47,014 CHG DMTEs, 67.8% were associated with hypermethylated DMRs. Though less frequent, 34.7% of CHH DMRs were TE-associated, and were predominantly (86.2%) associated with hypermethylation. In contrast to the other contexts, the most abundant CHH DMTE superfamilies were DTX (*unknown TIR*), XXX (unknown), DTT (*Mariner*), and DTH (*Harbinger*). TE abundance suggests that dominant TE families were shared across CG and CHG DMTEs, while CHH DMTEs were largely distinct with targeting for non-LTR transposons.

I mined the association between TEs and DMRs to identify 31 enriched TE families among DMRs of at least one context (**Figure 4.4A**). Where possible below, TE families are referred to by their *clariTeRep* identifier and legacy ID (in brackets).

Notable families enriched in more than one context included two members of the *LTR Copia* superfamily: *RLC_famc15* (*HORPIA*), which was enriched among CG (4.5x) and CHG (3.3x) DMRs, and *RLC_famc16*, which was also enriched among CG (7.1x) and CHG (2.7x) DMRs. Both were primarily hypomethylated. Enriched LTR Ty3-like families included *RLG_famc17*, which was enriched among CG (2.8x) and CHG (2.0x) DMRs, and *RLG_famc20*, which displayed enrichment with CG (4.4x) and CHG (2.5x) DMRs; both tended towards hypomethylation in CG but hypermethylation in CHG contexts. Of unknown LTR TEs, *RLX_famc9* was enriched among CG (4.3x), CHG (1.9x), and CHH (25.4x) contexts, while *RLX_famc3* was enriched in both CHG (1.7x) and CHH (4.4x) contexts. Both were predominantly hypermethylated in non-CG contexts, especially in CHH, while *RLX_famc9* was slightly more associated with hypomethylated CG DMRs. *RLX_famc9* was the only TE family to be enriched among all C contexts (**Figure 4.4A**).

RIX_famc7 (*Nadine*) and *RIX_famc9* (*Ramona*), both non-LTR transposons (LINEs), were enriched among CG (4.0x and 1.7x respectively) and CHG (4.1x and 2.1x) DMRs; similarly to the Ty3-likes, they displayed substantial overlaps with hypomethylated DMRs, though *RIX_famc7* was associated more strongly with hypermethylated CHG DMRs.

TE enrichment results suggested that some TEs were strongly associated with differential methylation before and after the drought period, identifying *RLX_famc9* as a prime candidate. Further enrichment results can be found in **Supplemental Data S4.4**.

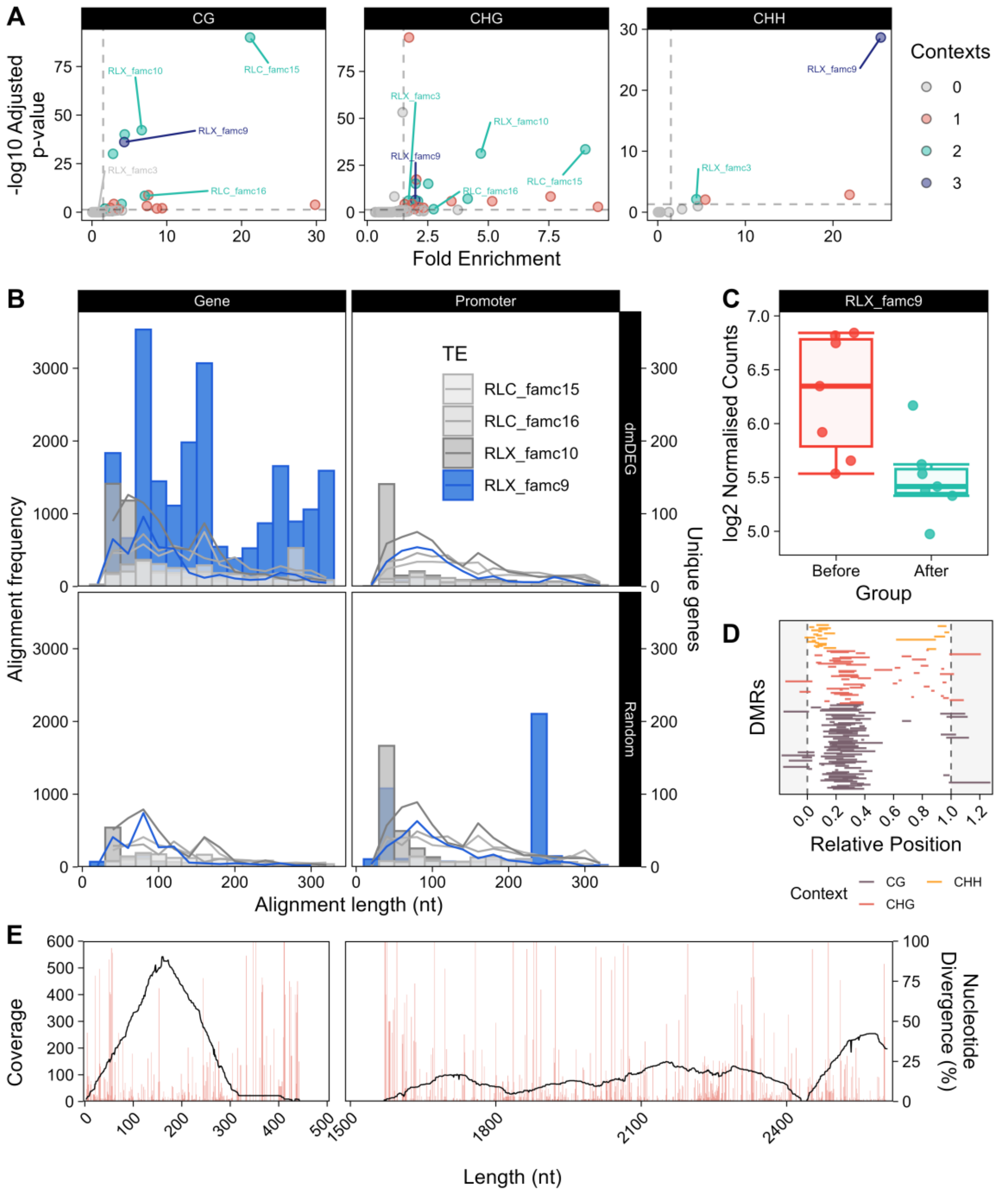

Figure 4.4: **Transposable element families enriched with DMRs were examined in detail, identifying families that could have *trans*-regulatory functions under drought conditions**. **(A)** DMR TE enrichment across all contexts showed several TE families to be enriched. Some TE families have been highlighted: families are coloured by their number of enriched DMR contexts (grey = 0, red = 1, green = 2, and blue = 3). **(B)** TE families enriched among DMRs were analysed for sequence similarity to the gene and promoter sequences of drought-associated genes (both methylome and transcriptome) and to random non-associated genes as a control. *RLX_famc9* was chosen due to its enrichment in all 3 C contexts, while *RLC_famc15*, *RLC_famc16*, and *RLX_famc10* were chosen due to their enrichment in 2 C contexts. Sequence similarity was plotted as a histogram of alignment lengths (left axis) and unique gene counts (right axis). **(C)** *RLX_famc9* transcript expression was compared before and after drought treatment. **(D)** Relative positions of DMRs overlapping with members of the *RLX_famc9* family were used to identify locations targeted for differential methylation. Relative positions were calculated for each DMR against the TE it was associated with. 0 represents the start of the TE; 1 represents the end. **(E)** Reads associated with *RLX_famc9* were mapped to a selected reference sequence; coverage (line chart, left axis) and percentage nucleotide divergence from the reference sequence (bar chart, right axis) were plotted against nucleotide position.

4.4.9. RLX_famc9 TEs show sequence similarity to differentially methylated and drought responsive genes

BLAST+ was used to identify sequence similarity between DMR-enriched TE families and 1,290 promoters/ gene bodies of genes that were both differentially methylated and differentially expressed after filtering. A random selection of 1,290 other genes and their promoters were used as a control set. Kolmogorov-Smirnov tests between the BLAST alignment distributions at each nucleotide length (**Figure 4.4B**) identified that the 3 most significant families for gene body alignments were *RLX_famc9* (FDR-adjusted *p = 1.06e-153*), *RLX_famc3* (*p = 4.85e-88*), and *RLC_famc16* (*p = 9.94e-56*), giving rise to the idea that they may be involved in their regulation in *trans*.

RLX_famc9, an unclassified LTR retrotransposon species, emerged as the most promising candidate from these significantly enriched TE families that also displayed significant homology to differentially methylated and expressed genes. It displayed significantly more homology to the target gene set than the random gene set and was enriched in DMRs across all three C contexts. It trended towards hypomethylation in the CG context, with 63.2% of DMRs overlapping with it displaying decreased methylation, while 63.1% of *RLX_famc9*-associated non-CG DMRs were hypermethylated. GO terms including '*chloroplast stroma'*, '*chloroplast thylakoid membrane'*, '*starch biosynthetic process'*, and '*response to absence of light'* were enriched in genes found to show gene body sequence similarity to *RLX_famc9,* suggesting that the TE family could act to influence photosynthesis under drought; 55% of these genes possessed hypomethylated CG DMRs under drought, indicating that they may be made more accessible to regulators.

Differential expression analysis of TE sequences identified 34 differentially expressed TEs. *RLX_famc9*-like transcripts were weakly but significantly transcriptionally downregulated ($\log_2$FC = -0.85, *FDR = 0.044*) (**Figure 4.4C**). DMRs mapping to *RLX_famc9* were concentrated within the first third of sequences across the family, with fewer mapping towards the end (**Figure 4.4D**); CHH DMRs predominantly mapped in the first 10% of each sequence. Relative position of the DMRs to the TEs were used to compare across the variety of family sequences. Alignments from all RNA samples to a representative *RLX_famc9* sequence were visualised to map transcriptionally active regions across the transposable element, identifying at least 2 transcriptionally detectable regions: a high coverage region, T1 (~0-400 nt) and a lower coverage region, T2 (~1600-2600 nt) (**Figure 4.4E**). Across all 1412 sites with associated reads, mean coverage was 128x, while mean divergence from the TE reference base was 7.94%, indicating high conservation of the transcripts.

TE-derived transcripts may play a role in wheat's drought response through cleavage into regulatory sRNAs. I queried the expression of endoribonuclease *Dicer* and *Argonaute*-encoding genes (**Supplemental Data S4.5**) that are involved in cleavage and post-transcriptional gene silencing. Of 26 *Dicer* family genes, 6 were DEGs; 3 (*DCL4-B, DCL4-D*, and *RTL3*-like) were transcriptionally upregulated after drought, while 3 (*DCL3a, DCL1-B*, and *DCL1-D*) were downregulated. Only 5/ 69 *Argonaute* family genes were deemed DEGs; 4 (*AGO2-A, AGO2-D, AGO18*, and *AGO4*) were transcriptionally upregulated after drought, while 1 (*AGO-PNH1*) was downregulated. The small proportion of genes identified as DEGs suggests that only some of the sRNA machinery was transcriptionally drought responsive.

4.4.10. HPS-dsRNA treatment with exogenous molecules and dsRNAs

The HPS-dsRNA treatment first needed to be provably able to introduce exogenous molecules to the plant leaf cells without inducing widespread senescence or causing significant lesions. This was tested using fluorescent 10 kDa FITC-dextran (12.5 mg/ mL) delivered to leaf cells at high pressure (8 bar), mediated by lesions created using 400-mesh silicon carbide (**Figure 4.5A**). FITC fluorescence can be observed clearly in the guard cell (central), with clear visualisation of the nucleus and visible cytoplasmic strands. Fluorescence in the lower half of the image is likely cells where the FITC-dextran penetrated vacuole space. Live imaging of the cells indicated that the treatment could deliver the FITC to cells without killing them due to the observation of continuous cytoplasmic streaming.

qPCR was used to determine the efficacy and effect of gene-derived dsRNA transcripts on gene expression. β-actin, a commonly used housekeeping gene, was targeted with dsRNAs (**Figure 4.5B**). After filtering, there were significant differences in relative quantification between timepoints (ANOVA*:* $p = 4.91e-07$) and treatments (ANOVA*:* $p = 7.45e-06$), as well as a significant effect of the interaction between the two (ANOVA*:* $p = 1.44e-05$). Post-hoc Tukey tests identified that while the 0 ($p = 0.998$) and 48-hour timepoints ($p = 0.987$) were not significantly different between the control and treatment group, 6 hours ($p = 0.0486$) and 24 hours ($p = 1.8e-06$) were. Relative quantification trended downwards over the course of the experiment until 24 hours, after which treated samples appeared to return to a similar expression level as untreated samples.

Treatment of YoGI_145 plants with RLX T1-derived dsRNAs (**Figure 4.5C**) was found to have a non-significant effect on normalised dry weight under drought conditions when compared to controls treated with water (ART ANOVA*:* $p = 0.695$), though T1-treated plants did display slightly reduced mean normalised dry weight (0.865) compared to plants treated with water (0.916), indicating that there may have been a small but non-significant effect of treatment.

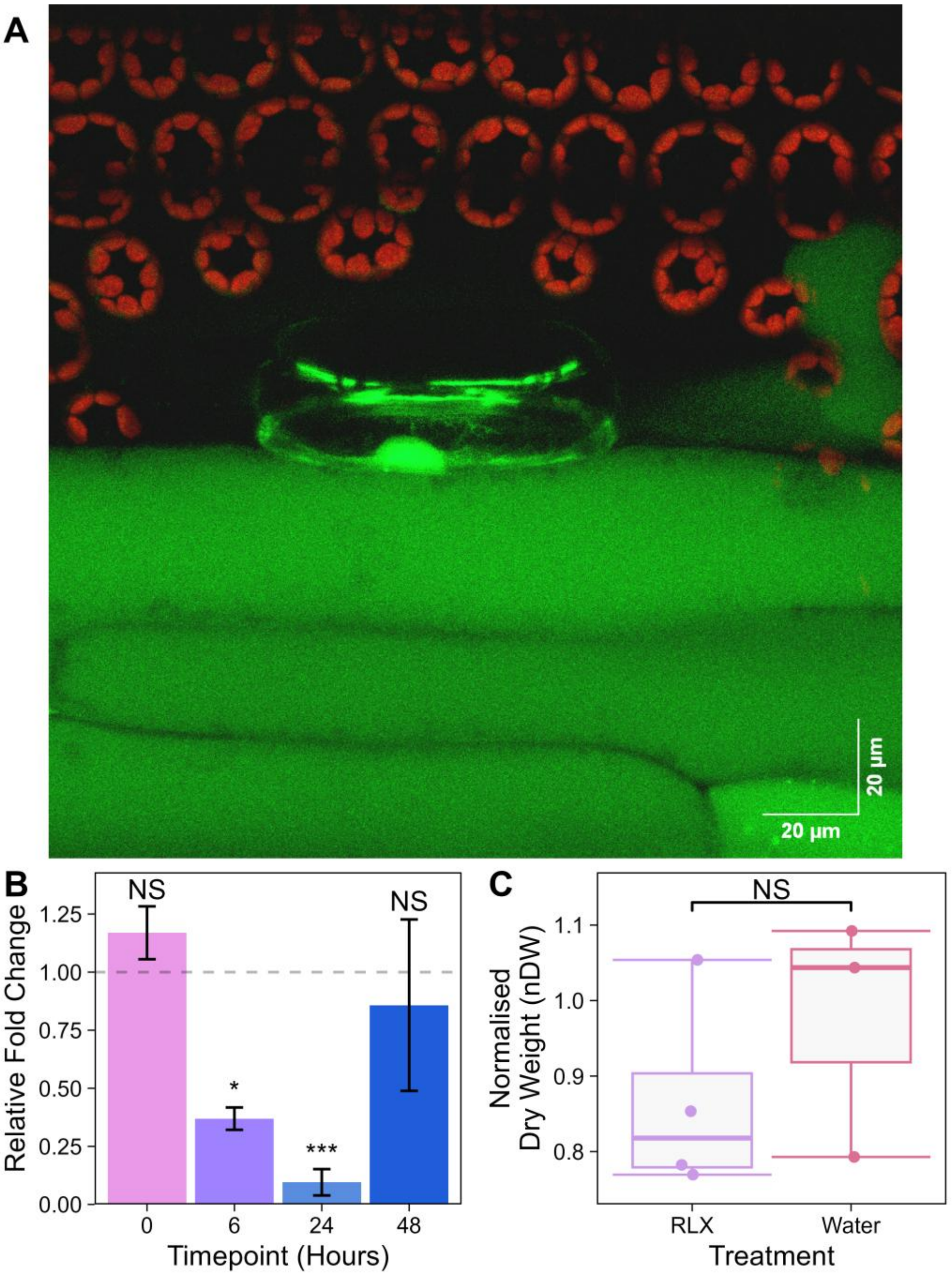

Figure 4.5: High-pressure sprayed dsRNAs may have a short-lived impact on gene expression and could promote drought tolerance. (A) Confocal microscopy was used to identify successful introduction of FITC-Dextran (10kDa; 12.5 mg/ mL) dissolved in nuclease-free water to cells by high-pressure spraying treatment. FITC dextran was used as a representative, stable molecule that could report successful introduction of exogenous molecules to the cytoplasm of leaf cells. FITC fluorescence can be observed in the cytoplasm of the guard cell (central). Below the guard cell FITC fluorescence can be observed in cell vacuoles and other organelles. Above the guard cell chlorophyll autofluorescence can be observed. **(B)** 24 Plants were sprayed at high pressure with *β-actin*-derived dsRNA to disrupt its expression through RNAi mechanisms, and samples were taken at 0, 6, 24, and 48 hours post treatment. There were 3 biological replicates (each with 3 technical replicates) per treatment per timepoint. Fold change represents qPCR-based relative fold change of *β-actin* gene expression in plants relative to plants treated with nuclease-free water, using *β-tubulin* as a reference gene. Asterisks represent significance compared to expression in control plants at that timepoint (* represents < 0.05; *** represents < 0.0001). Treatment was associated with a significant difference in *β-actin* expression at the 6-and 24-hour timepoints. **(C)** Normalised dry weight (above-ground biomass) of YoGI_145 plants treated with RLX_famc9 T1-derived dsRNAs, compared to plants treated with nuclease-free water. Each group contained 4 replicates.

4.5. Discussion

In wheat, the physiological and transcriptomic impacts of drought are well understood, but the methylome impacts are undercharacterised. DNA methylation likely influences gene expression under biotic stress conditions (Singh et al., 2022a; Hidvégi, Gulyás and Dobránszki, 2022), but its role in abiotic stress responses like drought has been overlooked. Pooling genomic DNA for analysis of the methylome has thus far been an underutilised technique despite its advantages in the analysis of complex crop genomes; pooling for WGBS has previously been done on both animal and plant samples (Gallego-Fabrega et al., 2015; Docherty et al., 2009; Vaughn et al., 2007; Eichten et al., 2011; Kaplow et al., 2015) but does not appear to have been used in genomes as large as that of *T. aestivum*. These results indicate that differential methylation analysis on pooled samples enables the identification of differences in methylation profiles before and after a stress event.

This effective and economical approach identified links between DNA methylation and gene expression shifts under drought, elucidating how the wheat drought response is regulated, and represents a step towards identifying epigenetic targets for crop improvement.

4.5.1. TaROS1a homoeologues may contribute differently to the wheat drought response

ROS1a emerged as the most drought-responsive demethylases, consistent with reports that demethylases like *ROS1* and DEMETER (*DME*) are positively associated with drought responses in various species (Silva et al., 2024; Kapazoglou et al., 2013). Substantial CG/CHG demethylation may suggest active demethylation plays an important role in wheat's drought response – something perhaps mediated by ROS1a, as suggested by the knockout TILLING lines for ROS1a-5A, - 5B, and -5D, potentially in a homoeologue-dependent way.

ROS1a-5A mutants appeared to exhibit significantly higher nDW than the wild-type allele-containing lines, while -5B and -5D mutants trended lower – though these comparisons were not significant – and all lines were more drought tolerant than WT Cadenza. The differences between these lines and Cadenza are likely due to the non-target mutation load present from the EMS, therefore I have primarily compared the ROS1a homozygous mutant lines to their homozygous wild-type out-segregants.

These contrasting results may suggest that the *ROS1a* homoeologues play distinct roles in mediating methylation under drought; I hypothesise that ROS1a-5A may preferentially demethylate regions involved in growth restraint under water deficit, with its knockout here relieving this repression, while the -5B and -5D homoeologues may demethylate loci mobilised under drought, resulting in reduced drought tolerance when disrupted. Overall impacts across all three were modest, with a 1.7% reduction in nDW, though this could be due to compensatory homoeologue behaviour in polyploids (Xiong, Gaeta and Pires, 2011), reducing the impact of detrimental mutations – though recent research has suggested that this assumption may not hold true in wheat for many genes (Dorussen *et al.*, 2025). Single homoeologue knockouts for high level processes like epigenetic modulation may also have limited impacts.

Several caveats underpin these results: firstly, the aforementioned redundancy of homoeologues may have allowed the functional copies of the other two homoeologues to compensate for the reduced abundance of demethylases, reducing the impact of possible changes; secondly, these lines all likely contain a nontrivial mutation load – additional rounds of backcrossing onto Cadenza would reduce the mutation load, maintain the mutation of interest, and potentially narrow the differences in inherent nDW between these lines and the Cadenza controls; thirdly, variable group sizes due to the uneven segregation of plants containing the mutant allele and those that did not will have resulted in reduced statistical power that could have confounded statistical testing.

Based on the results of this experiment with TILLING lines, I hypothesise that *ROS1a* may contribute to drought responses in a homoeologue-specific manner due to the differing results of the tested lines, with *ROS1a-5A* disruption appearing to increase nDW while disruption of its copies on the other sub-genomes may have resulted in reduced nDW. More advanced backcrosses, alongside double and triple knockouts of ROS1a homoeologues, are needed to clarify their role in wheat's drought response.

4.5.2. Drought prompts massive transcriptional reprogramming accompanied by subtle epigenetic reprogramming of genic regions

Wheat's transcriptomic response to drought has been substantially profiled at various growth stages (Barratt et al., 2023b; Niu et al., 2023; Kim, Kim and Lee, 2023); here we identified ~14,000 DEGs (~20% of expressed genes) between the BD and AD groups, indicating massive transcriptional reprogramming. By contrast, only ~9% of genes were associated with DMRs, with the majority of regions associated with TEs and intergenic regions, especially among non-CG contexts, a response also seen in the rice methylome under drought (Kou *et al.*, 2022). Given that methylation is just one of many regulatory mechanisms that may underpin stress responses this was not entirely unexpected. Under drought, hypermethylation was dominant in non-CG contexts; previous studies that have identified hypermethylation in wheat leaves following drought events (Duan et al., 2020; Kaur, Grewal and Sharma, 2018), though CG sites were also substantially hypomethylated, suggesting complex interplay or divergent roles between the cytosine contexts.

Average methylation levels at CG (*88.1-88.4%*), CHG (*55.1-56.2%*), and CHH sites (*1.64-1.75%*) were largely consistent with previous estimates in wheat (Singh et al., 2022a; Yuan et al., 2020), while methylation traces across genes and TEs were congruent with trends seen in other species (Li *et al.*, 2012, 2020; Weng *et al.*, 2023). I was able to affirm that in gene body CG methylation is significantly associated with gene expression, identifying that high methylation of the gene body is a strong predictor of high gene expression across genes. This is somewhat consistent with what has been observed in other species – methylation of the gene body is involved in maintaining high, stable expression (Wang et al., 2021e; Muyle et al., 2022). Similarly, high TSS methylation was a predictor of low gene expression, albeit with a weaker correlation than that of the gene body, which is congruent with analysis in mammals indicating that high TSS or first exon methylation is associated with reduced transcription (Brenet *et al.*, 2011).

Interestingly, genes with high $\log_2$FCs were associated with substantially lower methylation at the TSS/ gene body, suggesting reduced DNA methylation may enable greater access by other transcriptional regulators under stress. Prior studies have proposed that CG gene body methylation buffers transcriptional noise in plants (Zastąpiło et al., 2024; Horvath et al., 2019), and stress-responsive genes are thought to show significant levels of transcriptional noise in some organisms (Huh *et al.*, 2013; Cortijo *et al.*, 2019); I posit that low gene body methylation may be a characteristic of rapid stress response genes. DNA methylation's role in reducing transcriptional stochasticity could extend to cover transcriptional stress responsiveness, perhaps through methylation-mediated inhibition of the binding accessibility of target sequences to regulatory agents, or potentially through exclusion of histone H2A.Z (Takuno, Seymour and Gaut, 2017), ultimately shielding a gene's expression from environmental cues.

CG and non-CG DMRs displayed contrasting trends across the genome. Despite limited co-localisation, proximal DMRs across contexts were often positively correlated – especially CG and CHG DMRs – implying coordinated methylation control at those loci. CG and non-CG DMRs showed distinct methylation trends, primarily localised to different regions, and exhibited different traits, supporting a model in which each methylation context is governed by distinct mechanisms with distinct roles in controlling the genome

4.5.3. Genic regions undergo methylation changes in response to stress

Specific stress-associated functions were enriched in gene-body associated DMGs, suggesting that the RdDM and demethylation pathways may specifically target gene bodies under stress conditions. Enriched GO terms describing signal transduction, phosphorylation, hormone regulation were common across hypo- and hypermethylated gene sets, indicating methylation may be involved in modulating these high-level processes.

Hypermethylated DMGs included a substantial number of leucine-rich repeat domain-containing proteins involved in signalling and phytohormone responses. These were broadly grouped into nucleotide-binding site leucine-rich repeat (*NBS-LRR*) disease resistance proteins and leucine-rich repeat receptor-like serine/ threonine protein kinases (*LRR-RLKs*). These *NBS-LRR* genes included members of the *RGA* and *RPM* families; *NBS-LRRs* have previously been implicated in several stress-resistance pathways, including the gibberellic (GA), salicylic (SA), and abscisic acid (ABA) cascades (Chini et al., 2004; Li et al., 2024f; Wu et al., 2020), and are potentially involved in abiotic stress responses. Despite classically being thought as pathogen responders (Noman, Aqeel and Lou, 2019), their influence over stomatal movement may make them useful targets for mediating transpiration under water deficit. Among hypermethylated *NBS-LRR* genes, several *RGA5* copies were weakly transcriptionally upregulated; their weak but broad upregulation across the family suggests that RGA proteins may be a target of the methylation pathways under drought. Similarly, *LRR-RLKs* were variably transcriptionally upregulated, including members of the HPCA1 family; *HPCA1* genes code for plasma membrane *LRR-RLKs* implicated in the sensing of and response to increasing ROS levels within cells (Mishra et al., 2022; Liu et al., 2022a) and acclimatisation to stress conditions (Fichman *et al.*, 2022). *LRR-RLKs* are strongly associated with stress responses and growth mediation (Yan et al., 2023b) and are associated with stress-responsive methylation changes (Luo *et al.*, 2023).

Among transcriptionally upregulated hypermethylation-associated DMGs annotated with the enriched GO term ‘response to cold’, triplet homoeologues of AAA-ATPases were identified, suggesting targeting of sequence motifs by the RdDM pathways. AAA-ATPases have been found to play a role in salt and drought responses in maize by reducing ROS buildup (Xia *et al.*, 2013; Zhang *et al.*, 2014).

Gene-flanking DMRs were enriched in transcription factor binding motifs, suggesting that methylation may mediate gene expression by modifying transcription factor binding. Methylation at TF binding sites can influence the binding affinity of TFs (Yin *et al.*, 2017; Vatov, Zentgraf and Ludewig, 2022), while some TFs themselves are methylation-sensitive (Kribelbauer *et al.*, 2020; Kaluscha *et al.*, 2022; Rimoldi *et al.*, 2024); in some species TFBM methylation may be an important gene regulator, with methyl groups altering binding affinity by mediating steric hinderance, or by subtly altering the minor groove width of the DNA (Kribelbauer *et al.*, 2020). Despite its potential role in gene regulation, little work has been done to identify motifs targeted by methylation mechanisms.

Many stress-associated TFBMs were enriched among both hypo- and hypermethylated DMRs across the flanking regions – this may have been due motif variants within these large families being targeted by different arms of the RdDM pathway to tune TF activity, or due to motifs varying in cytosine content. Analysis of plant TFBMs in *A. thaliana* identified that up to >75% of TFs that were investigated were methylation-sensitive across all C contexts (O’Malley *et al.*, 2016), with the majority of these associated with binding inhibition. Several of the families identified as being methylation sensitive are reflected in this analysis: basic helix-loop-helix (bHLH) and basic leucine zipper (bZIP) motifs were found to be strongly enriched among CHH promoter DMRs, suggesting that there may be CHH-mediated repression of binding for these key stress-responsive TF families. MYB and ERF/DREB TF motifs, key in stress responses, were enriched among both hyper- and hypomethylated DMRs across all three contexts, suggesting that methylation may be used to fine-tune the expression of specific gene responses. MYB genes and their motifs are known to be regulated in part by methylation (Wang et al., 2020a), with methylation also playing a role in the recognition of target sequences by the TF; many ERF/DREB/ AP2-associated motifs have been found to be sensitive to inhibition from hypermethylation (O'Malley *et al.*, 2016), potentially due to the high GC content of the DREB/CRT (GCCGAC) and GCC motifs (AGCCGCC) (Wu et al., 2022b) associated with the superfamily.

Despite these notable genes, most gene body DMGs showed little change in expression, indicating that methylation may play a subtle role at the genic level. Combined with the hypothesis that gene body methylation is a promoter of high, stable, stress-insensitive expression, this implies that it may instead act to modulate the accessibility of other regulatory mechanisms to enhance or inhibit expression, rather than directly influencing expression itself. Sustained hypomethylation marks could perhaps prime genes to be more stress responsive (Catoni *et al.*, 2022), or gene body hypermethylation could shield genes from future environmental cues. I found little evidence of drought inducing targeted methylation changes in promoters at the biological process level, suggesting that promoter DMRs were not a major tool in wheat's drought response, and that genes regulated in this way would be better identified from a candidate-first approach.

4.5.4. Transposable element families appear targeted for methylation changes

DMRs were found to be significantly enriched in specific TE families. The role of TEs, or TE-derived transcripts, in wheat's drought response is understudied; *A. thaliana* is host to TE families that have been implicated in both *cis*- and *trans*-regulatory stress mechanisms (Ito et al., 2016; Xu, Thieme and Roulin, 2024; Wilkinson et al., 2023), but similar epigenetically controlled responses in grasses have been elusive.

Enriched families like *RLC_famc15, RIX_famc9*, and *RIX_famc7* are known to be overrepresented in promoter sequences (Wicker *et al.*, 2018); despite this enrichment, they were rarely accompanied by differences in the expression of proximal genes, suggesting that these TEs did not play a large *cis*-regulatory role. In wheat's incredibly TE-rich genome, almost all genes are likely to have nearby TEs, but despite the ubiquity of these TEs, previous studies in wheat have not identified strong associations between TE families and the *cis*-regulation of gene expression (Wicker *et al.*, 2018), suggesting that DMR-targeted TEs may instead act in *trans* to regulate other genes. It has been proposed that intron-localised TEs in *A. thaliana* and *Solanum lycopersicum* are associated with mostly enhanced gene expression of stress-associated genes (Deneweth, Van de Peer and Vermeirssen, 2022), suggesting that this could be an under-detected mechanism of cis-regulation by TEs in plants.

Intergenic TE-associated DMRs may play a role in genomic regulation, potentially through chromatin structure, or *trans*-acting small RNAs, rather than the cis-regulation of proximal gene expression. Although a substantial number of intergenic TEs overlapped with DMRs, suggesting that they may have potential *trans*-acting gene-regulatory or DNA structural roles, Circos visualisation (**Supplementary Figure 4.1**) indicated that DMR density was higher in gene-rich and TE-poor chromosome arm regions. This suggests that intergenic TE-associated DMRs may be involved in the regulation of potentially regulatory TEs families nearby gene-rich regions – though this does not necessarily mean they are *cis*-regulatory – rather than involved in the regulation of gene-distal or pericentromeric TEs. TE-associated intergenic DMRs are likely involved in regulating activity at the gene-rich chromosome arms, rather than in broad chromatin restructuring. Some intergenic TE-associated DMRs in gene-poor regions however may be involved in drought-responsive hetero- or euchromatinisation, as DMRs were still present in gene-poor TE-rich regions, though these are likely in the minority compared to the non-genic TE-associated DMRs in gene-rich regions.

4.5.5. Methylation may mediate *RLX_famc9*-derived siRNAs

Some transposable elements are known to be capable of *trans*-acting regulation of gene expression. Methylation-mediated *trans*-acting TEs could impact the expression of genes through different pathways: they could modulate the expression of nearby TFs, indirectly influencing the expression of downstream genes (Wilkinson *et al.*, 2019), they could alter chromatin accessibility, potentially reducing the accessibility of genic regions to proteins (Sun et al., 2022b; Liu and Chang, 2021), or they could influence distant genes through the production of siRNAs from TE-derived mRNAs (McCue, Nuthikattu and Slotkin, 2013; McCue et al., 2012; Wilkinson et al., 2023). I hypothesised that some wheat TE families, like *RLX_famc9*, produce mRNA transcripts that are then processed by the Post Transcriptional Gene Silencing (PTGS) pathways: DICER (DCL) endonucleases cleave the RNA into short 21 or 24 nt siRNAs, which are then incorporated into RISC complexes with AGO proteins, and delivered to complementary transcripts for degradation, or for recruitment of DNA methyltransferases and delivery to genomic sequences for the deposit of DNA methylation (Chow and Mosher, 2023), ultimately influencing their expression.

DEGs involved in the PTGS pathway led to a hypothesis of siRNA machinery reconfiguration under drought, with a shift towards *DCL4* and *AGO2*-mediated PTGS through mRNA transcript disruption and away from *DCL3*-mediated RdDM silencing through methylation of target genes. Under drought, key 21 nt sRNA-related genes were primarily transcriptionally upregulated: *AGO2*, *AGO18*, and *DCL4* all exhibited increased expression; *AGO2s* are known loaders of 21 nt siRNAs (Liu et al., 2022c; Liang et al., 2023), with *AGO18* known to load 21 nt phasiRNAs and some miRNAs (Das *et al.*, 2020), while *DCL4* genes, which are involved in the production of 21 nt siRNAs for the disruption mRNA transcripts (Dunoyer, Himber and Voinnet, 2005), were also weakly upregulated.

DCL1s, responsible for producing 21 nt miRNAs (Reinhart *et al.*, 2002; Gasciolli *et al.*, 2005), were found to be downregulated.

DCL3, the main *DCL* involved in cleaving transcripts – particularly from TEs (Wei et al., 2014; Cho et al., 2008) – to produce 24 nt siRNAs for RdDM, also showed a reduced transcript abundance. A shift away from 24 nt siRNA-mediated RdDM, normally a major mechanism of maintaining TE methylation, and towards rapid 21 nt-mediated PTGS may make sense under abiotic stress conditions, where the faster accumulation of mRNA-targeted siRNAs (Jay, Brioudes and Voinnet, 2023) may be advantageous for the silencing of detrimental stress-induced transcripts. It may also point towards a proportional decline in 24 nt abundance in the cell and an increase in hypomethylated sites as DNA methylation maintenance is scaled down.

The most promising of potentially *trans*-acting TE families was *RLX_famc9,* a TE family highlighted due to being the only TE family enriched in all methylation contexts. The RLX superfamily is characterised by its structure as a long terminal repeat retrotransposon, though unlike the major RLC or RLG superfamilies it lacks canonical gag or pol protein domains, and are generally considered to be more ancient and degenerated than other LTRs (Wicker *et al.*, 2018; Bariah, Keidar-Friedman and Kashkush, 2020). The lack of protein domain likely means that the family is unable to transpose (Wicker *et al.*, 2018), which accompanied by their age (~1.6 million years old) may have given rise to exaptation to become stable and functional regulatory elements within the genome (Joly-Lopez and Bureau, 2018; Capy, 2021). These results show that *RLX_famc9* exhibited significantly higher homology to 245 genes responsive to drought in both the methylome and the transcriptome when compared to random genes, indicating that it may play an important role in their regulation.

Given this high sequence similarity to drought-responsive genes, a *trans*-acting regulatory role on downstream genes under drought conditions becomes plausible for *RLX_famc9* elements. In this model, drought induces non-CG hypermethylation at *RLX_famc9* loci, repressing TE transcription and therefore the production of the TE-derived siRNAs. In turn, a reduction in the abundance of these siRNAs would limit RdDM activity at the high-similarity genes, leading to an increased degree of hypomethylation as *de novo* and maintenance methylation slow. Simultaneously, increased expression of the ROS1a DNA glycosylases could actively cause hypomethylation at these target genes, further enabling their transcriptional modulation under drought (**Figure 4.6**).

The model is supported by several different observations: evidence that RLX_famc9 may be transcriptionally active; indications that this TE family may be controlled by methylation, like many other TEs; and gene expression trends indicating that small RNA pathways may be shifting to prioritise PTGS over RdDM pathways.

Firstly, reads mapping to *RLX_famc9* sequences support the presence of at least one transcriptionally active region, with a substantial number of reads mapping within the first 500 nt of the TE sequence which contains the proposed T1 transcript. This T1 transcript was also well conserved among the reads that mapped to the TE, suggesting that this region is present across a variety of the members of the *RLX_famc9* family.

Secondly, transcription of *RLX_famc9* and its T1 region are likely regulated by methylation: by calculating the relative positions of *RLX_famc9*-associated DMRs (**Figure 4.4D**), it was observed that DMRs were densest within a similar range as the proposed transcript across the TE family, indicating that this may be a methylation-targeted region. Furthermore, non-CG *RLX_famc9* TE-associated DMRs saw a strong degree of hypermethylation, which may explain the transcriptional downregulation of *RLX_famc9* transcripts; non-CG methylation – especially CHG – has been suggested as a more

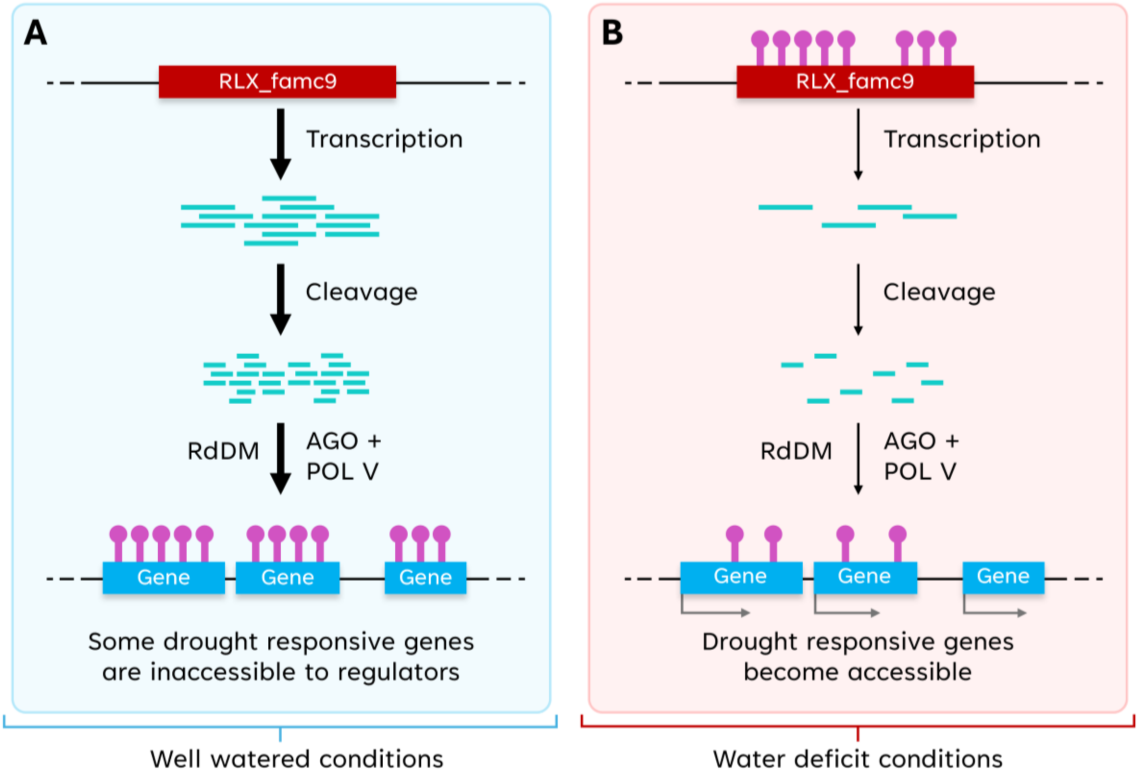

Figure 4.6: A proposed mechanism of *RLX_famc9* action. Under water-deficit conditions (right), *RLX_fam9* copies are hypermethylated to repress the expression of its transcriptionally active regions. As a result, fewer *RLX_famc9*-derived transcripts are cleaved into 24 nt siRNAs. Under well-watered conditions (left) these siRNAs would be loaded onto *AGO/Pol-V* complexes and recruit methyltransferases to deposit methylation at loci complementary to the siRNA sequence, but under drought the reduced abundance of these siRNAs allows for hypomethylation at the target genes and allows for the modulation of their expression in response to drought. *RLX_famc9*-derived transcripts are represented in blue, while drought-responsive gene transcripts are represented in red.

potent silencer of transposable elements than CG methylation (Domb et al., 2020), and is critical for TE silencing (Stroud *et al.*, 2014).

Finally, drought appears to shift small RNA pathways in a way that would support a reduction in TE-driven RdDM: shifts in expression were observed among genes involved in siRNA machinery (AGO2, AGO18, and DCL4 upregulation; DCL3 downregulation), suggesting that 24 nt siRNA-based RdDM could be deprioritised in favour of 21 nt PTGS. The potential reduction in 24 nt siRNAs involved in directing the RdDM pathway could result in a reduction of active TE self-silencing, which here could explain some of the demethylation occurring across *RLX_famc9* loci at CG sites, which are preferentially targeted by the transcriptionally upregulated *ROS1a* glycosylase (Ponferrada-Marín, Roldán-Arjona and Ariza, 2009).

The potential shift in the expression of ARGONAUTE genes however would also be consistent with a model in which AGO1 mediates transcriptional activation of downstream genes. Although AGO1 was not identified as a DEG, it is known as a key player in stress memory mediated by TE-derived siRNAs, and is tightly linked to jasmonate signalling (Wilkinson et al., 2023; Liu et al., 2018). AGO18, however, was upregulated here: in rice, AGO18 sequesters the AGO1-repressive microRNA miR168, therefore alleviating its suppression and permitting increased AGO1 activity (Wu et al., 2015b; Liu et al., 2020b). As a post-transcriptional regulation mechanism, this could enable increased AGO1 activity without an observed increase in expression. AGO1 has been reported to bind to chromatin, guided by 21 nt sRNA within the nucleus, and act as a transcriptional co-activator of jasmonate-responsive genes, in turn promoting stress responses. Taken together, these observations could be consistent with a model in which increased expression of miRNA/ 21 nt sRNA machinery enable greater AGO1 activity guided by *RLX-famc9*-derived small RNAs to activate the expression of complementary genes under drought. Testing this model would involve measuring miR168, AGO1 and AGO18 abundances, identification of RLX_famc9-derived 21 nt sRNAs, AGO1 loading, and chromatin assays to identify downstream target loci.

Both potential models posit that gene expression could increase, albeit through different mechanisms. Though this chapter does not directly deal with drought tolerance, and instead the drought response, this provides an interesting link back to **Chapter 3**; could TE-derived sRNAs be involved in the upregulation of JA-responsive genes, congruent with Chapter 3's hypothesis that higher jasmonate-associated gene expression can promote drought tolerance through a loss in hypersensitivity?

This proposed model comes with mechanistic and evolutionary caveats, however. *RLX_famc9*'s associated CG and non-CG DMRs were opposing, with CG DMRs tending towards hypomethylation, suggesting that there may be a degree of regulatory antagonism by the de novo, maintenance, and demethylation pathways, or perhaps cell-specific TE regulation. Two thirds of non-CG *RLX_famc9*-associated DMRs were hypomethylated, partly driven by the hypermethylation of 88% of *RLX_famc9* CHH DMRs, but two thirds of CG *RLX_famc9*-associated DMRs were hypomethylated, indicating a complicated lack in consistent directional bias across methylation contexts.

Additionally, not all members of the family were subject to differential methylation, and differential methylation was focused on specific regions of the TE family – though members of the same TE family are considered highly homologous (Wicker *et al.*, 2007, 2018), indicating that many members of the family could be involved in generating these transcripts, the lack of consistent association with differential methylation may suggest that the proposed role is not a family-wide exaptation. Conversely, this could be explained by the high degree of degeneration among the family leading to a loss of the T1 transcript, rather than the absence of a regulatory role for *RLX_famc9*.

This proposed mechanism, while similar to those identified in other species, requires further exploration and validation. Several experiments could be conducted to determine the role of different aspects of the model: the role of AGO proteins in siRNA-guided trans-regulatory methylation could be identified using TILLING or knockdown mutants under drought conditions, while epigenetic sequencing of ROS1a mutants could further elucidate the role of demethylation at the TE family's target genes. Other studies could be used to determine the loci targeted by the RdDM pathway, the data from which could then be overlapped with the hypothesised TE and gene targets.

4.5.6. HPS-dsRNA methods may present an opportunity to test TE-derived mechanisms

Our method, adapted from previous studies (Dalakouras and Ganopoulos, 2021; Dalakouras et al., 2016), was deemed successful at delivering exogenous molecules into the cytoplasm of cells (**Figure 4.5A**). The results demonstrate the introduction of fluorescent markers to the cytoplasm and cellular organelles using a high-pressure air brush and abrasive-induced lesions. qPCR also indicated that the introduction of exogenously synthesised dsRNAs may have been able to significantly influence gene expression, with the 6- and 24-hour timepoints indicating that expression of the target gene significantly decreased before trending back towards the baseline at 48 hours. Following on, I attempted to apply this same method to the putative *RLX_famc9*-derived transcript, T1. This attempt to mimic the proposed model through this method was somewhat inconclusive due to the observation of only small and non-significant differences between RLX-treated and water-treated plants under drought conditions. Despite this, slight reductions in nDW were observed among plants treated with RLX T1 compared to plants treated with water, potentially indicating that RLX T1 treatment may have had a slight negative impact under drought, congruent with the hypothesis that RLX_famc9-derived siRNAs are negative regulators of drought responsive genes.

The small differences observed in plants treated with *RLX_famc9*-derived dsRNAs are likely due to natural variation, though could also be influenced by small sample sizes, insufficient concentration of dsRNA applied, or due to inconsistencies in the spraying treatment.

This method may prove to be a useful reverse genetics approach to characterising gene or TE function in the future with substantial optimisation – further development should give rise to greater efficacy and help to elucidate the true function of these transposable element transcripts under stress conditions. Required optimisation steps would include developing a more resource-efficient and even spraying approach, further optimisation of the dsRNA synthesis steps, and identification of the optimal dsRNA concentration for foliar spraying.

4.6. Future steps

Further exploration and validation of this model is required, but I believe that it may represent a promising avenue of research for drought stress regulation in complex grasses like wheat. This model however exists with several caveats and unknowns: small RNAs associated with the *RLX_famc9* family have not yet been identified, nor has this mechanism been observed at the individual plant or landrace level; the it is derived from differential methylation results from 2 pooled samples, limiting its statistical power; and the drought regulome is very complex and other epigenetic mechanisms would also likely play a role.

Next steps would likely include sequencing of different epigenetic mechanisms across drought conditions: small RNA-seq, ATAC-seq, and landrace-specific WGBS. Small RNA-seq would hopefully identify novel small RNAs associated with responding to drought, hopefully also identifying an siRNA species associated with the TE family of interest. Wheat's catalogue of siRNAs is underexplored, with the majority of currently studied small RNAs being miRNA – identifying siRNAs in wheat is likely confounded by the majority of siRNAs being produced by TEs (Vaucheret and Voinnet, 2024), of which wheat is incredibly abundant, while studies in other grasses like rice have found that drought-induced siRNAs are largely heterogeneous and with weak evolutionary conservation (Jung et al., 2016), indicating that while the siRNA landscape in grasses is complex, it may have a breadth of novel regulatory molecules to identify. Small RNA sequencing would, at the very least, give a more comprehensive view of the epigenetic drought regulome in wheat landraces.

ATAC-seq would also be particularly useful for associating the proposed role of intergenic DMRs and DMR-associated distal TEs with chromatin structure. Under drought, one might expect to see overlaps between ATAC-seq peaks, transcriptional activation, and hypomethylation of drought-induced genes – this might enable the identification of functional methylation within promoter regions of genes where it was not possible to make confident predictions based on WGBS and transcriptomics alone. Drought-induced ATAC-seq peaks would also make any TE-regulating methylation clearer: observing *RLX_famc9* family members with changing accessibility through non-CG hypermethylation would give substantial support for the model. I would expect to see changes in chromatinization across regions rich in both TEs and genes, with the TEs acting as RdDM targets to modulate the accessibility and expression of nearby genes.

4.7. Conclusions

Together these findings highlight the multifaceted role of DNA methylation in controlling wheat's response to drought. *ROS1a*-mediated demethylation may play an important role in wheat's drought response. Gene body methylation appears to support stable, stress-insensitive expression, while promoter methylation appears to have limited regulatory impact, suggesting that genic methylation changes may be more specifically targeted and complex than previously assumed. Enrichment of transcription factor binding motifs in differentially methylated regions proximal to drought-responsive genes pointed towards a mechanism in which methylation modulates transcription factor binding affinity, especially among genes regulated by the MYB, bZIP, bHLH, and ERF/DREB TF families. The vast majority of differentially methylated regions, however, occurred outside of genes: transposable elements emerged as promising candidates for *trans*-acting regulation. RLX_famc9, an LTR retrotransposon, appeared to be targeted by differential methylation in all methylcytosine contexts and was differentially expressed under drought conditions, and displayed significant sequence similarity to drought responsive genes, suggesting that it may potentially act in trans to repress genes via PTGS under normal conditions, but may be silenced under drought conditions to relieve gene repression via siRNAs.

This analysis also demonstrates the benefits of pooled DNA methylation sequencing in complex polyploids like wheat, yielding high-resolution data at reduced cost, allowing the identification of regions with strong signals among a group of samples. Future work with more samples, accompanied by exploration of drought-responsive siRNAs, may further explore the proposed mechanisms and would be crucial for supporting *RLX_famc9*'s proposed role in the regulation of stress responsive gene expression.

4.8. Author Contributions

IJR and AH conceived and planned the project. LB and IJR conducted plant growth experiments and tissue sampling (see **Chapter 2**). IJR conducted WGBS DNA analysis from end-to-end (QC, alignment, methylation calling, differential methylation analysis, etc.). IJR designed and carried out validation experiments and analysis. IJR wrote the chapter in its entirety.

4.9. Acknowledgements

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5. Can drought priming improve water deficit responses among wheat landraces?

5.1. Abstract

Background: Drought is an enormous global environmental problem that drives up to 60% yield losses in wheat, affecting food security. Improvements in agricultural practices and infrastructure that ameliorate the deleterious effects of drought can be costly and slow to adopt, especially in low- and middle-income countries, indicating that cost-effective and novel approaches need to be taken. Plants that experience drought conditions have reportedly been able to generate 'stress memory', enabling advantages against that stress when experienced in the future, however the prevalence and effectiveness of this capability in crops like wheat remains unclear, as do the potential mechanisms that might underpin such a response.

Method: I examined whether early drought stress could prime drought responses both within a generation (where plants experienced priming stress at the three-leaf stage and secondary stress at anthesis) or across generations (where the parental and filial generations both experienced stress at the three-leaf stage) by imposing water deficit conditions for 10 days (three-leaf stress) and 8 days (anthesis stress). Head weight, seed number, above-ground biomass, developmental delay, and harvest index were all calculated and normalised against control plants to compare across groups.

Results: No clear indications of within- or across-generation priming resulting in improved drought responses were observed across different wheat landraces using a variety of traits (including head weight, seed quantity, biomass, and harvest index)

Conclusions: priming capability is specific to certain genotypes and not a broad-scale trait of wheat. Further screening of landrace panels and elite lines with a greater variety of drought stress treatments should be carried out to identify wheat cultivars and optimal treatments that can give rise to enhanced acquired drought tolerance through priming.

5.2. Introduction

Drought represents a critical stress that threatens the yields of essential crops worldwide; wheat yields can drop by 30-60% under water deficit conditions (Zhang et al., 2018b; Zhao et al., 2020; Nyaupane et al., 2024), threatening food security. Interventions like improved agricultural practices, the development of biotechnical strategies, and the introduction of more stress-tolerant genotypes have somewhat ameliorated these yield losses (Nyaupane *et al.*, 2024; Şimşek *et al.*, 2024), though adoption is not ubiquitous and remains unattainable for many farmers in low- and middle-income countries (LMICs) due to upfront costs and unsuitable infrastructure (Barrett, 2008; Holden and Shiferaw, 2004; OECD, 2025; Biswas et al., 2025). In many LMICs wheat yields are frequently outstripped by rising demand (Silva *et al.*, 2023), while improvement programmes for crops like wheat consistently report yield gains below the 1.5% increase needed to meet food security in 2050 (Rahman et al., 2021; Ray et al., 2013). Rising grain demand, increasingly hostile climates, and slow uptake of beneficial but expensive agricultural practices present a mounting problem for agriculture.

It is paramount that we identify novel sources of drought tolerance, especially those that can be introduced at low cost. Drought priming, where young plants are exposed to water deficit conditions, may induce a 'stress memory' that can enable faster and more effective responses if they or their progeny experience recurrent drought stress (Hilker and Schmülling, 2019), potentially offering a low-cost, easily adopted route to acquiring enhanced tolerance and reduced yield loss from adverse environmental conditions.

In recent years the idea of priming plants to better survive subsequent stresses has grown in interest. Various studies in model plants have suggested that abiotic stress priming can have a long-term impact on stress regulation; studies in *Arabidopsis thaliana* have demonstrated the role of histone demethylases in potentiating future activation of memory-related heat shock protein genes (Yamaguchi et al., 2021), while salt treatment delivered to *Sorghum bicolor* seedlings has been shown to convey acquired tolerance to saline conditions through reduced root Na+ uptake and increased photosynthetic rate compared to unprimed plants (Yan et al., 2015b). Mild drought priming in *Brassica napus* can activate salicylic acid-mediated modulation of drought-responsive pathways and improve tolerance to subsequent stresses (Muchlas et al., 2023). In wheat, drought priming has previously led to improved stomatal dynamics, improved nitrogen use efficiency, and ultimately reduced yield loss (Amini et al., 2023; Yang et al., 2023b; He et al., 2024a) under recurrent drought stress compared to unprimed plants, though these studies note that only some genotypes are 'priming-sensitive' and that drought-sensitive cultivars may exhibit greater priming potential.

Although the physiological and transcriptomic effects of stress priming treatments have been identified, the mechanisms that underpin them are relatively underexplored; current evidence suggests that this 'stress memory' is facilitated in part by epigenetic modifications to chromatin structure, histone methylation, small and non-coding RNAs, and DNA methylation (Liu, Able and Able, 2022; Aswathi *et al.*, 2025). As detailed in **Chapter 4**, it appears that DNA methylation is remodelled by and responsive to abiotic stresses like drought, while other studies have suggested that changes in DNA methylation are heritable and therefore memory could be transmissible across generations (Verhoeven *et al.*, 2010; Song *et al.*, 2016).

Recent studies in wheat have identified drought responsive methylation changes in the promoter regions of target genes during secondary drought events (Li et al., 2023c), though the regulatory basis of priming in wheat remains understudied. Large-scale omics explorations of priming-induced epigenetic changes are uncommon, though drought priming has been implicated in the mediation of ‘stress-memory’-related DNA methylation changes in intergenic and gene-body regions in *Boea* resurrection plants in (Sun et al., 2021a) and in the promoter regions of stress-responsive genes in *Medicago ruthenica* (Zi *et al.*, 2024).

Our methylome profiling of a selection of YoGI lines in **Chapter 4** identified a substantial number of potential targets for sources of drought priming mechanisms – a large proportion of non-genic differentially methylated regions (DMRs) were localised to unannotated intergenic regions which could potentially act as enhancers or *trans*-regulate gene expression, while many of the genic DMRs were not associated with clear changes in gene expression. Priming-induced DMRs at intergenic sites have previously been associated with potentiating gene expression through an association with histone-3-lysine-4 trimethylation (H3K4me3) histone marks, effectively altering chromatin structure (Yung et al., 2024, 2022), while changes at genic regions are frequently not associated with changes in gene expression during initial stresses (Sun et al., 2021a; Zi et al., 2024; Yung et al., 2024), with both priming responses associated with facilitating the future regulation of stress response genes, suggesting that the marks observed in Chapter 4 could potentially be involved in conveying a priming benefit against drought. However, to first explore the epigenetic basis of priming, we must first identify evidence of priming ability in our chosen lines.

I sought to ask whether drought-responsive DNA methylation marks could be associated with within- or across-generation ‘stress memory’, and whether this could enable plants that have previously experienced drought conditions – or their progeny – to exhibit acquired enhanced drought tolerance phenotypes. I aimed to examine the effects of a sustained drought priming event on our YoGI lines of interest, and whether they were able to demonstrate improved phenotypes under water deficit conditions compared to unprimed plants both within and across generations; phenotypic evidence for beneficial drought priming could imply the existence of ‘memory’ mechanisms, potentially providing an explanation for interesting regions identified in **Chapter 4** and an advantageous mechanism to promote drought resistance in wheat crops.

5.3. Methods

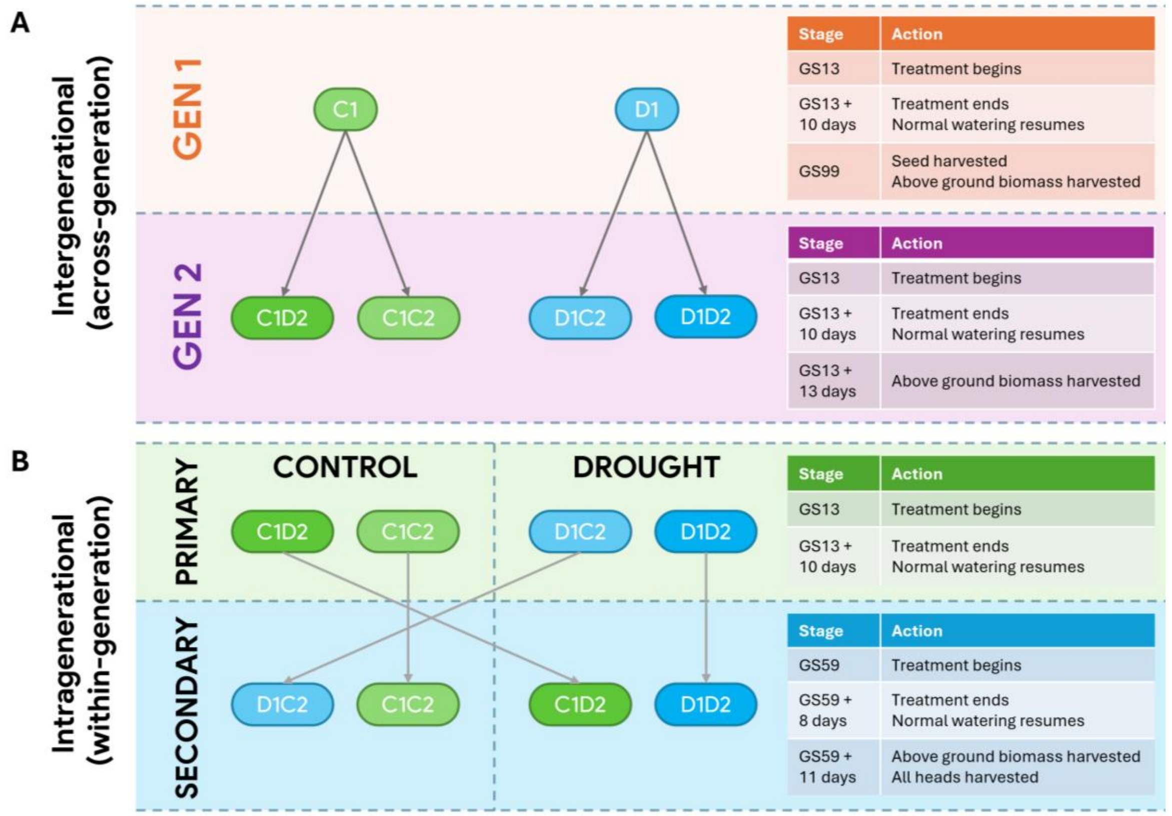
5.3.1. Plant Selection & Growth

Ten landraces with a variety of drought tolerance phenotypes were selected from the initial 20 used in **Chapter 2** (*Transcriptomic and co-expression network analyses on diverse wheat landraces identifies candidate master regulators of the response to early drought*). All eight of these lines are used in the intergenerational study: of these, six were deemed moderate, exhibiting a normalised dry weight of 0.6-0.7 in response to drought, with one tolerant line that exceeded the upper limit (0.7 nDW) and one susceptible line that was below the lower limit (0.6 nDW). Normalised dry biomass (see '*Normalisation of Traits*') was used as the barometer of drought tolerance. Only four were used in the intragenerational study: two tolerant and two susceptible lines. Lines selected are collated in **Table 5.1**.

Table 5.1: *Lines used from the YoGI panel spanned a variety of locations and drought tolerance phenotypes. Only two lines were used in both experiments. All lines were of Spring habit.*

Line	**Origin**	**Drought Tolerance Phenotype**	**Tested priming regimes**
YoGI_002	China	Susceptible	Intragenerational
YoGI_007	Nepal	Tolerant	Intergenerational + Intragenerational
YoGI_010	Kenya	Susceptible	Intergenerational + Intragenerational
YoGI_030	Egypt	Moderate	Intergenerational
YoGI_034	South Africa	Moderate	Intergenerational
YoGI_073	Mexico	Moderate	Intergenerational
YoGI_114	India	Moderate	Intergenerational
YoGI_152	India	Moderate	Intergenerational
YoGI_256	Greece	Moderate	Intergenerational
YoGI_261	Iran	Tolerant	Intragenerational

In each regime plants were sown in Levington Advance Seed & Modular F2S compost mixed with Aggregate Industries Garside Sands 16/30 sand in a 4:1 ratio. Seeds were sown at 1cm deep in a small pit made with the end of a 1.5 ml Eppendorf tube. Plants were treated with entomopathogenic nematodes (*Steinernema feltiae* & *S. carpocapse*) weekly during normal watering to prevent herbivory. Plants were grown in long day glasshouse conditions (16/8h, 20°C/14°C). Soil moisture was recorded using an ML3 Thetaprobe Soil Moisture Sensor with an HH2 Moisture Meter (Delta-T Devices, Cambridge, United Kingdom) to ensure that soil moisture was in the expected ranges for control and drought conditions, and that each group received similar levels of drought stress. Soil moisture content was compared using an ANOVA and Tukey post-hoc tests. ANOVAs and, where necessary due to data not meeting parametric assumptions, Aligned Rank Transformed ANOVAs (ART-ANOVAs) have been used for statistical testing of all plant traits, followed up with Tukey post-hoc testing for pairwise group comparison within the models.



Stage	Action
GS13	Treatment begins
GS13 + 10 days	Treatment ends Normal watering resumes
GS99	Seed harvested Above ground biomass harvested

Stage	Action
GS13	Treatment begins
GS13 + 10 days	Treatment ends Normal watering resumes
GS13 + 13 days	Above ground biomass harvested

Stage	Action
GS13	Treatment begins
GS13 + 10 days	Treatment ends Normal watering resumes

Stage	Action
GS59	Treatment begins
GS59 + 8 days	Treatment ends Normal watering resumes
GS59 + 11 days	Above ground biomass harvested All heads harvested

Figure 5.1: Intergenerational (A) and intragenerational (B) priming experiments differed in structure. Though all used a common onset of drought at the three-leaf stage – GS13 – the intergenerational priming experiment aimed to identify any drought tolerance benefits in the progeny after the parent generation was exposed to harsh drought stress at the seedling stage, while the intragenerational priming experiment aimed to identify any drought tolerance benefits at heading in the same plant after exposure to drought at the seedling stage.

Seeds taken from the YoGI panel had been through 5 selfing generations under well-watered conditions in glasshouse conditions, suggesting that they should be highly homozygous for alleles. Seeds had been stored for up to 2 years by time of sowing. Plants were under the same watering regimes and similar conditions within each generation, reducing any potential additive impact of adverse environmental conditions on the epigenome. Seeds were stored in a 4°C cold room in paper seed packets within opaque black boxes, with silica packets to reduce air moisture and prevent early germination. All seeds were treated in the same way.

5.3.2. Intergenerational Priming (Across generations)

Eight replicates of each line per group were sown (8 replicates per 8 lines per 2 groups; n = 128). Plants were split into two groups in the priming generation, C1 (control) and D1 (drought) (**Figure 5.1A**). Plants were well watered (twice-daily watering) until GS13 (three leaf stage; (Zadoks, Chang and Konzak, 1974)). At GS13 C1 plants continued with the well-watered regime; conversely water was withheld from D1 plants for a ten-day period, after which normal watering resumed. Half of the replicates of each line were harvested after three days of recovery to record a baseline drought phenotype for the priming generation (baseline n = 64). Above-ground biomass was harvested from all plants 13 days after the onset of the treatment period, weighed, and then dried in an oven at 65°C for two days, after which dry biomass was weighed. The remaining half of each line and group (full grown n = 64) was grown until seeds were ripe (GS99), after which they were harvested in the same manner. Time to flower, seed quantity, number of heads, and biomass were all recorded at harvest.

Seeds harvested from the primed and unprimed parents were sown in the same conditions as the first generation, though the second generation contained more groups: D1 (unprimed and droughted), C1C2 (control in each generation), D1D2 (droughted in each generation), and D1C2 (droughted in the first and under control conditions in the second generation). Four replicates for each line per group were sown. Plants entered drought or control conditions according to their group at the onset of GS13 for 10 days, followed by 3 days recovery, and finally all above ground biomass was harvested as previously described. All data was normalised against average values from C1C2 (see '*Normalisation of Traits*') to compare across lines and groups.

5.3.3. Intragenerational Priming (Within a generation)

Four replicates of each line were sown per line per group (4 replicates per 4 lines per 4 groups; n = 64). Four groups were used: C1C2 (control conditions at primary and secondary stages), D1D2 (drought conditions at primary and secondary stages), D1C2 (drought conditions at the primary stage and control conditions at the secondary stage), and C1D2 (control conditions at the primary stage and drought conditions at the secondary stage) (**Figure 5.1B**). Primary treatments were carried out as previously described at GS13.

Secondary stress was carried out at full head emergence (GS59), just prior to flowering. Water was withheld from droughted groups in the same manner as the primary stress, though the secondary stress period only lasted 8 days. Larger plants were expected to have higher water usage than smaller, younger plants, leading to a shorter secondary stress period compared to the primary stress period. After the secondary treatment phase plants were left to recover and above-ground biomass was harvested at GS99. Time to head, total above-ground biomass, main and total head weight, and Harvest

Index (HI) were all measured and calculated. All data was normalised against average values from C1C2 (see below) to compare across lines and groups.

5.3.4. Normalisation of Traits & statistical tests

Normalised traits were used to compare tolerance for traits across the panel, and were calculated using the formula ($normalised\ trait = \frac{trait_{drought}}{trait_{control}}$), where $trait_{drought}$ represents an individual plant's value for a given trait under drought conditions and $trait_{control}$ represents the mean value of a given trait for that line under control conditions. Normalised traits were expressed as a proportion. All traits were normalised to allow for comparison between groups and lines.

5.4. Results

5.4.1. Drought priming shows no improvement in sustained growth across generations

Through the first generation, plants received a harsh drought treatment upon reaching GS13 and then were grown under well-watered conditions until seed and above-ground biomass were harvested at GS99.

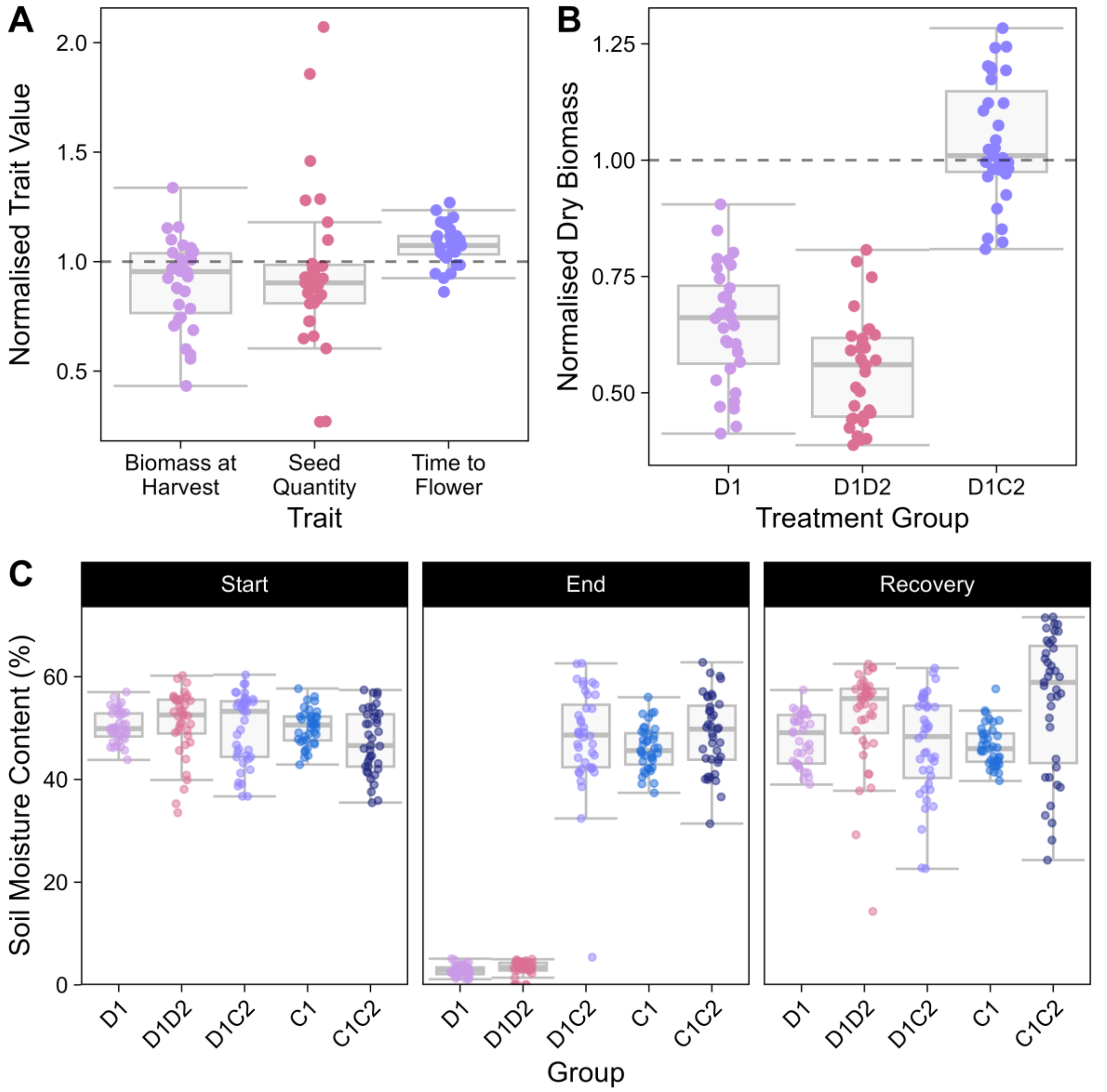


Figure 5.2: Little or no improvement was observed among the progeny of plants primed against drought after normalisation against their control counterparts. (A) First generation drought treatment identified a small but significant reduction in normalised dry biomass at harvest (GS99) (ART ANOVA: p = 0.0252) as well as a small reduction in seed yield. Normalised flowering time was significantly increased compared to control plants. **(B)** Second generation plants exhibited a reduced normalised dry biomass compared to their unprimed plants. Some D1C2 plants displayed greater biomass in the second generation under control conditions. **(C)** Soil moisture was within expected ranges and consistent between treatments and generations.

Early drought stress had a substantial impact upon several raw traits within the first generation. Drought was found to have a line-specific impact on dry biomass at harvest in the first generation (Aligned Rank Transformed (ART) ANOVA: $p = 0.0252$), though no individual line was significantly different by itself by Tukey post-hoc testing. Though marginally insignificant (ART ANOVA: $p = 0.0507$), treatment groups (C1 and D1) showed differences between the 8 lines tested, with all lines bar YoGI_152 showing a mean decrease in biomass at harvest, suggesting persistent deleterious effects.

Analysis of normalised traits (**Figure 5.2A**) identified that drought treatment had a significant effect on normalised dry biomass between the C1 and D1 groups (ART ANOVA: $p = 0.0173$), while line was also a significant factor (ART ANOVA: $p = 0.0254$). The D1 group produced a mean of 90.3% of the biomass of the C1 group. Normalised time to flowering was also significantly different between the two treatment groups (ANOVA: $p = 1.14e\text{-}07$), something that differed in a line-dependent manner as per the significant interaction term (ANOVA: $p = 4.57e\text{-}04$). Droughted lines displayed a mean increase of 9% in time to flower compared to the control group. Normalised seed quantity was not significantly different between treatment groups (ART ANOVA: $p = 0.197$), though it was significantly different between lines (ART ANOVA: $p = 0.0302$). The D1 group produced a mean of 95.8% seed content compared to its controls. Normalised traits suggest that drought caused a delay in development and reduced growth but did not have a significant effect on seed quantity.

Following to the second generation, no significant increase was seen in drought primed plants against unprimed plants and their control counterparts (**Figure 5.2B**). D1D2 (droughted in each generation), D1C2 (droughted in the first generation but kept in control conditions in the second) and D1 (droughted once) groups were all normalised against their respective control groups. I observed that treatment group had a significant effect on normalised dry biomass (ART ANOVA: $p < 2.22e\text{-}16$); post-hoc Tukey tests revealed the D1 and D1D2 groups were significantly different by normalised dry biomass ($p = 1.30e\text{-}05$). D1D2 displayed slightly lower mean normalised dry biomass overall compared to the D1 group, with a 9.7% decrease across all lines. YoGI_007, previously identified as a drought tolerant line in **Chapters 2 and 3**, exhibited the largest decrease in nDW (22.7%) between the first and second generation, while YoGI_073 showed the smallest decrease at 2.1%. Interestingly, some lines in D1C2 displayed increased biomass compared to their control group in the second generation, though the mean was only 4% higher than the control group and was not significantly different (ART ANOVA: $p = 0.538$). YoGI_073 also showed the largest increase in nDW in the D1C2 group compared to its control group. Intergenerational priming did not appear to induce acquired tolerance benefits.

To ensure that each group received suitable watering treatment, and that drought was sufficiently applied, soil moisture was compared across generations and groups (**Figure 5.1C**). Pairwise testing via post-hoc Tukey tests indicated that there was no significant difference between the soil moisture content for D1 and D1D2 at the end of the drought treatment (Tukey: p = 1.00). Soil moisture was also not significantly different between D1C2 and C1C2 groups (Tukey: p = 0.617). At the end of treatment periods, soil moisture in drought-treated groups was significantly different to that of control groups (Tukey: p = 5.042e-10), indicating sufficient droughting.

5.4.2. Within-generation drought stress provides no obvious benefit for plants experiencing later stress

Drought priming was subsequently explored within a single generation. I hypothesised that a primary drought stress may induce acquired enhanced tolerance to secondary drought stress delivered later in its life cycle.

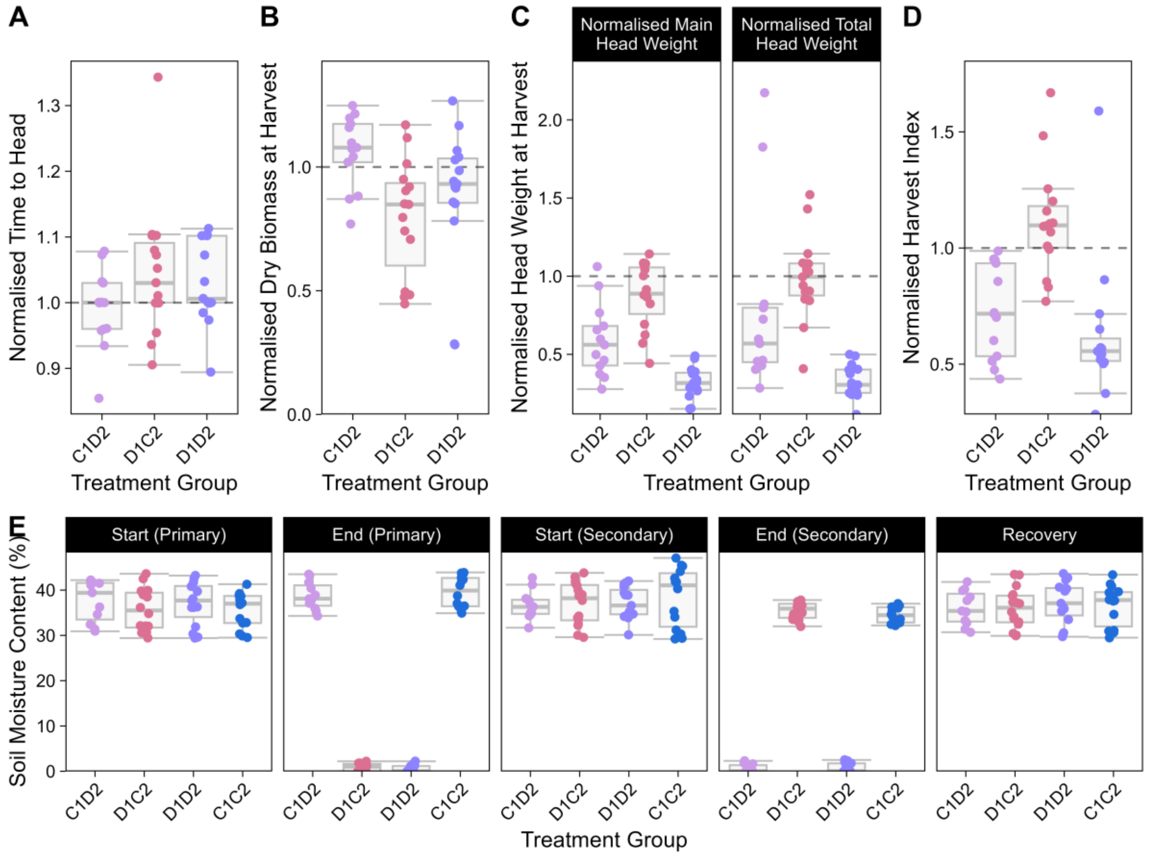


Figure 5.3: Within-generation drought priming appeared to have no benefit to agronomic traits. (A) Plants that experienced drought at the primary treatment appeared to exhibit a slight developmental delay. **(B)** Normalised dry biomass at harvest was reduced in both groups that experienced drought at the primary treatment. **(C)** All droughted groups exhibited reduced main and total weight except for total head weight in D1C2. **(D)** Normalised harvest index analysis identified D1D2 as possessing the lowest value of any group. **(E)** Soil water moisture content significantly varied between treatments (ART ANOVA: p < 2.22e-16), though all remained in the expected ranges for well-watered and drought treatment regimes.

Much like the across-generation effect, primary drought treatment had a significant impact (ANOVA: *p* = 2.64e-07) on raw dry biomass using treatment group and YoGI line as factors. Treatment group (C1C2, C1D2, D1C2, and D1D2) was also found to have a significant effect on dry biomass (ANOVA: *p* = 6.68e-3). Treatment group (ANOVA: *p* = 3.84e-03) and line (ANOVA: *p* = 0.0312) both had significant effects on nDW, though the interaction term (*group x line*) was not significant (ANOVA: *p* = 0.672).

Further analysis was conducted using values for each group and line normalised against their C1C2 counterparts, as they represent the control group for both the primary and secondary stresses. This allowed the direct comparison across treatment groups and lines.

Primary drought treatment appeared to cause slight developmental delays. Primary treatment (D1 or C1) was associated with a significant difference (ART ANOVA: *p* = 0.0272) in normalised time to head (**Figure 5.3A**). D1 plants displayed delayed floral transition compared to C1 plants, though the effect was small, with just 3.4% difference observed between normalised time to head means. Overall treatment group had no significant effect on normalised time to heading (ART ANOVA: *p* = 0.0978), though lines tended to exhibit slightly increased means compared to C1C2, with D1C2 and D1D2 5% and 3% higher respectively. C1D2 was within 0.9% of the C1C2 mean. This suggests that primary drought treatment caused an increase, albeit small, in time to head.

Treatment group again had a significant effect on normalised dry biomass (ANOVA: *p* = 0.00384), as did line (ANOVA: *p* = 0.0312), though the interaction between the two factors was not significant (ANOVA: *p* = 0.673) (**Figure 5.3B**). Tukey post-hoc testing identified that while C1C2 was significantly different to D1C2 (*p* = 0.0359), C1D2 (*p* = 0.834) and D1D2 (*p* = 0.427) were not when including all lines. C1D2 showed a mean increase of 6% compared to C1C2, while D1D2 exhibited a 12.2% decrease and a 20% decrease in biomass was observed in D1C2 plants. Although D1D2 plants displayed a smaller reduction in biomass compared to C1C2 than D1C2 plants, D1D2 and D1C2 were not significantly different (Tukey: *p* = 0.565). This suggests that perhaps D1C2 plants directed more biomass towards head and seed development than D1D2 plants.

Normalised main and total head weight were also significantly different between treatment groups. The normalised weight of the main head – the head of the main productive shoot – was found to be significantly different between treatment groups (ANOVA: *p* = 9.82e-19), something that differed in a genotype-specific way (ANOVA: *p* = 7.03e- 3). Similarly, the total head weight was found to be significantly different between treatment groups (ART ANOVA: *p* = 2.86e-13), accompanied a significant interaction between group and line (ART ANOVA: *p* = 0.0152). Drought-primed and droughted plants (D1D2) exhibited the lowest normalised main and total head weight across all groups (**Figure 5.3C**), possessing only 31.8% and 32.6% of its C1C2 counterparts; D1C2 produced 87.5% and 99% respectively, while C1D2 produced 58.9% and 76.9%

respectively. All droughted plants exhibited a decrease in yield by seed weight. Secondary drought treatment was significantly deleterious to seed weight, but most so in plants droughted twice.

Harvest Index (HI) was also calculated and normalised to the C1C2 group (**Figure 5.3D**). Group had a significant effect on normalised HI (ART ANOVA: p = 4.65e-08); both groups that were under water deficit conditions at the secondary treatment stage were significantly lower than C1C2, with C1D2 and D1D2 showing 27.8% (Tukey: p = 7.01e-04) and 37.7% (Tukey: p = 2.18e-05) decreases in HI. D1C2 displayed a slight but insignificant increase in HI compared to the C1C2 (+11%, Tukey: p = 0.777). Although D1D2 expressed a substantially lower mean normalised HI (0.623) than its unprimed C1D2 counterpart (0.722), the two groups were not significantly different (Tukey: p = 0.789), suggesting that primary drought treatment was not significantly damaging to HI, but secondary treatment was.

Watering regimes were significantly different between groups: there were significant differences (ART ANOVA: $p < 2.22e-16$) across treatment groups and timepoint (onset and end of both treatment period), mean soil moisture content values remained in expected bounds for each group and treatment, with treatment onset soil moisture ranging from 29.2 – 43.2% across all groups, and treatment end values for plants in control conditions ranging from 32.2 – 43.9% and in droughted plants from 0 – 2.5% (**Figure 5.3E**). All treatments were within expected ranges.

5.5. Discussion

'Stress memory' – where pre-stressed plants are better able to respond to subsequent stresses compared to plants experiencing that stress for the first time – presents a beneficial opportunity for obtaining more stress-tolerant crops. It is this 'memory' that underpins the idea of stress priming; previous studies have identified that prior stresses may prime wheat against future abiotic stresses like drought (He et al., 2024a; Li et al., 2024b; Ru et al., 2025; Amini et al., 2023), salinity (Feghhenabi *et al.*, 2020), and heat (Wang et al., 2016b). Priming effects have been observed both across and within generations (Tabassum *et al.*, 2017; Amini *et al.*, 2023) from a variety of sources, though most evidence lies in the impact of recurrent stresses. I hypothesised that differentially methylated regions identified in **Chapter 4**, many of which with unknown functions, could be involved in conferring heritable or maintained 'stress memory'. Although stress priming has shown promise in other model and crop systems, with some identifying the role of epigenetics in mediating it in the long-term, I found no clear evidence for priming-induced benefits across these small panels.

Here I made two hypotheses: in the intergenerational experiment, drought treatment at the seedling stage would provide an inherited adaptive benefit to its progeny that experienced the same drought conditions at the same growth stage; in the intragenerational experiment, drought treatment at the seedling stage would convey an adaptive benefit to the same plant experiencing a similar drought stress at the heading stage. In neither experiment was a positive response to initial priming treatment observed, indicating that drought priming – at least at the seedling stage – is not able to induce a priming benefit for plants experiencing drought, though this may have been due to various limitations or assumptions made in this study. As a result, the capacity of these wheat landraces to be primed remains unknown and no further observations can be made about the epigenetic marks identified in **Chapter 4**.

Considering the results of **Chapter 4**, the potential for jasmonate-associated tolerance through various mechanisms discussed in **Chapters 3** and **4**, and the previous studies which have identified a potential capacity for priming in wheat (Amini et al., 2023; Tabassum et al., 2017) and intimated this may be due to epigenetic inheritance across generations, it was plausible that an epigenetically-controlled priming ability could be observable within the YoGI panel. Despite these results, it remains plausible that some genotypes of wheat may possess the capacity to be primed against drought, or that this study may not have provided the correct environmental or stress conditions to stimulate this response.

5.5.1. Drought priming does not appear to induce enhanced tolerance acquisition in wheat landraces

Exploration of drought priming in wheat has reported that drought priming at the seed and seedling stages can enhance enzymatic antioxidant efficiency (Amini *et al.*, 2023), stomatal control, and water use efficiency (Yang et al., 2023b) at the vegetative and jointing stages, ultimately leading to faster and more effective drought responses and less affected yields under stress conditions. Despite these findings, I was unable to find evidence for either within- or across-generation priming mechanisms in wheat that were able to rescue growth or yield traits under stress among the tested landraces from the YoGI panel.

Prior work on the functional basis of priming has suggested that DNA methylation and other epigenetic mechanisms may underpin how ‘stress memory’ is maintained and proliferated within and across generations; it is likely that many of the epigenetic marks previously observed are not associated with conveying temporal ‘stress memory’, within or across generations. Some observed DMRs may have simply been markers of age, development, or growth (Osabe et al., 2014; Candaele et al., 2014), and not evidence of drought priming mechanisms.

Across multiple traits, including normalised dry biomass, seed quantity, head weight, and harvest index, primed plants – whether primed in the previous generation or earlier in the lifecycle – did not outperform their unprimed counterparts or improve relative to the previous generation. Conversely, across several traits primed plants exhibited reduced performance compared to unprimed plants in both experiments, indicating that drought had maladaptive costs as opposed to an adaptive priming response. Normalised dry weight was reduced in intergenerationally primed plants relative to its unprimed siblings while primed plants saw reduced normalised biomass, harvest index, and head weight compared to the unprimed group under drought conditions. Early drought stress likely imposed lasting developmental penalties on plants droughted twice within the same generation, as opposed to adaptive benefits, but the significant reduction in mean normalised dry weight of droughted plants across generations was unexpected: possible explanations could include developmental penalties in the first generation resulting in negative maternal effects on seedling vigour, or inherited negative drought-tolerance traits. Maternal effects are distinct from priming mechanisms: maternal effects are broad impacts on the progeny of the environmental influences on the mother plant, usually due to changes in seed resources, size, and hormones, while priming mechanisms are more to do with a specific preparedness to a stressor based in ‘memory’.

Importantly though, the reduction in performance should not be interpreted as true increased susceptibility or a negative priming effect, but rather as a likely consequence of developmental penalties, stress exposure, or detrimental maternal effects.

Our findings somewhat align with previous reports that priming responses are highly genotype specific: one major priming study in wheat used synthetic wheat cultivars while another used cultivars known to be sensitive to priming strategies (He et al., 2024a; Amini et al., 2023), suggesting that I was unlikely to identify priming-sensitive varieties in the landrace panel despite the broad genetic diversity that it offers. This also suggests that priming may not be a ‘drop-in’ solution to moderating the impact of drought on wheat farming, as many commercial and specialised cultivars may lack any priming ability.

5.5.2. Developmental delays, yield penalties, and resource distribution

Primary drought stress in both experiments resulted in small but significant delays in developmental time. These delays are likely maladaptive costs of the early drought exposure, rather than elements of any adaptive priming response – a true priming response would be expected to enhance future drought tolerance, rather than show sustained developmental effects. Though these were also line-dependent, the per-group effect was consistent enough to suggest that early harsh drought stress can disrupt developmental timing and subsequently yield traits; wheat conventionally employs drought escape methods to quickly produce seeds (Nyaupane et al., 2024; Shavrukov et al., 2017), though this suggests that harsh intermittent drought could compound yield losses and developmental delays instead of conveying a priming benefit. Developmental delays in the field, if severe enough, could also be damaging to harvests through crop abandonment (Shahbazi, Shahbazi and Zare, 2025) as farmers focus on less disrupted crops.

Primary drought had a modest effect on normalised biomass at harvest in the intragenerational study: treatment group was significant, as was line, with no priming benefit observed. Plants that were droughted twice within the same generation exhibited a non-significant but slightly higher mean nDW than plants that were only droughted at the primary treatment: wheat and other cereals have previously been found to accumulate more biomass in response to mild drought events at various growth stages (Ma et al., 2024b; Gao et al., 2024; Fang et al., 2024), potentially a sign that wheat plants may redirect energy into vegetative growth after initial stress to better enable later-developing tillers to produce seed, even beyond the initial floral transition. Slight floral delay could also contribute to this small increase in biomass by maintaining vegetative growth. Nevertheless, this slight biomass gain did not result in increased reproductive success, with total head weight and harvest index remaining lowest in primed and droughted plants, suggesting that primed plants performed similarly poorly as unprimed plants, if not more so, at the grain-filling stage.

Under these conditions, I hypothesise that early drought exposure had several impacts: it likely delayed or reduced tillering development, constraining yield potential, and may also prevent root growth and development, which would be critical in these small pots. These would represent developmental penalties of the drought treatment. A long-term impact of drought on the plants' hormonal profile – especially if gibberellic acid production was modulated – would suppress growth and delay floral transition, inducing the delay seen in some of the lines, as well as likely moving resource allocation from growth to stress survival, reducing the energy available for processes like grain-filling.

These developmental costs induced within the first-generation plants likely resulted in a degree of maternal impact on the progeny: reduced seed size, seed nutrient content, or hormone content could convey reduced seedling vigour which would plausibly explain the reduction in performance in the second generation. Within-generation costs are likely explained by changes to hormone profiles and root and shoot development, as opposed to a material change in drought susceptibility, or priming response.

5.5.3. Limitations

From these results I was unable to identify any 'stress memory' that was able to overcome the imposed water deficit – if anything, recurrent stress exacerbated growth penalties. This could be due to several factors, including the combination of stress duration, intensity, and timing, due to the genotypes selected, or the growth conditions of the plants themselves – or simply because these mechanisms do not exist in many wheat landraces. If the initial drought was too intense, it could have imposed costs on later development – something potentially seen in the results for D1C2 plants in the intragenerational experiment, which exhibited reduced biomass and main head yield compared to their control group – and ultimately limit the growth and yield potential of the plant by affecting leaf and root architecture in a way that outweighed any priming benefits (Zhang et al., 2025e; Shazadi, Christopher and Chenu, 2024). I selected lines with a variety of drought stress phenotypes based on prior studies using the YoGI panel, but may not have tested enough genotypes in the current study: priming benefits appear to be highly genotype- and treatment-specific (Dueñas et al., 2024; Amini et al., 2023), suggesting that a larger screen of the YoGI panel could sift out landraces that do exhibit priming benefits under moderate stress conditions.

Further work is needed to identify lines capable of stress priming in order to begin to dissect the mechanistic basis of such responses. Future attempts should examine a broader panel of wheat landraces to capture more genetic diversity and identify drought-sensitive genotypes, test a variety of priming intensities and regimes, and pinpoint critical periods for 'stress memory' formation. Additionally, sampling tissue for transcriptomic and methylomic profiling would help elucidate the mechanisms behind priming responses, should they arise.

It is also important to note that the phenotypic data here, had it allowed for the successful observation of drought priming, would not alone be evidence of epigenetic inheritance, especially when considering potential maternal effects or other adaptive mechanisms. Identifying epigenetic inheritance – or epigenetic marks – as a mechanism that can enable drought priming responses would require the observation of sustained epigenetic marks linked to beneficial phenotypes and gene expression profiles. These marks may also not necessarily be observable in DNA methylation; priming could be conveyed through any of the other epigenetic mechanisms.

5.6. Conclusions

Despite evidence pointing towards wheat cultivars possessing the capacity for drought priming responses, the subset of lines tested from the YoGI panel did not demonstrate any benefit in drought tolerance after priming with drought stress applied at the seedling stage, and in some cases exhibited negative outcomes, suggesting that cumulative stress imposed long-term developmental penalties rather than conferring 'stress memory'. This may have been due to growing conditions, stress conditions, genotype, or a combination of the three. Further work is required to identify landraces from the YoGI panel that possess drought priming capabilities and to optimise priming conditions, and to elucidate any mechanisms behind drought priming in major crops like wheat that might be exploited to help them acquire greater stress tolerance.

5.7. Author Contributions

IJR and AH conceived and planned the project. IJR conducted plant growth experiments and tissue sampling. IJR conducted phenotypic analysis in R. IJR wrote the chapter in its entirety.

6. Discussion

From the outset, this body of work has fundamentally been an exploration of the genomic and transcriptomic regulation of the wheat drought regulome, and how these regulatory mechanisms affect phenotypes both physiologically and temporally. Across the chapters of this thesis, I have aimed to explore the way in which wheat landraces respond to drought at the transcriptomic level (**Chapter 2)**, followed by an examination of the trends in gene expression that may be linked to drought tolerance (**Chapter 3)**, before diving further into the epigenetic regulation of the drought response (**Chapter 4)**, and finally aiming to identify whether these epigenetic marks may be sustained and heritable in a way that positively benefits wheat's drought resilience (**Chapter 5)**. In this thesis, I have identified candidate genes that may be key parts of wheat's drought response and basal tolerance levels, and uncovered potential epigenetically controlled transposable element-driven drought response mechanisms; all of which could be of interest to crop breeders aiming to produce more drought-resilient wheat. Though **Chapter 5** was unable to observe drought priming mechanisms, and **Chapter 4** was under significant statistical and economical constraints, this thesis represents significant steps forward for our understanding of wheat's drought response, and the ways in which it is regulated.

By utilising the YoGI landrace panel and its broad phenotypic and genetic diversity, I sought to identify key regulators that determine how both drought-sensitive and drought-tolerant wheat landraces respond to early water deficit events, the inherent tolerance traits that propel growth under stress, the overarching regulatory mechanisms that might control these responses, and explore these regulatory mechanisms might be beneficial in the long term.

Application of underutilised analyses in wheat have formed the basis of this work: though publications using co-expression network analyses to parse transcriptomic patterns in response to stresses have increased in recent years, using networks across landraces with diverse genetic and phenotypic variation alongside stress-induced transcriptomic reprogramming is rare. Furthering this through examination of wheat's methylome using whole-genome bisulphite sequencing and paired transcriptomes in response to early drought events represents a first for the field.

The use of landraces has proved to be an effective approach to studying the regulatory basis of the wheat drought response and the identification of potentially novel genes in the regulation of stress tolerance. It has also been useful for capturing the clearest signals among broad variation in methylation signatures in response to drought. The YoGI landrace panel captures an enormous amount of genetic and phenotypic diversity due to its accumulation of mutations from environmental selection pressure as it spread beyond the Fertile Crescent, which in turn makes it a valuable genetic pool from which to introduce novel beneficial alleles into elite crop varieties. Wheat landraces have rarely been used to identify candidate transcriptomic master regulators to stresses like drought through WGCNA – extant studies are mostly from the Harper lab – marking a significantly underutilised scheme of study. This thesis, as mentioned previously, marks a first in the genome-wide profiling of the wheat drought methylome, but the use of landraces in studies like this is also novel, potentially capturing a wider variety of methylomic responses that may be applicable to a wider range of wheat genotypes than just one specific variety.

In summary, this thesis can hopefully contribute not only to the wheat scientific community's understanding of stress regulation and the transcriptomic and epigenetic changes under stress at a critically understudied period of growth and inform the wider scientific community of potential methods that could be applied to future studies in other commercial crops. Beyond this, these findings may also provide wheat breeders with potential targets for marker-assisted breeding programmes and a greater understanding of the mechanisms that govern these targets.

6.1. Key insights into the drought regulome of wheat landraces

In **Chapter 2**, this body of work first examined the way in which wheat landraces respond to drought stress in the early vegetative stages, with the aim of understanding how the wheat transcriptome is reshaped under harsh water-deficit conditions. Establishing this transcriptional understanding of how wheat seedlings respond to drought provided a base for the subsequent chapters to build upon: once we understood *how* wheat responded at the transcriptomic level, I could examine the role of inherent drought tolerance and how it interacts with the drought response, potentially identifying novel traits among these landraces, before investigating the role of DNA methylation in mediating the response to drought among tolerant landraces, and finally exploring the potential of drought priming as a mechanism for improving drought tolerance.

From the wider YoGI panel, 20 landraces were selected based on prior work carried out by **Chapter 2**'s co-author, Liam J. Barratt, that measured seedling drought phenotypes across the panel. Fourteen lines were carried forward after determining their drought tolerance phenotypes for use across **Chapter 2-4**. Plants underwent a harsh drought stress event at the three-leaf stage, with water withheld for 10 days, letting average soil moisture reduce below detectable levels. Tissue was sampled before and after the drought event and sequenced, allowing for quantification of gene expression per landrace and timepoint. As expected, early drought stress had significant adverse effects on growth.

We utilised this data to identify genes that were significantly different both before and after drought (**Chapter 2**) and between phenotypically drought-tolerant and susceptible landraces (**Chapter 3**). We constructed a weighted gene co-expression network to further elucidate the expression patterns between our groups of interest. We observed massive transcriptional reprogramming in response to drought treatment, with over

10,199 genes found to be differentially expressed between the before drought (BD) and after drought (AD) groups. Upregulated genes were enriched for water deprivation and oxidative stress responses, cellular maintenance, and the regulation of gene expression, while downregulated genes were largely associated with photosynthetic processes and machinery or involved in cellular homeostasis. It must be noted that the experiment targeted differences in relation to drought over a short time span; though drought can induce developmental arrest as a resource-reallocation or DNA-damage-mitigation strategy (Zhang, Zhao and Zhu, 2020; Serrano-Mislata *et al.*, 2025), some of these differentially expressed genes will likely have been involved in enabling continued development under drought, especially among more tolerant lines, something that was taken into account with both this and future analyses.

Differential expression analysis alone however cannot fully resolve the relationships between drought-associated genes in the dataset. Construction of the co-expression network enabled us to further dissect the transcriptional relationship between these genes, resulting in the identification of 23 drought-associated modules, the hub genes of which were determined to be good potential master regulators of the early drought response. Several modules provided promising candidate hub genes, with two standing out most: *TaDHN4* (*TraesCS5D02G379200*), an upregulated dehydrin-encoding gene, and an uncharacterised gene (*TraesCS3D02G361500*) potentially involved in stomatal control. We hypothesised that *TaDHN4*, beyond its conventional role as a ROS scavenger, DNA- and protein-binding molecular chaperone, and hydrophilic water carrier (Liu et al., 2017b; Zhang et al., 2018a), could also be involved in the regulation of downstream dehydrins and homoeologues by maintaining transcription factor stability or through direct DNA-binding activity. The uncharacterised hub gene however appeared to be involved in controlling stomatal aperture, cell turgidity, and stomatal morphogenesis under water deficit conditions, though this was inferred from its connected genes and weak orthologues.

Chapter 3 built upon the understanding gained from Chapter 2 by shifting focus to the genes in the network that may underpin inherent drought tolerance. Comparing differential expression analyses between tolerance-associated and response-associated DEGs revealed a substantial overlap, with 27.1% of the 560 tolerance DEGs also found to be drought-responsive, exemplifying the contribution of tolerance genes to the drought response rather than isolated tolerance traits and demonstrating the importance of examining traits in their full context. Of the genes more highly expressed in tolerant lines, several jasmonic acid and water-deprivation associated GO terms were found to be enriched, while genes with lower expression in tolerant lines were enriched among biotic and other osmotic stress GO terms. The two tolerance hub genes, *TaWRKY19* (*TraesCS5D02G145800*) and *TaJOX1* (*TraesCS1D02G072700*), were both found to be both tolerance and response DEGs: while both were positively associated with drought tolerance, the former was upregulated under drought conditions while the latter was downregulated. I hypothesised that both hub genes were involved in modulating jasmonic acid signalling, preventing JA hypersensitivity in tolerant lines and therefore enabling greater growth under drought stress conditions. Interestingly, a homoeologue of *TaJOX1* was connected to the uncharacterised hub gene from **Chapter 2**, suggesting a possible link between the JA hydroxylation hub gene and stomatal aperture regulation.

Exploring the drought regulome's control at higher epigenetic levels made for a logical next step: **Chapter 4** built upon the understanding of **Chapters 2** and **3** through the understudied lens of DNA methylation, with the aim of identifying links between changing DNA methylation and changing gene expression and better understanding the trends occurring across wheat's enormous genome – observations that could not made from transcriptomics alone. This work represents the first high-resolution genome-wide analysis of wheat's drought methylome and the second whole-genome bisulphite sequencing-based methylome study in wheat under any stress (Singh et al., 2022a). Using pooled before- and after-drought samples, I identified localisation-specific methylation profiles that were associated with gene expression, finding that CG methylation in gene bodies was significantly positively correlated with expression, but significantly negatively correlated with $\log_2$FoldChange under drought; similar trends were observed in CHG methylation, albeit at a reduced magnitude, while CHH methylation showed no significant associations. These observations were largely congruent with previous studies showing that gene body methylation can buffer transcriptional noise (Huh *et al.*, 2013; Zastąpiło *et al.*, 2024), and support emerging evidence that gene body methylation plays a role in regulating the ability of genes to respond to stress (Wang et al., 2021e; Lee et al., 2023), perhaps by inhibiting deposition of the histone variant H2A.Z (Zilberman et al., 2008; Miao et al., 2024; Sura et al., 2017; Long et al., 2023). This interpretation was furthered by stress-associated functional enrichment of gene body DMR-associated genes, though interestingly very few of the candidate hub genes from **Chapter 2/ 3** were proximal to DMRs, and few DMR-associated genes were found to also be differentially expressed, suggesting changes in gene-body methylation may result in subtle or temporally delayed effects on expression.

Among transposable elements I identified several families overrepresented among DMRs. The most notable family was *RLX_famc9*, a long terminal repeat (LTR) retrotransposon family lacking extant protein domains that was weakly downregulated under drought conditions. *RLX_famc9* was found to show significant sequence similarity to DMR-associated genes, suggesting a potential *trans*-regulatory mechanism governed by DNA methylation-mediated silencing through the RdDM pathway; the family was found to be overrepresented in DMRs across all three methylation contexts, the DMRs of which tended to cluster in the first third of the TE sequence. Comparison of the reads that mapped to the TE sequence and the DMR localisation suggested the presence of a transcriptionally active region that I hypothesised could generate double-stranded RNAs, which could ultimately be cleaved into siRNAs which would target genes with sequence similarity to the TE. These genes were enriched among chloroplastic GO terms, suggesting that RLX_famc9 may play a role in regulating photosynthetic genes.

Comparatively few conclusions could be drawn from differential methylation associated with promoter, downstream, or intergenic regions. Gene-flanking regions failed to show functional enrichment, though they did appear to contain targeted drought-associated transcription-factor binding motifs, while the under-annotation of non-genic or non-TE regions in wheat limited the interpretation of intergenic DMRs. Despite this, I hypothesised that these regions must have some function – changing methylation states is likely energetically expensive, so they must be occurring for a reason – and that this function might be involved in priming genes to respond better to stressors in the future.

With this hypothesis in mind, in **Chapter 5** I aimed to explore drought priming and 'stress memory' in wheat. Drought broadly had impacts on agronomic traits, with significant negative effects on normalised biomass and yield: I found no evidence that drought priming treatment was able to improve biomass or yield across generations. Within-generation priming produced similar trends, with primed landraces exhibiting reductions in yield, biomass, and harvest index compared to unprimed landraces – priming treatment appeared to compound the deleterious effects of water deficit at the secondary drought event, rather than ameliorate them. Interestingly, I observed a consistent but slight delay in floral phenology following harsh early drought stress, as opposed to the conventional drought-escape mechanism, suggesting that developmental arrest as a result of harsh early drought stress may cause long-term effects on wheat that are not recovered, unlike some traits which largely catch up to their control counterparts by harvest.

Broadly, this thesis charts a progression from defining the transcriptional landscape of the early drought response in wheat landraces, pinpointing major players in the drought transcriptome, to identifying genes associated with promoting a form of drought tolerance under stress conditions, to elucidating the nuanced role of DNA methylation in mediating genome-wide transcriptional activity of genes and TEs, and finally testing the potential temporal effects of DNA methylation changes by phenotypically testing landraces for drought priming responses. This body of work finds that the drought response to wheat is governed by widespread and coordinated changes at both the transcriptional and epigenetic levels, with certain genes and phytohormones – like dehydrins and the JA-signalling pathway – emerging as key players in the drought response and in determining tolerance. Methylation at genes and TEs was found to be associated with transcriptional activity and stability, though its effects appear nuanced, subtle, and context dependent. Priming responses were found to be absent among the landraces under the experimental conditions used, suggesting that early drought may not convey benefits to wheat under recurrent stress events. Together, these findings further our understanding of the molecular framework that underpins drought amelioration in wheat, highlighting promising regulatory targets for wheat breeders and providing an impetus for further study of the wheat methylome.

6.2. Experimental design: limitations, approaches, and considerations

The work in this thesis came with several broad limitations: practicality has constrained the number of replicates able to be used for some experiments – like the analysis in **Chapter 4** – which ultimately limits the confidence in their interpretation. TILLING validation experiments in **Chapters 3** and **4** and the priming experiments in **Chapter 5** were similarly under replication limitations, with the former held back by seed availability for plants with a TILLING mutation load (both mutant and WT for the allele of interest) and the latter held back by the aim of keeping a manageable plant population size. The wheat genome itself has also proved to be somewhat of a limitation, constraining the scope of **Chapter 4**'s analysis both through its size making WGBS computationally/ financially costly and its complexity complicating analysis. Additionally, our use of different landraces was in some way a limitation: the reference genome assembly was based on the landrace Chinese Spring which may possess substantial genomic differences from those in the YoGI panel. Furthermore, validation experiments were constrained by the inherent mutation load of TILLING lines, which ideally would have been backcrossed more than once and selfed further to reduce the mutation load and increase homozygosity. These limitations only serve as boundaries on interpretation however, and do not substantially diminish the conclusions of this thesis. With this in mind, several other considerations were made across the experimental design of this thesis.

The use of landraces in this study enabled us to maximise the amount of diversity captured across each experiment while maintaining cost-effectiveness. The dataset used in **Chapters 2** and **3** represented over 14 different landraces with a variety of drought tolerance phenotypes, with each sample itself a pool of 3 replicates to control for variation within each landrace. We then considered each landrace a biological replicate for the purpose of the differential expression analysis, with the full diversity of this data then observed in the co-expression network. This could be argued to be a limitation of the data used in this thesis, though evidence like the PCA present in **Chapter 2** suggests that the expression changes seen in high-variance genes in response to drought were consistent enough to cleanly segregate between the BD and AD groups, with a smaller proportion of variance attributed to differences between genotypes.

WGBS is considered to be a 'gold standard' approach to profiling the methylome (Kernaleguen *et al.*, 2018; Olova *et al.*, 2018), though continually developing technologies like Nanopore are becoming more accurate and performant (Sigurpalsdottir et al., 2024; Angeloni, Ferguson and Bogdanovic, 2022). The trade-off for using technologies like WGBS in complex polyploids is largely cost, with extensive sequencing time required to achieve desired genome coverage and read depth (Adusumalli *et al.*, 2015; Wreczycka *et* *al.*, 2017), something mitigated in this study by foregoing individually-sequenced biological replicates and high statistical power in favour of pooling samples to make the sequencing viable. DNA samples from phenotypically tolerant lines were pooled in equimolar proportions into two groups (BD and AD) that were paired with the individually sequenced RNA samples from **Chapter 2**. This pooling approach has previously been applied in various scenarios (Kaplow et al., 2015; Docherty et al., 2009, 2010) though still remains underutilised in the study of large plant genomes. This pooling approach at a suitable sequencing depth and genome coverage allowed me to capture diversity in methylation across 7 different landraces, with the idea that equal individual contribution from each line would enable identification of the strongest signals – the ones that were consistent across the constituent samples – and that inconsistent sites would tend towards middling methylation levels. With some stringent pre-filtering and sense checks, I determined that the data was appropriate for differential methylation analysis. Despite this, as mentioned previously, this approach can massively reduce statistical power at individual sites: by using the DSS package, which takes into account spatial methylcytosine correlation (Wu et al., 2015a), I were able to effectively use region-based information to profile wheat's drought methylome.

This thesis has also touched on the differences between a plant responding to drought, and a plant being inherently more tolerant to it: tolerance here was defined as the relative ability of a plant to grow under stress, quantified as normalised biomass, the dry biomass of plants under stress conditions expressed as a proportion of the dry biomass of their control counterparts, though this is only one facet of tolerance. Not all would agree that it is directly representative of stress tolerance, though I posit that it is at least a significant proxy for tolerance and therefore is an appropriate phenotype to study, if perhaps a simplification of several individual tolerance traits. Previous studies have used similar measures of tolerance for exploring the impact of various stresses (de Jong and Lin, 2017; Henderson et al., 2020; Li et al., 2022a; Sherstneva et al., 2024), though others have framed drought tolerance in a more holistic manner as the capacity of plants to carry out transcriptional reprogramming and physiological remodelling to escape drought conditions (Bashir *et al.*, 2021; Haghpanah *et al.*, 2024) or as a combined trait measured through changes in stomatal conductance, osmolyte accumulation, and metabolism (Farooq *et al.*, 2024). I also observed a slight negative correlation between control biomass and normalised dry weight per landrace under drought conditions, perhaps exposing a weakness of this measure of tolerance: seedlings with greater foliar biomass likely have a greater transpiration rate, potentially meaning that drought conditions would be more deleterious. Despite this, the correlation was weak and did not account for the majority of variation between our landraces with distinct drought phenotypes. Nonetheless, I posit that the candidate transcriptional master regulators identified in **Chapter 3** represent genes involved in drought tolerance due to their putative role in facilitating continued growth under drought stress conditions.

6.3. Drought-responsive gene regulation in wheat is likely controlled by complex interplay between transcriptomic and epigenetic sources

This thesis has explored the role of both transcriptomic and methylomic changes that are induced in response to water deficit conditions, finding massive transcriptional reprogramming accompanied by overlapping but nuanced genome-wide changes in DNA methylation. Use of co-expression networks in **Chapters 2** and **3**, and the examination of the genic methylome in **Chapter 4**, exemplify the importance of understanding how genes interact and regulate each other at all levels.

Our results suggested that DNA methylation plays a role in subtle *cis* regulation of gene expression under drought, with the majority of functional changes in genic regions focused on the gene bodies. I determined that gene body DNA methylation was positively associated with gene expression levels, and negatively associated with responsiveness under drought, supporting the idea that DNA methylation promotes a stable high baseline expression (Pisupati et al., 2023; Zastąpiło et al., 2024; Wang et al., 2021e). I hypothesise that DNA methylation state at the gene body, and perhaps transcription factor binding motifs, may act similarly to chromatin structure, inhibiting binding of regulator proteins, while hypomethylation instead may 'open up' the DNA for regulation by transcription factors and other facilitative proteins or by influencing transcriptional efficiency. In *A. thaliana* gene body methylation has been suggested to explain substantial amounts of gene expression variance, with gene body methylation alongside histone modifications linked to the control of genes like *FLC* (Shahzad et al., 2025; Sheldon et al., 2000; Jean Finnegan et al., 2005), and potentially also playing a role in the stress-responsiveness of genes to facilitate rapid adaptation to the environment (Shahzad *et al.*, 2025), as well as being associated with regulation of lowly expressed genes, which would likely include stress-responsive genes under normal conditions (Pisupati *et al.*, 2023). The concept of DNA as a subtle modulator of gene expression is furthered by the observation that genes that were associated with differential methylation in **Chapter 4** were often at the periphery of the co-expression network modules from **Chapter 2**, exhibiting weaker degree scores than transcriptional hub genes. Of the hub genes identified in this thesis, only one was found to be associated with differential methylation, suggesting that epigenetic regulation may have broader but more subtle effects on gene expression compared to both direct and indirect transcriptomic regulators.

Promoter regions, however, were less forthcoming with conclusions than gene bodies; I was unable to identify any enrichment of biological functions across all three methylation contexts, suggesting that changes in promoter methylation may not be a major *cis*-acting target in wheat's drought response, or that it only regulates a small proportion of genes. The latter is supported by the fact that only a small portion of DMRs appear to localise to promoter regions under drought stress in **Chapter 4**, the broad lack of high promoter methylation in model species (Zhang, Lang and Zhu, 2018) and the large proportion of proximal TEs in large polyploids interrupting promoter regions (Wicker et al., 2018; Sabot et al., 2005), though these TEs may act as demethylation targets under stress to activate expression of their nearby genes (Le *et al.*, 2014; Secco *et al.*, 2015). DNA methylation however is known to be variable – though may broadly follow the same trends – between plants, even of the same or closely related genotypes and under the same conditions (Wilkinson et al., 2023; Ronchi et al., 2025; Vanden Broeck et al., 2018; Becker et al., 2011), suggesting that promoter-specific variation could have been plant-specific and therefore not captured in this pooling approach, something that would be improved by increased sample sizes. Ultimately, the lack of clear promoter targeting supports the idea that changes in gene-body methylation may be the primary target of *cis*-regulatory genic DNA methylation under harsh drought conditions in wheat.

As previously mentioned, other epigenetic mechanisms can also have an impact on transcriptional control. One major mechanism proposed in **Chapter 4** was the RdDM-dependent siRNA activity of *trans*-regulatory TEs at complementary genes; TE-generated siRNAs have been implicated in RNA interference processes and stress regulation (Wilkinson et al., 2023; Tanurdzic et al., 2008; Panda and Slotkin, 2020; Gill et al., 2021), suggesting that responsiveness of such a mechanism is plausible under drought conditions. Recent studies have explored the role of miRNAs in the wheat drought response (Sharma et al., 2025; Ulu, Unel and Baloglu, 2025; Gómez-Martín et al., 2023), though siRNAs are understudied. Landraces may house novel sRNAs or have genotype-specific sRNA responses (Bizabani, Rogans and Rey, 2021; Wu *et al.*, 2025), suggesting that the YoGI landrace panel may be a promising source of small RNA diversity under drought conditions, and exploration of this other epigenetic mechanism may support **Chapter 4**'s findings.

One aspect of wheat's drought response identified in **Chapter 4** was the vast intergenic regions that held a substantial proportion of the identified DMRs across all three cytosine contexts. Stress-induced methylation changes at intergenic regions have previously been identified in *A. thaliana*, rice, and poplar (Wang et al., 2016a; Vining et al., 2012; Yong-Villalobos et al., 2015), suggesting in plants the intergenic spaces may contain a wealth of as-yet unannotated regions, harbouring features like enhancer regions, long noncoding RNAs, or transposable elements. These DMRs – especially over large genomic spans – may also be involved in 3D chromatin structure, with DNA methylation known to play a role in mediating chromatin accessibility and forming topologically associating domains (TADs) (Buitrago et al., 2021; Sun et al., 2022b). This could be investigated using a Transposase-Accessible Chromatin (ATAC-seq) assay that would enable the profiling of regions of open chromatin and comparison to the WGBS methylome data, identifying regions rich in DMRs that also have an open or closed structure. This would help to associate these intergenic DMRs with potential functions and help to identify both *cis*- and *trans*-regulation of gene expression from intergenic sources.

Regulation of plant stress responses should be considered in a holistic manner: each regulatory layer or epigenetic mechanism has some level of interplay with others. The stacked drought regulatory system, comprised of hormone signals, transcriptional networks, small RNAs, DNA methylation, chromatin structure, and more, will undoubtedly have links and nexus points that are yet to be uncovered. The dissection of wheat's drought response in this thesis touches upon the role of hormone signalling in coordinating the transcriptional network, and vice-versa, likely through the activation of transcription factor and TF-like proteins, a relationship which is well trodden; ABA and JA signalling pathways, for example, are known to promote the expression of core stress-responsive TFs (Barratt, Franco Ortega and Harper, 2023; Yu et al., 2020a; Ren et al., 2022) which can make strong candidate transcriptional master regulators. Similarly, there is a strong link between epigenetic mechanisms: TE-derived siRNA production is known to be involved in self-silencing and silencing of nearby regions through the recruitment of DNA methylation and increased heterochromatinisation (Zhang, Lang and Zhu, 2018). Research also supports the role of DNA methylation in modulating gene expression, linking back towards the transcriptional networks, with known temporal effects on the expression of genes in stress hormone pathways (Zhang et al., 2025b; Shahzad et al., 2025; Zastąpiło et al., 2024; Wilkinson et al., 2023). Taken altogether, I propose a model in which wheat's drought response is governed by DNA methylation and chromatin structure by acting as a gatekeeper to regulatory elements, reinforced through TE-derived siRNAs and the RdDM pathway, which then influences the structure of stress-responsive gene networks that interact with stress hormones in a feedback loop.

Despite the support for a model like this, the analysis in this thesis is unable to identify direct causal relationships between the transcriptional and epigenetic regulatory layers: while I can identify correlations and associations between differentially methylated regions, stress-induced gene expression, changes at TFBSs, and TEs, there are too many unknowns to infer actionable and causative relationships without functional validation and further exploration. The identified methylation patterns give rise to potential mechanisms, but whether these methylation changes are causative to changes in TE or gene expression remains to be seen, especially given the finding that DMRs were not strongly associated with changing gene expression. While current evidence from the field and this thesis supports a potential model in which stress stimuli and hormone signalling can activate genes within a drought-responsive transcriptional network potentially shaped by DNA methylation, small RNAs, and chromatin, the data in **Chapter 4** only reveals correlative links between these regulatory layers and is better considered as exploratory.

6.4. Drought has potentially contradictory influences on floral phenology

Floral development and genes underpinning it represent a subtle but recurring theme in all chapters of this thesis. Drought stress is known to alter wheat floral phenology or flowering-related gene expression (Riboni *et al.*, 2016; Gu *et al.*, 2022; Nyaupane *et al.*, 2024), with **Chapters 2, 4,** and **5** all corroborating this; early drought treatment was found to induce a slight but consistent delay in flowering compared to plants under control conditions (**Figures 5.2 & 5.3**).

Examination of potential drought-responsive master regulators in **Chapter 2** identified an orthologue of *RICE FLOWERING LOCUS T 1-like* (*TraesCS3D02G144500; FT*) as a putative hub gene. It was identified as the upregulated (response LFC: +3.3) hub gene of the midsized midnightblue module, with almost uniform expression across the drought-susceptible and drought-tolerant groups. In wheat, *TraesCS3D02G144500* has been identified as *FT2*, a subfunctionalised copy of *FT* involved in spike development (Tan et al., 2025; Yang et al., 2021). *FT-like* genes are positive regulators of flowering, are known to be upregulated following drought, and may potentially play a role in integrating stress signalling and the flowering pathway (Su *et al.*, 2013; Gu *et al.*, 2022). *FT* and flowering genes would return in the results from **Chapter 4**: of the hub genes identified, the *FT* hub was the only one associated with differential methylation, containing CG and CHG hypermethylated DMRs in its 3' downstream region. Stress-responsive downstream DMRs have previously been identified in response to drought in rice (Kou *et al.*, 2022), though the function of downstream DNA methylation is somewhat unclear; downstream DMRs may influence RNA polymerase II transcription termination, be involved in gene-proximal TE silencing, or promote heterochromatinisation downstream of *FT*, potentially to counteract early *FT* expression, or to act as a marker to promote recruitment of methylation upstream.

DNA methylation was further implicated in the control of floral development: the GO term 'inflorescence morphogenesis' was enriched in hypermethylated gene-body DMRs (**Supplemental Data 4.3**), suggesting that the plant may be acting to initiate floral development as part of a drought escape mechanism, much like the hub gene is hypothesised to. Several DMGs annotated with this GO term were identified as *LRR-RLKs* involved in meristem development and phytohormone signalling. Transcription factor-binding motif enrichment of gene-flanking DMRs also identified both hypo- and hypermethylation of motifs related to the *REM, VRN*, and *LAV* transcription factors, known positive regulators of flowering (Liu et al., 2025a; Chen and Dubcovsky, 2012; Yu et al., 2020b), suggesting nuanced regulation of flowering TF targets via the RdDM pathway. *REM* and *VRN* TF expression is positively correlated with *FT,* linking back to the hub gene identified above (Chen and Dubcovsky, 2012; Deng et al., 2015; Yu et al., 2020b), indicating a role for drought-induced modulation of DNA methylation in regulating floral development. Some form of methylation playing a role in floral development is not new: additive histone methylation is key to the vernalisation pathway and control of the flowering repressor *FLC* (Bastow *et al.*, 2004) and DNA methylation is increasingly thought to play a role in both vernalisation and photoperiod-associated flowering through *FT* and *FLC* (Shi et al., 2023; Duan et al., 2017; Yang et al., 2022b).

Upregulated expression of genes like *FT* would normally be expected to accelerate heading, yet identical treatments to that used in **Chapters 2-4** were found to bring about a degree of floral delay. This may suggest that harsh drought events can induce competing pathways, simultaneously activating an *FT*-driven drought escape response and promoting floral development while also bringing about an arrest in wider growth and development. Profiling the expression of *FT*-like genes across this period of growth – both under normal well-watered conditions and drought conditions – would help to understand how drought stress can affect this critical developmental period, as *FT*-like genes may have been upregulated between the two sampling points anyway. Consequently, early harsh drought may cause temporal changes to floral phenology that could be of agricultural significance: better understanding the way in which these floral changes are brought about may be of interest to wheat breeders and farmers who may seek to mitigate these changes if the same trends are seen among elite cultivars.

Ultimately, understanding the true impact of early drought treatment on floral phenology would require repeated sampling and sequencing of plant tissue at regular intervals, up until the floral transition: identifying early drought induction of consistently identified methylation signatures at genes involved in floral development, transcription factor binding sites, and consistently altered expression of genes like *FT* would inform on the molecular impact of drought on floral development. Meristem dissections and sequencing of meristem cells may also provide a greater understanding of the role of these changes on floral development.

6.5. Polyploidy, epigenetic diversity, and the unidentified basis of stress priming

The complex allohexaploid nature of wheat's genome has been a recurring theme throughout several chapters of this thesis. Ploidy has clearly played a substantial role in wheat's agricultural success, contributing to many of its desirable traits and enabling its radiation out from the Fertile Crescent. Polyploidy has provided wheat with a wealth of genic content: many genes have one or two homoeologues, enabling neofunctionalisation, functional redundancy, and potentially compensatory behaviour, but it has also shaped gene regulation and the methylome.

The hub genes identified in **Chapters 2** and **3** tended not to behave in isolation: their homoeologues showed similar patterns of expression, and were either directly connected in the network, or showed similar patterns of expression in very closely related modules. TaDHN4 was connected to its homoeologues, suggesting that they may work in concert, while JOX1 was connected to both homoeologues and paralogues. Analysis in **Chapter 4** identified stress-responsive homoeologue triplets that were similarly differentially methylated, suggesting conserved rather than subgenome-specific responses – though there were still a substantial number of genes that were differentially methylated while their homoeologues were not. This aligns with reports that a many triplet homoeologues may express a degree of subgenomic dominance (Leach et al., 2014; Feldman et al., 2012), something which may be influenced by DNA methylation patterns and TE localisation (Bird et al., 2021; Wang et al., 2022b). Evenly expressed homoeologues may contribute additively, underpinning one of the agricultural benefits of polyploidy, while divergent homoeologues may reflect a degree of neofunctionalisation or specialisation of pleiotropic genes. Where multiple homoeologues are housed within the same module, it is likely that they would be better analysed as a collective; multiple well-connected hub genes within a module would comprise a more robust marker for drought response control or tolerance than individual genes, accounting for more nuance in phenotypic variation across the dataset (Barratt et al., 2023a), though this effect is likely diluted by noise of weaker co-expressed genes when using the entirety of a large module.

Genome dominance in polyploids like wheat is thought to have played a substantial role in their domestication and contributed to this homoeologue expression bias (Wang et al., 2022b). DNA methylation and chromatin accessibility are thought to be associated with controlling genomic dominance and mediating gene dosage (Jia et al., 2025; Li et al., 2023b; Alger and Edger, 2020). In **Chapter 4** the three subgenomes appeared to exhibit subtly different methylation patterns: analysis of genome-wide differentially methylated CG sites (**Appendix 1.2**) indicated that the BB genome was significantly overrepresented in differential methylation relative to the overall genome, suggesting that the genomes may display subgenome-specific DNA methylation patterns under drought conditions. One genome being able to undergo more extensive DNA methylation remodelling than the others, or genome-specific DNA methylation patterning, has been observed in other polyploids (Edger *et al.*, 2017; Ziegler *et al.*, 2023), and appears to be associated with relative TE level – though the three subgenomes of the wheat genome have similar proportions of TEs (Wicker *et al.*, 2018), the BB genome is most abundant in TEs, which may give rise to its greater proportion of DNA methylation plasticity.

Chapter 5 identified no substantial benefit of drought priming on drought tolerance to secondary stresses across any of the landraces, contrary to observations in other varieties and species, raising the question: what contributes to a plant's ability to exhibit a drought priming response? 'Exotic' cultivars of wheat – synthetic polyploids and varieties grown outside of their niche – have been shown to exhibit greater benefits from priming approaches than more conventional lines (Amini *et al.*, 2023), suggesting that both genetic diversity and unfamiliar stress regimes may play a role. Genetic and epigenetic diversity only sometimes show significant correlation (Wang et al., 2020c; Chen et al., 2024c; Venetsky et al., 2015), suggesting that genetic diversity may not directly convey a level of epigenetic plasticity that could underpin stress priming responses; some findings may indicate that a malleable epigenome instead compensates for low genetic diversity in the population (Medrano et al., 2020; Schrey et al., 2012; Boquete, Muyle and Alonso, 2021). Nonetheless, greater genetic diversity in a population has been linked to more effective 'stress memory' responses in polyploid grasses (Safari and Majidi, 2025; Habibabadi et al., 2025), suggesting that identification of novel polyploid lines formed from highly diverse progenitors may provide a path towards identifying prime-able lines in the YoGI panel. Screening tile plots of the YoGI panel (Barratt et al., 2023a) for interesting rearrangement, regional deletions, or homoeologous exchanges may help to identify landraces with novel genomic profiles for further priming study.

6.6. Future directions and implications for crop improvement

6.6.1. Validation and characterisation

Further work is needed to properly characterise and validate candidate transcriptional master regulators of the drought response and drought tolerance. Our exploration of the drought response and drought tolerance using co-expression networks identified four genes of particular interest: two response genes (*TaDHN4* and the uncharacterised hub gene) and two tolerance genes (*TaWKRY19* and *TaJOX1*). While these genes, identified through their expression profiles, co-expressed genes, and putative functions, are strong candidates for master regulators, their exact role and functions are largely yet to be examined in depth.

The uncharacterised drought response hub gene is by far the most in need of validation and further study, with weak sequence orthologues and its function mainly derived from its predicted localisation and its connected genes. Identification of any domains or structures displaying similarity with other proteins was explored using AlphaFold Server and FoldSeek (van Kempen et al., 2024; Abramson et al., 2024) with default settings; by predicting and comparing the 3D protein structure of the uncharacterised hub gene to other known structures, I was able to identify weak similarity (29.1%, e-value = 1.08e-11) to a tetratricopeptide repeat (*TPR*)-like superfamily protein in *Glycine max* (soybean) (**Appendix 1.1**). The *TPR*-like superfamily is broad with many uncharacterised proteins, though the *TPR* domain is known to be involved in mediating protein-protein interactions (Schapire, Valpuesta and Botella, 2006; D'Andrea and Regan, 2003) and genes containing these domains have been identified as playing a substantial role in the drought stress response (Liu et al., 2024a; Rosado et al., 2006; Slawin, Ajayi and Mahalingam, 2024), though it must be noted that the hub gene here was downregulated under drought, as were its connected genes, suggesting that it may be a negative regulator of drought or govern processes only beneficial under control conditions.

To further the hypothesis that the uncharacterised hub is involved in protein-protein interactions, construction of a STRING protein interaction network might comprise an appropriate next step, with comparison to the co-expression network potentially identifying key interactions with the hub gene and improving functional inferences (Szklarczyk et al., 2023; Pardo-Diaz et al., 2021). Any findings could be experimentally validated using co-immunoprecipitation: Co-IP could help test the hypothesis that the hub gene's protein interacts with other proteins and help identify those targets.

As we hypothesised that the uncharacterised hub was involved in controlling stomatal aperture, TILLING knockout or transgenic overexpression lines could be generated, or viral-induced gene silencing could be employed, to examine the effects of altered gene expression on the phenotype under drought; overexpression (OE) lines would be expected to perform significantly worse during drought conditions under this hypothesised function. The stomatal density phenotype of mutant plants could be assessed to identify any differences in stomatal density compared to their WT siblings. Assessment of the hub gene's putative role in stomatal aperture control could also be carried out through measurement of stomatal conductance under drought with these mutants using porometer assays or thermal imaging (Batke et al., 2020; Vialet-Chabrand and Lawson, 2020; Craparo et al., 2017). Single-cell RNA sequencing (scRNA-seq) could be used alongside genetic transformation techniques with Green Fluorescent Protein (GFP) used as a reporter for imaging to confirm the transcriptional profile and localisation of the hub gene transcript and its translated protein to the guard cells.

Given the proposed function of the *DHN4* regulating the expression of its co-expressed dehydrins, STRING, Co-IP, and TILLING knockouts should also be utilised in its validation. Identification of the downstream proteins, like transcription factors, that it can chaperone under drought conditions would potentially identify other key drought genes for study.

Exploration of the drought tolerance-associated hubs using TILLING lines produced some interesting results. *WRKY19* validation failed to identify a significant effect of its knockout on drought tolerance, though this study was time- and replicate-limited. The effects of the *WRKY19* mutant should be re-assessed with a larger panel; in the current examination only three mutant individuals were in the control group, potentially skewing the data. This also could have been due to functional redundancy with or transcriptional compensation by its homoeologues, two of which were housed in a closely related module and displayed very similar expression patterns. In addition to repeating and expanding the screening, chromatin-immunoprecipitation sequencing (ChIP-seq) could be used to identify genomic loci that *WRKY19* may bind to, as could prediction of transcription-factor binding sites in the promoter regions of the connected genes.

JOX1, like the two drought response hub genes, is yet to be validated. Though *JOX1* is a putative regulator of drought tolerance, a TILLING knockout line was not thought to be an effective way of exploring its role under drought conditions due to its downregulation under drought conditions and likely nuanced role. I determined that knocking it out would likely be deleterious to normal conditions and it would be difficult to disentangle its role across the tolerance and response comparisons, unlike *WRKY19's* positive association with both. Despite this, OE lines may display an interesting drought phenotype: *JOX1* genes are known to downregulate plant immunity (Caarls *et al.*, 2017a), something ordinarily negatively correlated with drought-induced gene expression, and overexpression of other JA catabolism genes has been found to induce increased osmotic stress tolerance though reduced JA-hypersensitivity in rice (Ndecky et al., 2025; Kurotani et al., 2015; Ndecky et al., 2023). Collectively, these validation strategies should help clarify the regulation of the wheat's drought response and tolerance traits and hopefully support these hub genes and mechanisms as prime targets for wheat breeders and crop improvement programmes.

6.6.2. Improved genomic resources

Similarly, further characterisation of wheat's drought methylome based on improved genomic resources would be incredibly beneficial for wheat improvement and our understanding of abiotic stress regulation. Resources of non-model species like wheat lag behind those of species like *A. thaliana*, especially with regards to the epigenome – as detailed previously, whole-genome approaches to studying the wheat methylome are exceedingly rare for various reasons. Studies on *A. thaliana* have resolved epigenomes of methylation machinery, chromatin, and histone modification mutants, as well as maps of loci targeted by RdDM (Stroud et al., 2013; Ito et al., 2015; Lee et al., 2023), and a plethora of datasets across different growth stages and environmental conditions. Future directions for studying the wheat epigenome should aim to tackle this disparity, generating more epigenetic maps under different conditions.

Chapter 5's lack of evidence for drought priming benefits raised many questions about what contributes to a plant's ability to be primed, and how we might further explore this. The possibility of greater epigenetic plasticity giving rise to priming ability and greater stress tolerance provides an interesting point of future study: has the bottlenecking of intense domestication limited epigenetic diversity in the same way as genetic diversity? Wild crop relatives are thought to potentially exhibit greater epigenetic diversity with novel epialleles than their elite counterparts (Varotto et al., 2022; Venetsky et al., 2015; Medrano et al., 2020), yet the epigenomic landscape of elite crop varieties – especially in species like wheat – is largely uncharacterised, leaving the possibility that domestication has narrowed epigenetic plasticity and therefore adaptive potential. Future research into priming and the stress-responsive methylome should examine elite cultivars alongside diverse populations like landraces.

6.6.3. Mechanistically dissecting the drought methylome and transcriptome

Wheat's methylome could be further mechanistically dissected through the use of networks, much in the same way as the transcriptome: co-methylation networks could enable better exploration of genotype x environment effects on the DNA methylome. Modified WGCNA and co-methylation network approaches like Comethyl and iNETgrate have been somewhat successful at highlighting co-methylated genes and genomic regions associated with traits (Mordaunt et al., 2022; Mouat et al., 2023; Chhetri et al., 2020; Tremblay et al., 2019; Sajedi et al., 2023), and can be used to construct comparative networks to test for module preservation and identify genes that are both co-methylated and co-expressed, which would suggest that they are co-regulated. This approach is seldom used in plants, likely due to the cost required to obtain sufficient samples and difficulties in data preparation and network construction. One major difficulty in the use of current techniques is selection of regions to correlate: barring those that exhibit TE-like methylation patterns, genes are rarely uniformly methylated from beginning to end (**Chapter 4**; (Zhang *et al.*, 2016; Zi *et al.*, 2024)), with the partitioning of that methylation seemingly important, as early genic methylation is negatively associated with gene expression while later methylation is positively associated with it (Brenet *et al.*, 2011), meaning that simply taking an average of methylation across the gene is likely inappropriate. Promoter methylation appears to be a poor choice of region selection for network approaches in complex polyploid species like wheat due to their high genomic transposable element content: a large proportion of genes are very close to TE regions – the median distance between genes and upstream TEs is 1.52 Kb (Wicker et al., 2018) – and promoter regions are highly variable in length (Hammond-Kosack *et al.*, 2021), suggesting that without a solid annotation of the wheat promoter regions, quantifying per-sample promoter methylation state for use in a network approach may yield spurious correlations. Methylation networks, while potentially powerful for examining the role of co-methylation, likely need to examine genes in their wider context – somehow incorporating information of the methylation profile from its upstream to downstream regions – to maximise effectiveness. Multi-omic approaches using networks or correlations between gene expression and DNA methylation across samples remains rare, limiting exploration of the functional role of DNA methylation in plants, but may prove to be worth exploring in larger datasets.

6.6.4. Translational marker development

The hub genes, mechanisms, and regions of differential methylation identified in **Chapters 2, 3**, and **4** represent strong candidate translational markers for further development in wheat crop improvement strategies. The expression and responsiveness of drought-associated hub genes, stress-associated TFBSs with differential methylation, and TE-associated methylation changes could all form the basis of molecular markers that could be used to predict stress tolerance across wheat populations and potentially be used for selective breeding to generate progeny with increased drought tolerance traits.

Hub gene DEGs identified in **Chapters 2** and **3** are clear candidates for translational marker development: the tolerance hub genes in Chapter 3 were significantly associated with tolerant drought phenotypes, and therefore their expression could potentially be used to suggest whether a given genotype is drought tolerant within a screen. Similarly, profiling the expression of the dehydrin hub gene in **Chapter 2** could be used in crop selection as a rapid screen for the vigour of a plant's drought-responsive capacity. Despite this potential however, all hub genes need further validation before they could be used as part of reliable screening criteria.

Further sequencing across more genotypes and conditions would help to identify DMRs consistently associated with the drought response. Reproducible and persistent DMRs across individuals or groups undergoing drought exposure could act as epigenetic markers for drought, as demonstrated for rice under other abiotic stress conditions (Song et al., 2025), and enable selection for or against them to enhance the population. Beyond this, DMRs at drought-associated TEs could be good translational markers: with further sequencing, it would be possible to ascertain whether RLX_famc9's hypothesised methylation-controlled transcriptionally active region is a consistent feature of wheat's methylome response to drought, and if so the methylation state at that region could be used as part of a marker-assisted approach. Together, these transcriptomic and epigenomic features identified in this thesis could provide the foundation of marker development that could benefit future crop populations.

6.7. High pressure spraying of dsRNAs may be a promising technique for inducing RNAi and RdDM responses

Chapter 4 involved a step towards trying a novel RNAi technique to support the proposed mechanism of action of the identified methylation-responsive TE family *RLX_famc9*. The use of high-pressure spraying techniques to introduce exogenous dsRNAs and bring about RNA interference through the RdDM or post-transcriptional gene silencing pathways has previously been shown to induce DNA methylation and affect gene expression (Dalakouras and Ganopoulos, 2021; Uslu *et al.*, 2022; Zhao *et al.*, 2024), while a recent preprint has successfully shown the expression of exogenous mRNA molecules sprayed on leaves (Uslu *et al.*, 2025). The varying level of success of these published attempts reflects the varying level of success observed in **Chapter 4**; exogenous molecules were successfully introduced to leaf cells through abrasion-mediated high pressure spraying, and spraying of *β-actin*-derived dsRNAs appeared to have a visible and significant effect on *β-actin* gene expression, but spraying of RLX_famc9-derived dsRNAs may only have had a slight but nonsignificant effect on growth under drought conditions.

This may be down to the treatment itself: little time was available for optimisation and so these attempts very much mark the first steps in trying to utilise high pressure spraying mechanism for spray-induced gene silencing (SIGS) approaches. Though largely unsuccessful, this approach can be significantly optimised, with better dsRNA synthesis and spraying protocols. Due to time constraints, I was unable to quantify and optimise the efficiency of the spraying treatment: a degree of overspray results in some dsRNA lost to the spraying chamber, while some will fail to enter the leaf and sit on the surface, while others will likely be removed from the cell through efflux. The initial input of dsRNA application could also have been overly high – though less than 50 ng/ µL was applied in each treatment, introduction of exogenous mRNAs was successful with a concentration of only 2 ng/ µL (Uslu *et al.*, 2025), suggesting that a much lower concentration could be used without sacrificing efficacy. The spraying technique itself is also open to substantial optimisation: the present treatment was only applied to one side of a single leaf, but more viable and consistent treatments would likely require whole-plant spraying: a spray tower design (Kallavus et al., 2024) connected to the compressor would likely be a more effective system, both able to contain a whole wheat seedling and to deliver dsRNA treatment more uniformly across the leaf surface area while minimising overspray. This would better enable a standardised and repeatable approach for applying exogenous dsRNAs to plants.

The mechanism of action once in the cell is also somewhat unknown: while previous studies have indicated that methylation can be induced through the RdDM pathway post-spraying (Dalakouras and Ganopoulos, 2021), the mechanistic details regarding cleavage into siRNA, resultant siRNA size, or localisation of action are all yet unidentified. It is possible that dsRNAs could be engineered to target specific aspects of the RdDM or PTGS pathways.

Nonetheless, it is clear that there is interest in this technique for gene knockdown or transcript introduction in plants. This technique has potential agricultural benefits, and is of commercial interest: companies like Monsanto have patented approaches for the introduction of dsRNAs and siRNAs to seeds and plants to regulate both endogenous genes and transgenes (Sammons *et al.*, 2011; Avniel *et al.*, 2015), suggesting that it could serve as a useful tool in crop breeding programmes focusing on the DNA methylome, or as field applications for targeted gene expression changes on commercial crops. It would be critically useful in research for gene function testing, bypassing the time-consuming and costly process of backcrossing TILLING lines or the setup of CRISPR-Cas9 platforms. This technique has a lot of potential, but is far from ready for broader applications, and hopefully will be further developed by others.

6.8. Final thoughts

The work contained in this thesis across **Chapters 2, 3, 4, and 5** represents a substantial step forward in our understanding of the transcriptional, physiological, and epigenetic responses to drought stress in spring hexaploid wheat. It has generated novel datasets that can be utilised by others in the wider wheat genomics community, provided candidate genes and mechanisms for future pursuit and experiments that could be used to profile their mechanism of action, as well as explored potential techniques that could be used to benefit future research. These findings and proposed next steps provide a framework for dissecting how complex polyploids combat environmental stress, and highlight practical next steps for future study, including functional validation, further sequencing, and novel bioinformatic approaches.

This thesis provides a foundation for future wheat improvement strategies, and may inform current ones, but there is still more work to be done before the mechanisms and genes discussed in these chapters can be used in the field. The identification of drought-response and -tolerance hub genes provides candidates for marker-assisted selection and breeding strategies and opens up the possibility of targeted gene editing approaches, while the hypotheses of drought-associated DMRs and TE methylation indicate epigenetic markers could enhance and complement existing genomic markers, especially when trying to identify plants with high stress responsiveness. While a lack of priming benefit was observed, suggesting that priming capacity is highly genotype-specific, this could indicate that high-throughput screening of existing cultivars could identify prime targets for future priming studies. Additionally, the work in this thesis to compare transcriptomic and epigenomic signatures that are associated with drought responses could provide insights for future research that will further inform future wheat improvement strategies.

Ultimately, this work should be taken in the context of urgency: the current wheat germplasm is not sufficient to provide for booming populations under a harshening climate, but hopefully the work included in this thesis can contribute to the long-term goals of developing high-yielding and climate-resilient varieties of wheat that can help sustain global food security.

7. Appendices

Appendix 1.1

Appendix 1.1: AlphaFold 3 Server (Abramson *et al.*, 2024) and FoldSeek (van Kempen *et al.*, 2024) were used with default settings to predict the protein structure of the Uncharacterised Hub Gene (*TraesCS3D02G361500*) and compare it to the structures of other known proteins. The AFDB-PROTEOME database was used to identify proteins that exhibited structural similarity. The top 12 hits are shown above, the majority of which are associated with tetratricopeptide repeat (TPR)-like domains.

Target	Description	Scientific Name	Prob.	Seq. Id.	E-Value	Score	Query Pos.	Target Pos.
AF-K7KAR0-F1-model_v4	Uncharacterized protein	Glycine max	1	30.9	4.78E-18	398	11-480 (480)	100-473 (482)
AF-K7M568-F1-model_v4	Uncharacterized protein	Glycine max	1	29.2	1.88E-17	376	4-474 (480)	96-468 (477)
AF-I1NHZ1-F1-model_v4	TPR_REGION domain-containing protein	Glycine max	1	29.1	1.66E-07	290	323-471 (480)	230-383 (387)
AF-A0A1D6KRF0-F1-model_v4	Tetratricopeptide repeat (TPR)-like superfamily protein	Zea mays	1	22.2	1.08E-11	260	1-480 (480)	1-346 (357)
AF-A0A0R0EYT8-F1-model_v4	TPR_REGION domain-containing protein	Glycine max	1	23	5.88E-08	257	231-480 (480)	1-202 (213)
AF-C0PAC9-F1-model_v4	Tetratricopeptide repeat (TPR)-like superfamily protein	Zea mays	1	22.9	1.20E-08	253	222-480 (480)	7-253 (278)
AF-Q10RZ6-F1-model_v4	Expressed protein	Oryza sativa Japonica Group	1	22.7	1.08E-08	251	222-480 (480)	66-316 (339)
AF-I1L4Y2-F1-model_v4	TPR_REGION domain-containing protein	Glycine max	1	22.3	3.22E-08	250	222-474 (480)	4-234 (253)
AF-Q337F8-F1-model_v4	Expressed protein	Oryza sativa Japonica Group	1	22.2	3.41E-11	250	1-476 (480)	10-354 (363)
AF-Q69Q43-F1-model_v4	Os06g0606700 protein	Oryza sativa Japonica Group	1	20.5	1.86E-07	245	214-474 (480)	51-253 (278)
AF-I1LZW9-F1-model_v4	TPR_REGION domain-containing protein	Glycine max	1	20.3	8.62E-08	240	222-474 (480)	2-226 (257)
AF-A0A1D6GNK7-F1-model_v4	Tetratricopeptide repeat (TPR)-like superfamily protein	Zea mays	1	21.6	5.29E-11	239	6-480 (480)	1-351 (363)

Appendix 1.2

Appendix 1.2: One-proportion Z tests in R were used to determine whether each chromosome (above) and each genome (below) possessed a significantly greater proportion of CG differentially methylated loci (DMLs) than expected if CG DMLs were distributed evenly across the genome. P-values were then adjusted using the p.adjust package in R with a 'BH' (Benjamini-Hochberg) correction applied. Significant entries (p < 0.05) are italicised. The table is ordered by *p* value.

Genome/ Chromosome	**DMLs**	**Total CG Sites**	**FDR**
1A	*51843*	*36007367*	*0*
1B	*59193*	*42035271*	*0*
7A	*67585*	*45634401*	*0*
3B	*68691*	*50644714*	*8.94E-240*
6B	*57925*	*42244911*	*9.63E-239*
5B	*59534*	*44282927*	*2.90E-180*
2B	*64123*	*48024178*	*2.94E-172*
7D	*57664*	*43383139*	*8.29E-143*
7B	*57721*	*45999882*	*3.97E-31*
1D	36191	32929380	1
2A	56763	48024178	1
2D	45594	49466404	1
3A	49826	48054048	1
3D	43317	41858038	1
4A	50613	46201867	1
4B	41430	43699737	1
4D	26002	35214411	1
5A	50637	44981001	1
5D	42819	38487076	1
6A	40139	37717926	1
6D	34085	32287749	1
B (total)	*408617*	*316931620*	*0*
Un	*26672*	*13097758*	*0*
A (total)	367406	306620788	0.1254313
D (total)	285672	273626197	1

8. Supplementary Data

Supplementary data is provided where appropriate for all chapters. Data tables, and figures that are suitable for inclusion in the text version of the thesis are included below. Entries that are unsuitable for inclusion in this version of the thesis due to size, data type, or format are marked with [EXT], signifying their inclusion solely in the external .zip file accompanying this thesis.

Supplementary Data S2.1: Landraces selected to examine the impact of drought stress on the early spring wheat transcriptome with the country of origin, collection and plant ID.

Collection	Plant ID	Plant Name	Origin	YoGI ID	Habit
CIMMYT	BW 7112	RA SHIH PAI Pïl	China	YoGI_002	Spring
CIMMYT	BW 19498	LOHARI Y91-92 NO.123	Nepal	YoGI_007	Spring
CIMMYT	CWI 2165	K7155.22	Kenya	YoGI_010	Spring
CIMMYT	CWI 3924	ROOI INDIES	South Africa	YoGI_017	Spring
CIMMYT	CWI 3926	ROOI SPITSKOP	South Africa	YoGI_018	Spring
CIMMYT	CWI 6075	KOELZ W 9375:AE	India	YoGI_021	Spring
CIMMYT	CWI 6118	KOELZ W 9660:AE	India	YoGI_026	Spring
CIMMYT	CWI 13432	WHITE FIFE	Japan	YoGI_047	Spring
CIMMYT	CWI 15005	LAGEADINHO	Brazil	YoGI_059	Spring
Watkins	1190224	Red wheat	China	YoGI_145	Spring
Watkins	1190254		Morocco	YoGI_153	Spring
Watkins	1190292	Asprokoutsoullon	Cyprus	YoGI_161	Spring
Watkins	1190305		Egypt	YoGI_164	Spring
Watkins	1190705	Kooseh	Iran	YoGI_261	Spring

Supplementary Data S2.2: [EXT] Dry weight (g) and soil moisture content (SMC%) for all lines in both the drought and control groups. Visualisation of this data is included in Figure 1A and 1B.

Supplementary Data S2.3: [EXT] Gene normalised counts of the 14 selected landraces before **(B)** and after **(A)** a 10-day drought stress.

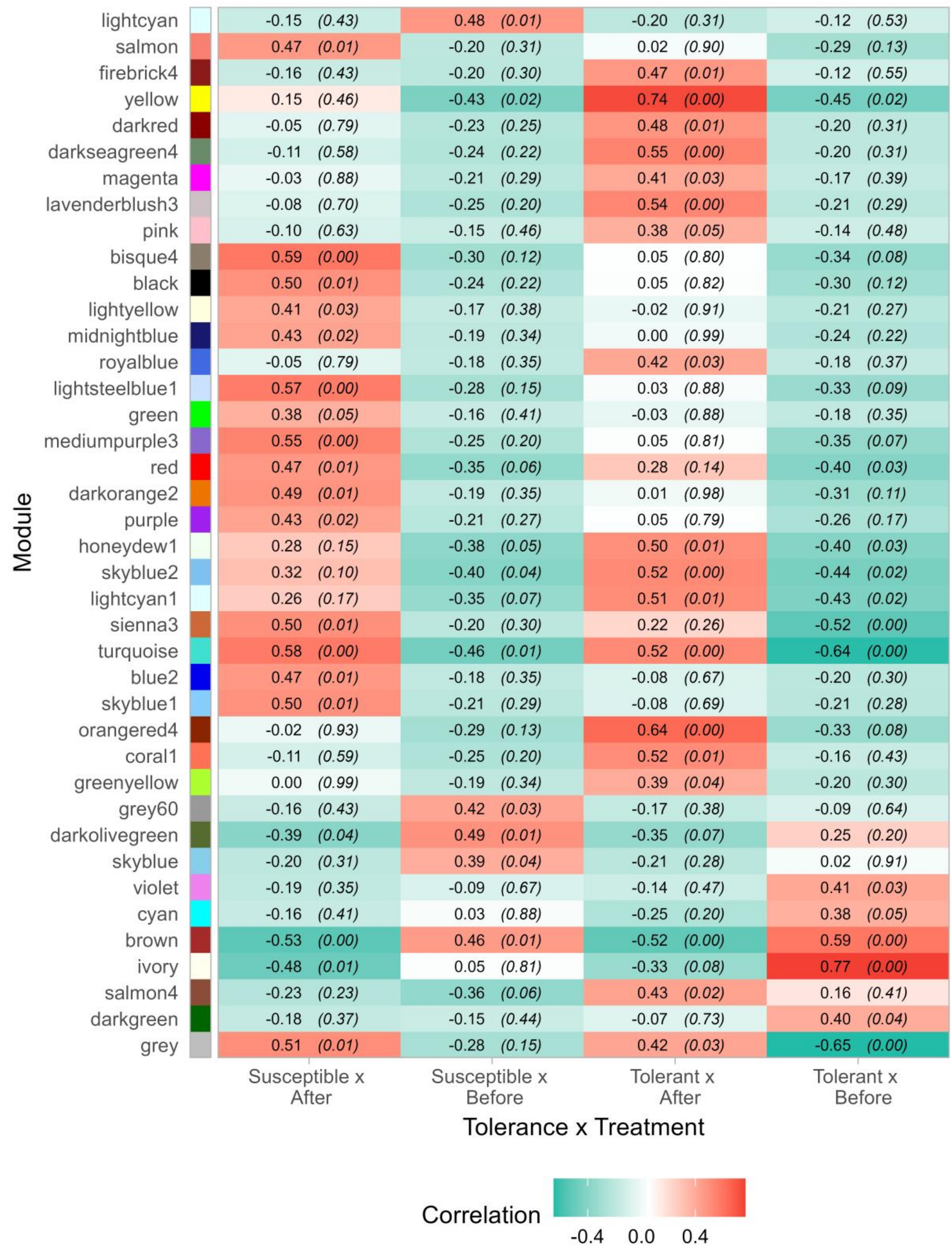
Supplementary Data S2.4: [EXT] Differentially expressed genes (DEGs) between samples before and after 10 days of drought stress. Genes were deemed to be DEGs when their FDR-adjusted p-value < 0.05, and their log2FoldChange > 1.5 or <-1.5.

Supplementary Data S2.5: [EXT] Gene Ontology (GO) enrichment of the upregulated differentially expressed genes before and after drought stress was carried out using agriGO Singular Enrichment Analysis. 231 enriched GO terms were identified.

Supplementary Data S2.6: [EXT] Gene Ontology (GO) enrichment of the downregulated differentially expressed genes before and after drought stress was carried out using agriGO Singular Enrichment Analysis. 258 enriched GO terms were identified.

Supplementary Data S2.7: [EXT] Genes included in co-expression network construction, and their module membership.

Supplementary Figure S3.1



Module	Susceptible x After	Susceptible x Before	Tolerant x After	Tolerant x Before
lightcyan	-0.15 *(0.43)*	0.48 *(0.01)*	-0.20 *(0.31)*	-0.12 *(0.53)*
salmon	0.47 *(0.01)*	-0.20 *(0.31)*	0.02 *(0.90)*	-0.29 *(0.13)*
firebrick4	-0.16 *(0.43)*	-0.20 *(0.30)*	0.47 *(0.01)*	-0.12 *(0.55)*
yellow	0.15 *(0.46)*	-0.43 *(0.02)*	0.74 *(0.00)*	-0.45 *(0.02)*
darkred	-0.05 *(0.79)*	-0.23 *(0.25)*	0.48 *(0.01)*	-0.20 *(0.31)*
darkseagreen4	-0.11 *(0.58)*	-0.24 *(0.22)*	0.55 *(0.00)*	-0.20 *(0.31)*
magenta	-0.03 *(0.88)*	-0.21 *(0.29)*	0.41 *(0.03)*	-0.17 *(0.39)*
lavenderblush3	-0.08 *(0.70)*	-0.25 *(0.20)*	0.54 *(0.00)*	-0.21 *(0.29)*
pink	-0.10 *(0.63)*	-0.15 *(0.46)*	0.38 *(0.05)*	-0.14 *(0.48)*
bisque4	0.59 *(0.00)*	-0.30 *(0.12)*	0.05 *(0.80)*	-0.34 *(0.08)*
black	0.50 *(0.01)*	-0.24 *(0.22)*	0.05 *(0.82)*	-0.30 *(0.12)*
lightyellow	0.41 *(0.03)*	-0.17 *(0.38)*	-0.02 *(0.91)*	-0.21 *(0.27)*
midnightblue	0.43 *(0.02)*	-0.19 *(0.34)*	0.00 *(0.99)*	-0.24 *(0.22)*
royalblue	-0.05 *(0.79)*	-0.18 *(0.35)*	0.42 *(0.03)*	-0.18 *(0.37)*
lightsteelblue1	0.57 *(0.00)*	-0.28 *(0.15)*	0.03 *(0.88)*	-0.33 *(0.09)*
green	0.38 *(0.05)*	-0.16 *(0.41)*	-0.03 *(0.88)*	-0.18 *(0.35)*
mediumpurple3	0.55 *(0.00)*	-0.25 *(0.20)*	0.05 *(0.81)*	-0.35 *(0.07)*
red	0.47 *(0.01)*	-0.35 *(0.06)*	0.28 *(0.14)*	-0.40 *(0.03)*
darkorange2	0.49 *(0.01)*	-0.19 *(0.35)*	0.01 *(0.98)*	-0.31 *(0.11)*
purple	0.43 *(0.02)*	-0.21 *(0.27)*	0.05 *(0.79)*	-0.26 *(0.17)*
honeydew1	0.28 *(0.15)*	-0.38 *(0.05)*	0.50 *(0.01)*	-0.40 *(0.03)*
skyblue2	0.32 *(0.10)*	-0.40 *(0.04)*	0.52 *(0.00)*	-0.44 *(0.02)*
lightcyan1	0.26 *(0.17)*	-0.35 *(0.07)*	0.51 *(0.01)*	-0.43 *(0.02)*
sienna3	0.50 *(0.01)*	-0.20 *(0.30)*	0.22 *(0.26)*	-0.52 *(0.00)*
turquoise	0.58 *(0.00)*	-0.46 *(0.01)*	0.52 *(0.00)*	-0.64 *(0.00)*
blue2	0.47 *(0.01)*	-0.18 *(0.35)*	-0.08 *(0.67)*	-0.20 *(0.30)*
skyblue1	0.50 *(0.01)*	-0.21 *(0.29)*	-0.08 *(0.69)*	-0.21 *(0.28)*
orangered4	-0.02 *(0.93)*	-0.29 *(0.13)*	0.64 *(0.00)*	-0.33 *(0.08)*
coral1	-0.11 *(0.59)*	-0.25 *(0.20)*	0.52 *(0.01)*	-0.16 *(0.43)*
greenyellow	0.00 *(0.99)*	-0.19 *(0.34)*	0.39 *(0.04)*	-0.20 *(0.30)*
grey60	-0.16 *(0.43)*	0.42 *(0.03)*	-0.17 *(0.38)*	-0.09 *(0.64)*
darkolivegreen	-0.39 *(0.04)*	0.49 *(0.01)*	-0.35 *(0.07)*	0.25 *(0.20)*
skyblue	-0.20 *(0.31)*	0.39 *(0.04)*	-0.21 *(0.28)*	0.02 *(0.91)*
violet	-0.19 *(0.35)*	-0.09 *(0.67)*	-0.14 *(0.47)*	0.41 *(0.03)*
cyan	-0.16 *(0.41)*	0.03 *(0.88)*	-0.25 *(0.20)*	0.38 *(0.05)*
brown	-0.53 *(0.00)*	0.46 *(0.01)*	-0.52 *(0.00)*	0.59 *(0.00)*
ivory	-0.48 *(0.01)*	0.05 *(0.81)*	-0.33 *(0.08)*	0.77 *(0.00)*
salmon4	-0.23 *(0.23)*	-0.36 *(0.06)*	0.43 *(0.02)*	0.16 *(0.41)*
darkgreen	-0.18 *(0.37)*	-0.15 *(0.44)*	-0.07 *(0.73)*	0.40 *(0.04)*
grey	0.51 *(0.01)*	-0.28 *(0.15)*	0.42 *(0.03)*	-0.65 *(0.00)*

Supplemental Figure S3.1: Binary trait associations carried out in R identified 18 modules significantly positively correlated with Tolerant samples after drought, while 5 modules were significantly positively correlated with tolerant samples before drought. Correlation strength (R^2) is given alongside the correlation *p* value (*in brackets*).

Supplementary Data 3.1: [EXT] All genes connected to the hub of the darkred module (TraesCS5D02G145800) with their DE status and GO annotation.

Supplementary Data 3.2: [EXT] All genes connected to the hub of the darkgreen module (TraesCS1D02G072700) with their DE status and GO annotation.

Supplementary Data 3.3: [EXT] Differential expression results between the phenotypically Drought Susceptible (DS) and Drought Tolerant (DT) groups. The DS group was used as the reference level: positive log2FCs represent genes more highly expressed in tolerant lines.

Supplementary Data 3.4: [EXT] Differential expression results between the phenotypically Drought Susceptible (DS) and Drought Tolerant (DT) groups. The DS group was used as the reference level: positive log2FCs represent genes more highly expressed in tolerant lines.

Supplementary Data 3.5: [EXT] Differential expression results between the phenotypically Before Drought (BD) and After Drought (AD) groups. The BD group was used as the reference level: positive log2FCs represent genes more highly expressed after drought.

Supplementary Data 3.6: [EXT] Genes identified as significantly differently expressed in both the ST and BA comparisons were grouped by their expression profiles. Here genes upregulated in both comparisons are listed with their log2FoldChange for both comparisons and their GO annotation.

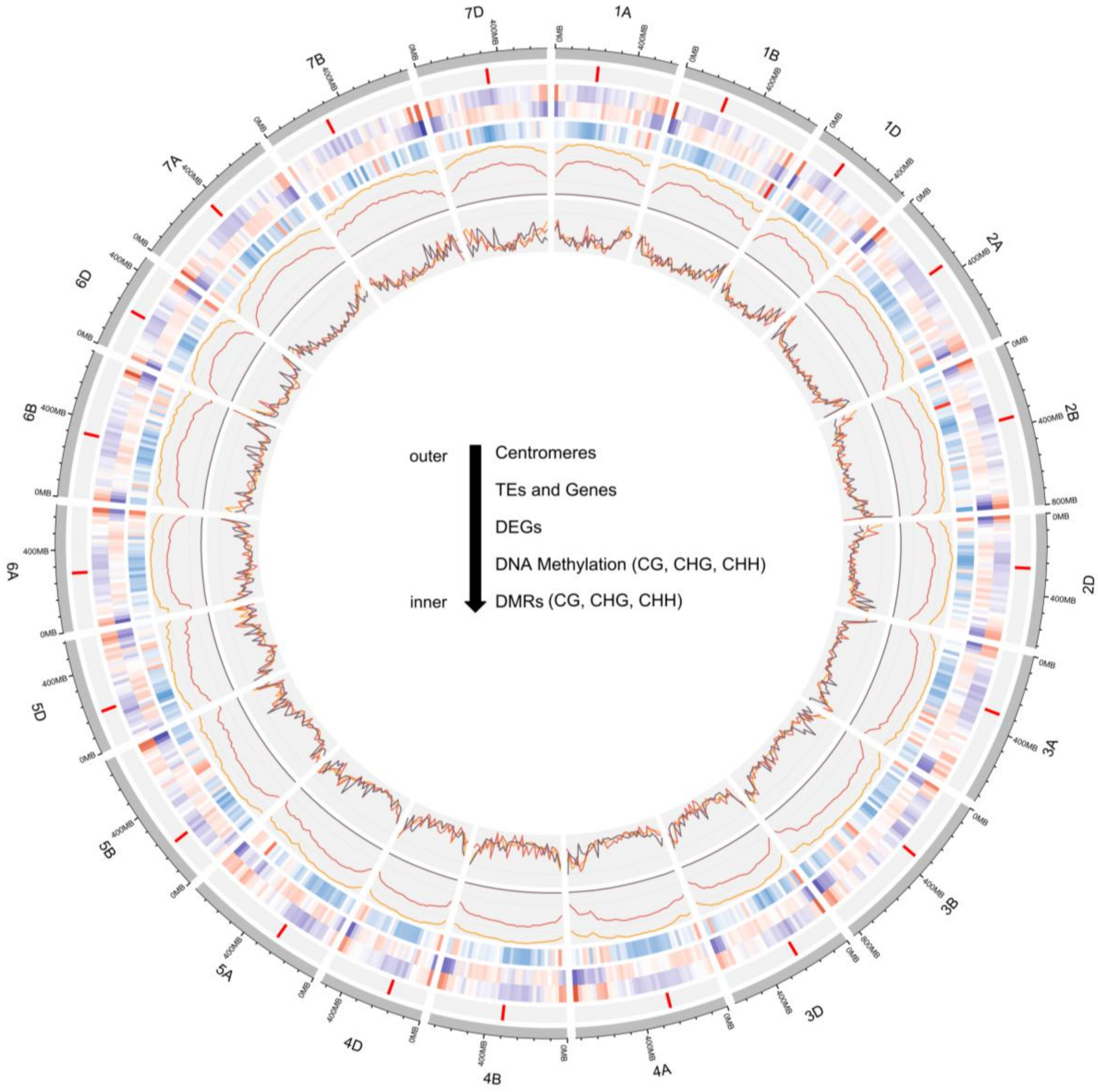
Supplementary Data 3.7: [EXT] Genes identified as significantly differently expressed in both the ST and BA comparisons were grouped by their expression profiles. Here genes downregulated in both comparisons are listed with their log2FoldChange for both comparisons and their GO annotation.

Supplementary Data 3.8: [EXT] Genes identified as significantly differently expressed in both the ST and BA comparisons were grouped by their expression profiles. Here genes with lower expression in tolerant lines that are upregulated after drought are listed with their log2FoldChange for both comparisons and their GO annotation.

Supplementary Data 3.9: [EXT] Genes identified as significantly differently expressed in both the ST and BA comparisons were grouped by their expression profiles. Here genes with higher expression in tolerant lines that are downregulated after drought are listed with their log2FoldChange for both comparisons and their GO annotation.

Supplementary Data 3.10: TILLING line and Primer details used for the WRKY19 hub gene validation.

Gene	*TaWRKY19-5D*
Gene ID	TraesCS5D02G145800
TILLING Line	Cadenza1792
WT Sequence	CCGGCCAA**[G]**CGAACGG
Mutant Sequence	CCGGCCAA**[A]**CGAACGG
Putative Effect on Drought Response	Negative
FW Primer	ACCTGAATCGCTCTTCCATCA
RV Primer	CCGAGATGTACACCACCGTA
PCR Annealing Temp.	64
Dimers	No
Hairpins	Very Weak



Supplementary Figure S4.1: DMRs tended to be densest in gene-rich, TE-poor regions at the arms of chromosomes. Centromeric regions (outermost track), Z-scored gene and transposable element density (second track), Z-scored DEG density (third track), average DNA methylation (fourth track), and Z-scored DMR density (innermost track) were visualised using a Circos plot in R using Circlize. Heatmap tracks (gene density, TE density, and DEG density) are visualised with blue tiles representing below-average density, white tiles representing average density, and red tiles representing above-average density. DNA methylation and DMR density tracks contain CG (yellow), CHG (orange), and CHH (brown) traces. DMR density appeared to be highest in the comparatively gene-rich and TE-poor chromosome arms, rather than the projected centromeric regions; in contrast, DNA methylation was highest in all three contexts at the centromeres. All values were calculated per 20 Mb bin. BD and AD methylation values were averaged together for the fourth track. Centromere positions were inflated by 1.5x to span a greater region for visualisation purposes.

Supplementary Data S4.1: [EXT] Primers used are listed, including primers for the ROS1a TILLING mutants and SIGS. T7 promoters are included (noted by -T7promoter suffix).

Gene	Gene ID	TILLING Line	WT Sequence	Mutant Sequence	Putative Effect of Mutation on Drought Response	FW Primer	RV Primer	PCR Annealing Temp.	Dimers	Hairpins
ROS1a-5A	TraesCS5A02G169000	Cadenza0002	CCCTG[G]AAAGG	CCCTG[A]AAAGG	Negative	AATGGTTTGGGTTGCACTGT	AGTCACAAACAGCTTCCAGT	58.7	No	None
ROS1a-5B	TraesCS5B02G165800	Cadenza1580	ATGTC[C]AAAGC	ATGTC[T]AAAGC	Negative	AACACACATCCTGGATCCCA	CCATCTGATTGGCTGAACTGT	64	No	Weak
ROS1a-5D	TraesCS5D02G173300	Cadenza1622	ATGTG[G]AAGTT	ATGTG[A]AAGTT	Negative	CATGGTGCTCTTGTCCCTTAC	CCCTTCGGAACCATCAACCA	63	No	None
b-Actin	TraesCS1A02G274400	NA	NA	NA	NA	GGAGAAGCTCGCTTACGTG	GGGCACCTGAACCTTTCTGA	59	No	Weak
b-Actin-T7promoter	TraesCS1A02G274400	NA	NA	NA	NA	TAATACGACTCACTATAGgg-GGAGAAGCTCGCTTACGTG	TAATACGACTCACTATAGgg-GGGCACCTGAACCTTTCTGA	59	No	Weak
RLX_famc9-T1	NA	NA	NA	NA	NA	CGGTAGGTTACAGTTGGACTCT	TCACACAAGCACGACACC	58.7	No	Weak
RLX_famc9-T1-T7promoter	NA	NA	NA	NA	NA	TAATACGACTCACTATAGgg-CGGTAGGTTACAGTTGGACTCT	TAATACGACTCACTATAGgg-TCACACAAGCACGACACC	58.7	No	Weak

Supplementary Data S4.2: [EXT] Gene ontology enrichment was carried out on DEGs identified between samples before and after drought. Upregulated and downregulated genes were analysed separately.

Supplementary Data S4.3: [EXT] Gene ontology enrichment was carried out on genes associated with DMRs identified between samples before and after drought. Hypo- and hypermethylated DMR-associated genes were analysed separately, as were genic regions.

Supplementary Data S4.4: [EXT] TE enrichment of DMRs was carried out using TEENA. Some TEs were enriched in more than one context. Contexts were analysed individually.

Supplementary Data S4.5: [EXT] Differential Expression Results of DICER, ARGONAUTE genes

9. References

Abdalla, M. et al. (2022). Stomatal closure during water deficit is controlled by below-ground hydraulics. *Annals of botany*, 129 (2), pp.161–170. [Accessed 6 September 2025].

Abdallah, N. A. et al. (2025). Multiplex CRISPR/Cas9-mediated genome editing to address drought tolerance in wheat. *GM crops & food*, 16 (1), pp.1–17. [Accessed 16 September 2025].

Abdelrahman, M. et al. (2017). The 'STAY-GREEN' trait and phytohormone signaling networks in plants under heat stress. *Plant cell reports*, 36 (7), pp.1009–1025. [Accessed 5 March 2025].

Abdul Rahim, A. et al. (2024). CRISPR/Cas9 mediated TaRPK1 root architecture gene mutagenesis confers enhanced wheat yield. *Journal of King Saud University. Science*, 36 (2), p.103063. [Accessed 16 September 2025].

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.

Abramson, J. et al. (2024). Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature*, 630 (8016), pp.493–500. [Accessed 19 September 2025].

Adusumalli, S. et al. (2015). Methodological aspects of whole-genome bisulfite sequencing analysis. *Briefings in bioinformatics*, 16 (3), pp.369–379. [Accessed 21 September 2025].

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. [Accessed 6 September 2025].

AHDB. (2025). *AHDB arable crop report*. [Online]. Available at: https://projectblue.blob.core.windows.net/media/Default/Market%20Intelligence/cereals-oilseeds/survey-results/Crop%20Development%20Reports/May%202025/AHDB%20Arable%20Crop%20Condition%20Report%20May%202025%20FINAL.pdf.

Ahmad, N. et al. (2023). Targeted genome editing in polyploids: lessons from Brassica. *Frontiers in plant science*, 14, p.1152468. [Accessed 15 September 2025].

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.

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.

Ahmed, K., Shabbir, G. and Ahmed, M. (2025). Exploring drought tolerance for germination traits of diverse wheat genotypes at seedling stage: a multivariate analysis approach. *BMC plant biology*, 25 (1), p.390. [Accessed 6 September 2025].

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.

Akagi, T. et al. (2022). Polyploidy before and after domestication of crop species. *Current opinion in plant biology*, 69 (102255), p.102255. [Accessed 13 August 2025].

Akalin, A. et al. (2015). Genomation: a toolkit to summarize, annotate and visualize genomic intervals. *Bioinformatics* , 31 (7), pp.1127–1129.

Alam, M. M. et al. (2014). Exogenous jasmonic acid modulates the physiology, antioxidant defense and glyoxalase systems in imparting drought stress tolerance in different Brassica species. *Plant biotechnology reports*, 8 (3), pp.279–293. [Accessed 9 September 2025].

Alger, E. I. and Edger, P. P. (2020). One subgenome to rule them all: underlying mechanisms of subgenome dominance. *Current opinion in plant biology*, 54, pp.108–113. [Accessed 22 September 2025].

Al-Huqail, A. A. et al. (2023). The effect of gibberellic acid on wheat growth and nutrient uptake under combined stress of cerium, zinc and titanium dioxide nanoparticles. *Chemosphere*, 336 (139199), p.139199. [Accessed 10 September 2025].

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.

Amini, A. et al. (2023). Drought stress memory in a germplasm of synthetic and common wheat: antioxidant system, physiological and morphological consequences. *Scientific reports*, 13 (1), p.8569.

Anderson, J. P. et al. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. *The plant cell*, 16 (12), pp.3460–3479. [Accessed 10 September 2025].

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.

Andrews, S. (2010). *FastQC: A quality control analysis tool for high throughput sequencing data*. [Online]. Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Angeloni, A., Ferguson, J. and Bogdanovic, O. (2022). Nanopore sequencing and data analysis for base-resolution genome-wide 5-methylcytosine profiling. *Methods in molecular biology (Clifton, N.J.)*, 2458, pp.75–94. [Accessed 21 September 2025].

Apelbaum, A. and Yang, S. F. (1981). Biosynthesis of stress ethylene induced by water deficit. *Plant physiology*, 68 (3), pp.594–596. [Accessed 10 September 2025].

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.

Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature*, 408 (6814), pp.796–815. [Accessed 8 September 2025].

Arca, M. et al. (2023). Genotyping of DNA pools identifies untapped landraces and genomic regions to develop next-generation varieties. *Plant biotechnology journal*, 21 (6), pp.1123–1139. [Accessed 20 June 2025].

Armeev, G. A. et al. (2021). Histone dynamics mediate DNA unwrapping and sliding in nucleosomes. *Nature communications*, 12 (1), p.2387. [Accessed 10 September 2025].

Arribas-Hernández, L. and Brodersen, P. (2020). Occurrence and functions of m6A and other covalent modifications in plant mRNA. *Plant physiology*, 182 (1), pp.79–96. [Accessed 11 September 2025].

Asseng, S., Foster, I. and Turner, N. C. (2011). The impact of temperature variability on wheat yields. *Global change biology*, 17 (2), pp.997–1012. [Accessed 1 May 2025].

Assenov, Y. et al. (2008). Computing topological parameters of biological networks. *Bioinformatics* , 24 (2), pp.282–284.

Aswathi, K. P. R. et al. (2025). The plant mind: Unraveling abiotic stress priming, memory, and adaptation. *Physiologia plantarum*, 177 (4), p.e70372. [Accessed 5 September 2025].

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.

Audenaert, K., De Meyer, G. B. and Höfte, M. M. (2002). Abscisic acid determines basal susceptibility of tomato to Botrytis cinerea and suppresses salicylic acid-dependent signaling mechanisms. *Plant physiology*, 128 (2), pp.491–501. [Accessed 14 July 2025].

Avniel, A. et al. (2015). Compositions and Methods for Silencing Gene Expression. *US Patent*. [Online]. Available at: https://patentimages.storage.googleapis.com/49/1f/4b/68ba84ef9d1378/US20150096079A1.pdf [Accessed 23 September 2025].

Avramova, V. et al. (2015). Drought induces distinct growth response, protection, and recovery mechanisms in the maize leaf growth zone. *Plant physiology*, 169 (2), pp.1382–1396. [Accessed 6 September 2025].

Awadalla, R. A. et al. (2024). The role of salicylic acid in modulating phenotyping in spring wheat varieties for mitigating drought stress. *BMC plant biology*, 24 (1), p.948. [Accessed 10 September 2025].

Ayadi, M., Brini, F. and Masmoudi, K. (2019). Overexpression of a Wheat Aquaporin Gene, TdPIP2;1, Enhances Salt and Drought Tolerance in Transgenic Durum Wheat cv. Maali. *International journal of molecular sciences*, 20 (10). [Online]. Available at: doi:10.3390/ijms20102389.

Baek, D. et al. (2019). AtPR5K2, a PR5-like receptor kinase, modulates plant responses to drought stress by phosphorylating protein phosphatase 2Cs. *Frontiers in plant science*, 10, p.1146. [Accessed 14 July 2025].

Bain, L. E. et al. (2013). Malnutrition in Sub-Saharan Africa: burden, causes and prospects. *The Pan African medical journal*, 15, pp.120–120.

Baird, D. et al. (2018). Agricultural origins on the Anatolian plateau. *Proceedings of the National Academy of Sciences of the United States of America*, 115 (14), pp.E3077–E3086. [Accessed 17 April 2025].

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.

Bandurska, H., Stroiński, A. and Kubiś, J. (2003). The effect of jasmonic acid on the accumulation of ABA, proline and spermidine and its influence on membrane injury under water deficit in two barley genotypes. *Acta physiologiae plantarum*, 25 (3), pp.279–285. [Accessed 9 September 2025].

Bannister, A. J. and Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell research*, 21 (3), pp.381–395. [Accessed 10 September 2025].

Bariah, I., Gribun, L. and Kashkush, K. (2022). Transposable elements are associated with genome-specific gene expression in bread wheat. *Frontiers in plant science*, 13, p.1072232. [Accessed 25 July 2025].

Bariah, I., Keidar-Friedman, D. and Kashkush, K. (2020). Where the wild things are: Transposable elements as drivers of structural and functional variations in the wheat genome. *Frontiers in plant science*, 11, p.585515.

Barratt, L. J. et al. (2023a). Co-expression network analysis of diverse wheat landraces reveals markers of early thermotolerance and a candidate master regulator of thermotolerance genes. *The Plant journal: for cell and molecular biology*, 115 (3), pp.614–626.

Barratt, L. J. et al. (2023b). Transcriptomic and co-expression network analyses on diverse wheat landraces identifies candidate master regulators of the response to early drought. *Frontiers in plant science*, 14, p.1212559.

Barratt, L. J., Franco Ortega, S. and Harper, A. L. (2023). Identification of candidate regulators of the response to early heat stress in climate-adapted wheat landraces via transcriptomic and co-expression network analyses. *Frontiers in plant science*, 14, p.1252885. [Accessed 4 March 2025].

Barrett, C. B. (2008). Smallholder market participation: Concepts and evidence from eastern and southern Africa. *Food policy*, 33 (4), pp.299–317. [Accessed 5 September 2025].

Bashir, S. S. et al. (2021). Plant drought stress tolerance: understanding its physiological, biochemical and molecular mechanisms. *Biotechnology, biotechnological equipment*, 35 (1), pp.1912–1925. [Accessed 9 September 2025].

Bastow, R. et al. (2004). Vernalization requires epigenetic silencing of FLC by histone methylation. *Nature*, 427 (6970), pp.164–167. [Accessed 23 September 2025].

Batke, S. P. et al. (2020). Plant responses to decadal scale increments in atmospheric CO2 concentration: comparing two stomatal conductance sampling methods. *Planta*, 251 (2), p.52. [Accessed 19 September 2025].

Beacon, T. H. et al. (2021). The dynamic broad epigenetic (H3K4me3, H3K27ac) domain as a mark of essential genes. *Clinical epigenetics*, 13 (1), p.138. [Accessed 15 September 2025].

Becker, C. et al. (2011). Spontaneous epigenetic variation in the Arabidopsis thaliana methylome. *Nature*, 480 (7376), pp.245–249. [Accessed 22 September 2025].

Becker, S. R. et al. (2016). Root traits contributing to drought tolerance of synthetic hexaploid wheat in a greenhouse study. *Euphytica; Netherlands journal of plant breeding*, 207 (1), pp.213–224. [Accessed 16 September 2025].

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.

te Beest, M. et al. (2012). The more the better? The role of polyploidy in facilitating plant invasions. *Annals of botany*, 109 (1), pp.19–45.

Beharav, A., Pinthus, M. J. and Cahaner, A. (1992). Interaction effects of the Rht1 and Rht2 dwarfing alleles and background genes on the growth and grain yield of spring wheat (Triticum aestivum L.). *European journal of agronomy: the journal of the European Society for Agronomy*, 1 (4), pp.263–269. [Accessed 23 April 2025].

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.

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.

Bevacqua, E. et al. (2024). Direct and lagged climate change effects intensified the 2022 European drought. *Nature geoscience*, 17 (11), pp.1100–1107. [Accessed 7 May 2025].

Bewick, A. J. et al. (2017). The evolution of CHROMOMETHYLASES and gene body DNA methylation in plants. *Genome biology*, 18 (1), p.65. [Accessed 11 September 2025].

Bharath, P., Gahir, S. and Raghavendra, A. S. (2021). Abscisic acid-induced stomatal closure: An important component of plant defense against abiotic and biotic stress. *Frontiers in plant science*, 12, p.615114. [Accessed 9 September 2025].

Bhattacharyya, R. et al. (2015). Soil degradation in India: Challenges and potential solutions. *Sustainability*, 7 (4), pp.3528–3570. [Accessed 25 April 2025].

Bilgic, H. et al. (2016). Ancient DNA from 8400 year-old Çatalhöyük wheat: Implications for the origin of neolithic agriculture. *PloS one*, 11 (3), p.e0151974. [Accessed 17 April 2025].

Bird, K. A. et al. (2021). Replaying the evolutionary tape to investigate subgenome dominance in allopolyploid Brassica napus. *The new phytologist*, 230 (1), pp.354–371.

Birkenbihl, R. P. et al. (2017). Induced genome-wide binding of three Arabidopsis WRKY transcription factors during early MAMP-triggered immunity. *The plant cell*, 29 (1), pp.20–38. [Accessed 14 July 2025].

Birkenbihl, R. P., Diezel, C. and Somssich, I. E. (2012). Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward Botrytis cinerea infection. *Plant physiology*, 159 (1), pp.266–285. [Accessed 11 March 2025].

Biswas, A. et al. (2025). Water scarcity: A global hindrance to sustainable development and agricultural production – A critical review of the impacts and adaptation strategies. *Cambridge prisms. Water*, 3 (e4), p.e4. [Accessed 5 September 2025].

Bizabani, C., Rogans, S. J. and Rey, M. E. C. (2021). Differential miRNA profiles in South African cassava mosaic virus-infected cassava landraces reveal clues to susceptibility and tolerance to cassava mosaic disease. *Virus research*, 303 (198400), p.198400. [Accessed 22 September 2025].

Blevins, T. et al. (2006). Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic acids research*, 34 (21), pp.6233–6246. [Accessed 11 September 2025].

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.

Bodi, Z. et al. (2012). Adenosine methylation in Arabidopsis mRNA is associated with the 3' end and reduced levels cause developmental defects. *Frontiers in plant science*, 3, p.48. [Accessed 11 September 2025].

Bohra, A. et al. (2022). Reap the crop wild relatives for breeding future crops. *Trends in biotechnology*, 40 (4), pp.412–431. [Accessed 24 April 2025].

Bolger, A. M., Lohse, M. and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* , 30 (15), pp.2114–2120.

Bolwell, G. P. et al. (2002). The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *Journal of experimental botany*, 53 (372), pp.1367–1376. [Accessed 9 September 2025].

Boquete, M. T., Muyle, A. and Alonso, C. (2021). Plant epigenetics: phenotypic and functional diversity beyond the DNA sequence. *American journal of botany*, 108 (4), pp.553–558.

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.

Bossdorf, O. et al. (2010). Experimental alteration of DNA methylation affects the phenotypic plasticity of ecologically relevant traits in Arabidopsis thaliana. *Evolutionary ecology*, 24 (3), pp.541–553. [Accessed 16 September 2025].

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. [Accessed 12 May 2023].

Bousfield, C. G., Morton, O. and Edwards, D. P. (2024). Climate change will exacerbate land conflict between agriculture and timber production. *Nature climate change*, 14 (10), pp.1071–1077. [Accessed 2 May 2025].

Brenet, F. et al. (2011). DNA methylation of the first exon is tightly linked to transcriptional silencing. *PloS one*, 6 (1), p.e14524. [Accessed 24 July 2025].

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.

Buitrago, D. et al. (2021). Impact of DNA methylation on 3D genome structure. *Nature communications*, 12 (1), p.3243. [Accessed 24 September 2025].

Caarls, L. et al. (2017a). *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. [Accessed 4 September 2024].

Caarls, L. et al. (2017b). 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.

Cai, X.-T. et al. (2014). Arabidopsis ERF109 mediates cross-talk between jasmonic acid and auxin biosynthesis during lateral root formation. *Nature communications*, 5 (1), p.5833.

Calvin, K. et al. (2023). IPCC, 2023: Climate Change 2023: Synthesis Report, summary for Policymakers. 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). *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*, Intergovernmental Panel on Climate Change (IPCC)., pp.1–34. [Online]. Available at: doi:10.59327/ipcc/ar6-9789291691647.001.

Camacho, C. et al. (2009). BLAST+: architecture and applications. *BMC bioinformatics*, 10, p.421.

Camaille, M. et al. (2021). Advances in wheat physiology in response to drought and the role of plant growth promoting rhizobacteria to trigger drought tolerance. *Microorganisms*, 9 (4), p.687. [Accessed 28 February 2025].

Candaele, J. et al. (2014). Differential methylation during maize leaf growth targets developmentally regulated genes. *Plant physiology*, 164 (3), pp.1350–1364. [Accessed 4 September 2025].

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.

Cao, F. Y., Yoshioka, K. and Desveaux, D. (2011). The roles of ABA in plant-pathogen interactions. *Journal of plant research*, 124 (4), pp.489–499. [Accessed 20 August 2025].

Cao, J. et al. (2025). MYB47 delays leaf senescence by modulating jasmonate pathway via direct regulation of CYP94B3/CYP94C1 expression in Arabidopsis. *The new phytologist*, 246 (5), pp.2192–2206. [Accessed 9 May 2025].

Capy, P. (2021). Taming, domestication and exaptation: Trajectories of transposable elements in genomes. *Cells (Basel, Switzerland)*, 10 (12), p.3590. [Accessed 5 August 2025].

Carlson, K. D. et al. (2017). Natural variation in stress response gene activity in the allopolyploid Arabidopsis suecica. *BMC genomics*, 18 (1), p.653. [Accessed 7 May 2025].

Carminati, A. et al. (2020). Stomatal closure prevents the drop in soil water potential around roots. *The new phytologist*, 226 (6), pp.1541–1543. [Accessed 6 September 2025].

Castiello, M. E. et al. (2025). Understanding the spread of agriculture in the Western Mediterranean (6th-3rd millennia BC) with Machine Learning tools. *Nature communications*, 16 (1), p.678. [Accessed 17 April 2025].

Catoni, M. et al. (2022). Long-lasting defence priming by β-aminobutyric acid in tomato is marked by genome-wide changes in DNA methylation. *Frontiers in plant science*, 13, p.836326. [Accessed 4 August 2025].

Ceccarelli, S. and Grando, S. (2020). Evolutionary plant breeding as a response to the complexity of climate change. *iScience*, 23 (12), p.101815. [Accessed 24 April 2025].

Chakraborty, P. et al. (2023). Cytochrome P450 gene families: Role in plant secondary metabolites production and plant defense. *Journal of xenobiotics*, 13 (3), pp.402–423. [Accessed 9 September 2024].

Chan, J. C. S., Ooi, M. K. J. and Guja, L. K. (2021). Polyploidy but not range size is associated with seed and seedling traits that affect performance of Pomaderris species. *Frontiers in plant science*, 12, p.779651. [Accessed 13 August 2025].

Chen, A. and Dubcovsky, J. (2012). Wheat TILLING mutants show that the vernalization gene VRN1 down-regulates the flowering repressor VRN2 in leaves but is not essential for flowering. *PLoS genetics*, 8 (12), p.e1003134. [Accessed 22 September 2025].

Chen, F. et al. (2022). The response mechanism and threshold of spring wheat to rapid drought. *Atmosphere*, 13 (4), p.596. [Accessed 6 September 2025].

Chen, J. et al. (2024a). RNA-seq-based WGCNA and association analysis reveal the key regulatory module and genes responding to salt stress in wheat roots. *Plants*, 13 (2), p.274. [Accessed 10 March 2025].

Chen, L. et al. (2025). Global increase in the occurrence and impact of multiyear droughts. *Science (New York, N.Y.)*, 387 (6731), pp.278–284. [Accessed 4 August 2025].

Chen, Y. et al. (2024b). Multiomic analyses reveal key sectors of jasmonate-mediated defense responses in rice. *The plant cell*, 36 (9), pp.3362–3377. [Accessed 9 July 2025].

Chen, Y.-H. et al. (2024c). Correlations among genetic, epigenetic, and phenotypic variation of *Phragmites australis* along latitudes. *Diversity & distributions*, 30 (9), p.e13907. [Accessed 23 September 2025].

Cheng, S. et al. (2024). Harnessing landrace diversity empowers wheat breeding. *Nature*, 632 (8026), pp.823–831. [Accessed 28 February 2025].

Chhetri, H. B. et al. (2020). Genome-Wide Association Study of Wood Anatomical and Morphological Traits in Populus trichocarpa. *Frontiers in plant science*, 11, p.545748.

Chini, A. et al. (2004). Drought tolerance established by enhanced expression of the CC-NBS-LRR gene, ADR1, requires salicylic acid, EDS1 and ABI1. *The Plant journal: for cell and molecular biology*, 38 (5), pp.810–822. [Accessed 27 January 2025].

Cho, S. H. et al. (2008). Physcomitrella patens DCL3 is required for 22-24 nt siRNA accumulation, suppression of retrotransposon-derived transcripts, and normal development. *PLoS genetics*, 4 (12), p.e1000314. [Accessed 5 August 2025].

Choi, J.-Y. et al. (2025). Interactive effects of seed priming and genetic diversity on rice germination and early growth under drought stress. *All life*, 18 (1). [Online]. Available at: doi:10.1080/26895293.2025.2527623 [Accessed 14 September 2025].

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.

Choudhary, A. and Senthil-Kumar, M. (2022). Drought attenuates plant defence against bacterial pathogens by suppressing the expression of CBP60g/SARD1 during combined stress. *Plant, cell & environment*, 45 (4), pp.1127–1145.

Chow, H. T. and Mosher, R. A. (2023). Small RNA-mediated DNA methylation during plant reproduction. *The plant cell*, 35 (6), pp.1787–1800. [Accessed 4 August 2025].

Christopher, J. T. et al. (2016). Stay-green traits to improve wheat adaptation in well-watered and water-limited environments. *Journal of experimental botany*, 67 (17), pp.5159–5172. [Accessed 5 March 2025].

Clarke, S. M. et al. (2004). Salicylic acid dependent signaling promotes basal thermotolerance but is not essential for acquired thermotolerance in Arabidopsis thaliana. *The Plant journal: for cell and molecular biology*, 38 (3), pp.432–447. [Accessed 14 September 2025].

Claros, M. G. et al. (2012). Why assembling plant genome sequences is so challenging. *Biology*, 1 (2), pp.439–459. [Accessed 8 September 2025].

Cooper, R. (2015). Re-discovering ancient wheat varieties as functional foods. *Journal of traditional and complementary medicine*, 5 (3), pp.138–143. [Accessed 17 April 2025].

Corrado, G. and Rao, R. (2017). Towards the Genomic Basis of Local Adaptation in Landraces. *Diversity*, 9 (4), p.51. [Accessed 25 October 2022].

Cortijo, S. et al. (2019). Widespread inter-individual gene expression variability in Arabidopsis thaliana. *Molecular systems biology*, 15 (1), p.e8591. [Accessed 24 July 2025].

Craparo, A. C. W. et al. (2017). Application of thermography for monitoring stomatal conductance of Coffea arabica under different shading systems. *The Science of the total environment*, 609, pp.755–763.

Cruz de Carvalho, M. H. (2008). Drought stress and reactive oxygen species: Production, scavenging and signaling. *Plant signaling & behavior*, 3 (3), pp.156–165. [Accessed 8 September 2025].

Cseh, A. et al. (2021). Exploring the legacy of Central European historical winter wheat landraces. *Scientific reports*, 11 (1), p.23915.

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.

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.

Dalakouras, A. et al. (2016). Induction of silencing in plants by high-pressure spraying of in vitro-synthesized small RNAs. *Frontiers in plant science*, 7, p.1327. [Accessed 2 September 2025].

Dalakouras, A. and Ganopoulos, I. (2021). Induction of promoter DNA methylation upon high-pressure spraying of double-stranded RNA in plants. *Agronomy (Basel, Switzerland)*, 11 (4), p.789. [Accessed 2 September 2025].

D'Andrea, L. D. and Regan, L. (2003). TPR proteins: the versatile helix. *Trends in biochemical sciences*, 28 (12), pp.655–662. [Accessed 19 September 2025].

Dar, F. A. et al. (2022). Role of epigenetics in modulating phenotypic plasticity against abiotic stresses in plants. *International journal of genomics*, 2022, p.1092894. [Accessed 16 July 2025].

Das, M. K. et al. (2016). Genetic diversity among synthetic hexaploid wheat accessions (Triticum aestivum) with resistance to several fungal diseases. *Genetic resources and crop evolution*, 63 (8), pp.1285–1296. [Accessed 16 September 2025].

Das, S. et al. (2020). Loss of function of Oryza sativa Argonaute 18 induces male sterility and reduction in phased small RNAs. *Plant reproduction*, 33 (1), pp.59–73. [Accessed 5 August 2025].

Daszkowska-Golec, A. and Szarejko, I. (2013). Open or close the gate - stomata action under the control of phytohormones in drought stress conditions. *Frontiers in plant science*, 4, p.138. [Accessed 9 September 2025].

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 Flaviis, R. et al. (2022). Quantitatively unravelling the effect of altitude of cultivation on the volatiles fingerprint of wheat by a chemometric approach. *Food chemistry*, 370 (131296), p.131296. [Accessed 15 April 2025].

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.

Deal, R. B. and Henikoff, S. (2011). Histone variants and modifications in plant gene regulation. *Current opinion in plant biology*, 14 (2), pp.116–122. [Accessed 15 September 2025].

Delang, C. O. (2018). The consequences of soil degradation in China: a review. *GeoScape*, 12 (2), pp.92–103.

Demirel, S. et al. (2025). DNA methylation change and antioxidant enzyme activity of drought stress and putrescine treatment in ancient wheat (Triticum monococcum L.). *Cereal research communications*, 53 (2), pp.771–781. [Accessed 12 September 2025].

Deneweth, J., Van de Peer, Y. and Vermeirssen, V. (2022). Nearby transposable elements impact plant stress gene regulatory networks: a meta-analysis in A. thaliana and S. lycopersicum. *BMC genomics*, 23 (1), p.18. [Accessed 4 August 2025].

Deng, W. et al. (2015). Direct links between the vernalization response and other key traits of cereal crops. *Nature communications*, 6 (1), p.5882. [Accessed 22 September 2025].

Diallo, A. M. et al. (2016). Polyploidy can Confer Superiority to West African Acacia senegal (L.) Willd. Trees. *Frontiers in plant science*, 7, p.821. [Accessed 13 August 2025].

Ding, G. et al. (2022). DNA methylation correlates with the expression of drought-responsive genes and drought resistance in rice. *Agronomy (Basel, Switzerland)*, 12 (6), p.1445. [Accessed 2 December 2024].

Ding, L. et al. (2016). Root ABA accumulation enhances rice seedling drought tolerance under ammonium supply: Interaction with aquaporins. *Frontiers in plant science*, 7, p.1206. [Accessed 10 September 2025].

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.

Ding, Y. et al. (2024). Abiotic stress-induced DNA methylation in transposable elements and their transcripts reveals a multi-layered response in Moso bamboo. *Industrial crops and products*, 210 (118108), p.118108. [Accessed 2 December 2024].

Distefano, T. et al. (2018). Shock transmission in the International Food Trade Network. *PloS one*, 13 (8), p.e0200639. [Accessed 15 September 2025].

Docherty, S. J. et al. (2009). Bisulfite-based epityping on pooled genomic DNA provides an accurate estimate of average group DNA methylation. *Epigenetics & chromatin*, 2 (1), pp.3–3.

Docherty, S. J. et al. (2010). DNA methylation profiling using bisulfite-based epityping of pooled genomic DNA. *Methods*, 52 (3), pp.255–258. [Accessed 21 September 2025].

Dodd, I. C. and Ryan, A. C. (2016). Whole-plant physiological responses to water-deficit stress. *eLS*, Chichester, UK: John Wiley & Sons, Ltd., pp.1–9. [Online]. Available at: doi:10.1002/9780470015902.a0001298.pub3.

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.

Domb, K. et al. (2020). DNA methylation mutants in Physcomitrella patens elucidate individual roles of CG and non-CG methylation in genome regulation. *Proceedings of the National Academy of Sciences of the United States of America*, 117 (52), pp.33700–33710. [Accessed 25 July 2025].

Dorussen, D. et al. (2025). Homoeolog expression in polyploid wheat mutants shows limited transcriptional compensation. *bioRxiv*, p.2025.07.01.662569. [Online]. Available at: doi:10.1101/2025.07.01.662569 [Accessed 1 September 2025].

Du, J. et al. (2012). Dual binding of chromomethylase domains to H3K9me2-containing nucleosomes directs DNA methylation in plants. *Cell*, 151 (1), pp.167–180. [Accessed 11 September 2025].

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, Z. et al. (2010). agriGO: a GO analysis toolkit for the agricultural community. *Nucleic acids research*, 38 (Web Server issue), pp.W64-70.

Duan, H. et al. (2020). Responsive changes of DNA methylation in wheat (Triticum aestivum) under water deficit. *Scientific reports*, 10 (1), p.7938. [Accessed 26 November 2024].

Duan, W. et al. (2017). Role of vernalization-mediated demethylation in the floral transition of Brassica rapa. *Planta*, 245 (1), pp.227–233. [Accessed 23 September 2025].

Dubcovsky, J. and Dvorak, J. (2007). Genome plasticity a key factor in the success of polyploid wheat under domestication. *Science (New York, N.Y.)*, 316 (5833), pp.1862–1866. [Accessed 13 August 2025].

Dueñas, C., Jr et al. (2024). Genotype-specific germination behavior induced by sustainable priming techniques in response to water deprivation stress in rice. *Frontiers in plant science*, 15, p.1344383. [Accessed 3 September 2025].

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.

Dunoyer, P., Himber, C. and Voinnet, O. (2005). DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nature genetics*, 37 (12), pp.1356–1360. [Accessed 5 August 2025].

Duvnjak, J. et al. (2023). Morpho-physiological and hormonal response of winter wheat varieties to drought stress at stem elongation and anthesis stages. *Plants*, 12 (3), p.418. [Accessed 28 February 2025].

Edger, P. P. et al. (2017). Subgenome dominance in an interspecific hybrid, synthetic allopolyploid, and a 140-year-old naturally established Neo-allopolyploid monkeyflower. *The plant cell*, 29 (9), pp.2150–2167. [Accessed 22 September 2025].

Eekhout, J. P. C. and de Vente, J. (2022). Global impact of climate change on soil erosion and potential for adaptation through soil conservation. *Earth-science reviews*, 226 (103921), p.103921. [Accessed 7 May 2025].

Eh, T.-J. et al. (2024). The role of trehalose metabolism in plant stress tolerance. *Journal of advanced research*. [Online]. Available at: doi:10.1016/j.jare.2024.12.025 [Accessed 8 September 2025].

Eichten, S. R. et al. (2011). Heritable epigenetic variation among maize inbreds. *PLoS genetics*, 7 (11), p.e1002372. [Accessed 22 November 2024].

El Baidouri, M. et al. (2017). Reconciling the evolutionary origin of bread wheat (Triticum aestivum). *The New phytologist*, 213 (3), pp.1477–1486.

Eliazer Nelson, A. R. L., Ravichandran, K. and Antony, U. (2019). The impact of the Green Revolution on indigenous crops of India. *Journal of Ethnic Foods*, 6 (1), pp.8–8.

Emamverdian, A., Ding, Y. and Mokhberdoran, F. (2020). The role of salicylic acid and gibberellin signaling in plant responses to abiotic stress with an emphasis on heavy metals. *Plant signaling & behavior*, 15 (7), p.1777372. [Accessed 10 September 2025].

Environment Agency. (2025). *National Drought Group meets after driest spring in 132 years*. [Online]. Gov.uk. Available at: https://www.gov.uk/government/news/national-drought-group-meets-after-driest-spring-in-132-years [Accessed 6 February 2026].

Epule, T. E. et al. (2018). The determinants of crop yields in Uganda: what is the role of climatic and non-climatic factors? *Agriculture & food security*, 7 (1), pp.1–17. [Accessed 25 April 2025].

Erenstein, O. et al. (2022). Global trends in wheat production, consumption and trade. In: *Wheat Improvement*. Cham: Springer International Publishing. pp.47–66. [Accessed 2 December 2024].

Erenstein, O., Chamberlin, J. and Sonder, K. (2021). Estimating the global number and distribution of maize and wheat farms. *Global food security*, 30 (100558), p.100558. [Accessed 28 February 2025].

Evenson, R. E. and Gollin, D. (2003). Assessing the impact of the green revolution, 1960 to 2000. *Science (New York, N.Y.)*, 300 (5620), pp.758–762. [Accessed 23 April 2025].

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.

Fang, Q. et al. (2024). Water use strategies and shoot and root traits of high-yielding winter wheat cultivars under different water supply conditions. *Agronomy (Basel, Switzerland)*, 14 (4), p.826. [Accessed 3 September 2025].

FAO. (2024). *World food and agriculture – statistical yearbook 2024*. Fao. [Accessed 28 February 2025].

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.

Farooq, M. (2023). Tolerance against combined drought and heat stresses in wheat landraces of Omani origin: Morphological, physiological, biochemical, and grain yield assessment. *Journal of soil science and plant nutrition*, 23 (4), pp.6034–6047. [Accessed 4 March 2025].

Farooq, M. et al. (2024). Recent advances in plant drought tolerance. *Journal of plant growth regulation*, 43 (10), pp.3337–3369. [Accessed 20 September 2025].

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. [Accessed 28 February 2025].

Feghhenabi, F. et al. (2020). Seed priming alleviated salinity stress during germination and emergence of wheat (Triticum aestivum L.). *Agricultural water management*, 231 (106022), p.106022. [Accessed 19 August 2025].

Feldman, M. et al. (2012). Genomic asymmetry in allopolyploid plants: wheat as a model. *Journal of experimental botany*, 63 (14), pp.5045–5059. [Accessed 13 August 2025].

Feng, H. and Wu, H. (2019). Differential methylation analysis for bisulfite sequencing using DSS. *Quantitative biology (Beijing, China)*, 7 (4), pp.327–334.

Feng, S. et al. (2010). Conservation and divergence of methylation patterning in plants and animals. *Proceedings of the National Academy of Sciences of the United States of America*, 107 (19), pp.8689–8694. [Accessed 22 January 2026].

Ferrero-Serrano, Á. and Assmann, S. M. (2016). The α-subunit of the rice heterotrimeric G protein, RGA1, regulates drought tolerance during the vegetative phase in the dwarf rice mutant d1. *Journal of experimental botany*, 67 (11), pp.3433–3443. [Accessed 14 July 2025].

Fichman, Y. et al. (2022). HPCA1 is required for systemic reactive oxygen species and calcium cell-to-cell signaling and plant acclimation to stress. *The plant cell*, 34 (11), pp.4453–4471. [Accessed 27 January 2025].

Food and Agriculture Organization of the United Nations. (2002). *Bread wheat: Improvement and production*. Food & Agriculture Organization of the UN (FAO).

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.

Food and Agriculture Organization of the United Nations (FAO). (2025). *Production / Crops and livestock products*. [Online]. Available at: https://www.fao.org/faostat/en/#data/QCL [Accessed 15 April 2025].

Foyer, C. H. (2018). Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. *Environmental and experimental botany*, 154, pp.134–142. [Accessed 8 September 2025].

Fradgley, N. S. et al. (2023). Prediction of near-term climate change impacts on UK wheat quality and the potential for adaptation through plant breeding. *Global change biology*, 29 (5), pp.1296–1313. [Accessed 1 May 2025].

Friedrich, T. et al. (2021). Heteromeric HSFA2/HSFA3 complexes drive transcriptional memory after heat stress in Arabidopsis. *Nature communications*, 12 (1), p.3426. [Accessed 14 September 2025].

Fu, Y.-B. (2015). Understanding crop genetic diversity under modern plant breeding. *Theoretical and applied genetics*, 128 (11), pp.2131–2142. [Accessed 10 March 2025].

Fujii, H. et al. (2009). In vitro reconstitution of an abscisic acid signalling pathway. *Nature*, 462 (7273), pp.660–664. [Accessed 9 September 2025].

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. [Accessed 17 April 2025].

Gahlaut, V. et al. (2020). Quantitative epigenetics: A new avenue for crop improvement. *Epigenomes*, 4 (4), p.25. [Accessed 16 September 2025].

Gahlaut, V. et al. (2022). Identification and characterization of DNA demethylase genes and their association with thermal stress in wheat (Triticum aestivum L.). *Frontiers in genetics*, 13, p.894020. [Accessed 29 August 2025].

Gahlaut, V., Samtani, H. and Khurana, P. (2020). Genome-wide identification and expression profiling of cytosine-5 DNA methyltransferases during drought and heat stress in wheat (Triticum aestivum). *Genomics*, 112 (6), pp.4796–4807. [Accessed 12 September 2025].

Gallego-Bartolomé, J. (2020). DNA methylation in plants: mechanisms and tools for targeted manipulation. *The New phytologist*, 227 (1), pp.38–44.

Gallego-Fabrega, C. et al. (2015). DNA methylation levels are highly correlated between pooled samples and averaged values when analysed using the Infinium HumanMethylation450 BeadChip array. *Clinical epigenetics*, 7 (1), p.78. [Accessed 22 November 2024].

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). [Online]. Available at: doi:10.3390/ijms20205046.

Ganguly, D. R. et al. (2017). The Arabidopsis DNA methylome is stable under transgenerational drought stress. *Plant physiology*, 175 (4), pp.1893–1912. [Accessed 14 September 2025].

Ganguly, D. R. et al. (2025). mRNA ADENOSINE METHYLASE promotes drought tolerance through N6-methyladenosine-dependent and independent impacts on mRNA regulation in Arabidopsis. *The new phytologist*, 245 (1), pp.183–199. [Accessed 11 September 2025].

Gao, C. et al. (2024). Drought and rewatering practices improve adaptability of seedling maize to drought stress by a super-compensate effect. *Heliyon*, 10 (20), p.e39602. [Accessed 3 September 2025].

Gao, H. et al. (2018). Overexpression of a WRKY transcription factor TaWRKY2 enhances drought stress tolerance in transgenic wheat. *Frontiers in plant science*, 9. [Online]. Available at: doi:10.3389/fpls.2018.00997.

Gao, L. et al. (2016). Heritable alteration of DNA methylation induced by whole-chromosome aneuploidy in wheat. *The new phytologist*, 209 (1), pp.364–375. [Accessed 14 September 2025].

Gasciolli, V. et al. (2005). Partially redundant functions of Arabidopsis DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Current biology: CB*, 15 (16), pp.1494–1500. [Accessed 5 August 2025].

Gaupp, F. et al. (2020). Changing risks of simultaneous global breadbasket failure. *Nature climate change*, 10 (1), pp.54–57. [Accessed 15 September 2025].

Gelvin, S. B. (2003). *Agrobacterium* -mediated plant transformation: The biology behind the “gene-jockeying” tool. *Microbiology and molecular biology reviews: MMBR*, 67 (1), pp.16–37. [Accessed 16 September 2025].

Georgieva, M. and Vassileva, V. (2023). Stress management in plants: Examining provisional and unique dose-dependent responses. *International journal of molecular sciences*, 24 (6), p.5105. [Accessed 9 September 2025].

Ghaffar, A. et al. (2023). Photosynthetic activity and metabolic profiling of bread wheat cultivars contrasting in drought tolerance. *Frontiers in plant science*, 14, p.1123080. [Accessed 5 March 2025].

Ghazouani, H. et al. (2025). Projected long-term climate change impacts on rainfed durum wheat production and sustainable adaptation strategies. *Journal of cleaner production*, 494 (144980), p.144980. [Accessed 29 April 2025].

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.

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.

Gill, R. A. et al. (2021). On the role of transposable elements in the regulation of gene expression and subgenomic interactions in crop genomes. *Critical reviews in plant sciences*, 40 (2), pp.157–189. [Accessed 22 September 2025].

Godfray, H. C. J. et al. (2010). Food security: the challenge of feeding 9 billion people. *Science*, 327 (5967), pp.812–818.

Godfree, R. C. et al. (2017). Empirical evidence of fixed and homeostatic patterns of polyploid advantage in a keystone grass exposed to drought and heat stress. *Royal Society open science*, 4 (11), p.170934. [Accessed 13 August 2025].

Gohar, S. et al. (2022). Domestication of newly evolved hexaploid wheat-A journey of wild grass to cultivated wheat. *Frontiers in genetics*, 13, p.1022931. [Accessed 17 April 2025].

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.

Gómez-Martín, C. et al. (2023). Genome-wide analysis of microRNA expression profile in roots and leaves of three wheat cultivars under water and drought conditions. *Biomolecules*, 13 (3), p.440. [Accessed 11 November 2024].

Gong, Z. et al. (2024). Whole-genome bisulfite sequencing (WGBS) analysis of Gossypium hirsutum under high-temperature stress conditions. *Genes*, 15 (10), p.1241. [Accessed 12 September 2025].

Goossens, J., Mertens, J. and Goossens, A. (2017). Role and functioning of bHLH transcription factors in jasmonate signalling. *Journal of experimental botany*, 68 (6), pp.1333–1347.

Grondin, A. et al. (2015). Aquaporins Contribute to ABA-Triggered Stomatal Closure through OST1-Mediated Phosphorylation. *The Plant cell*, 27 (7), pp.1945–1954.

Gross, K. and Schiestl, F. P. (2015). Are tetraploids more successful? Floral signals, reproductive success and floral isolation in mixed-ploidy populations of a terrestrial orchid. *Annals of botany*, 115 (2), pp.263–273. [Accessed 8 September 2025].

Grote, U. et al. (2021). Food security and the dynamics of wheat and maize value chains in Africa and Asia. *Frontiers in sustainable food systems*, 4, p.617009. [Accessed 15 April 2025].

Gu, H. et al. (2022). OsFTL4, an FT-like gene, regulates flowering time and drought tolerance in rice (Oryza sativa L.). *Rice (New York, N.Y.)*, 15 (1), p.47. [Accessed 10 September 2025].

Gu, Z. et al. (2014). circlize Implements and enhances circular visualization in R. *Bioinformatics (Oxford, England)*, 30 (19), pp.2811–2812. [Accessed 3 December 2024].

Gui, Y. et al. (2021). Differentiate responses of tetraploid and hexaploid wheat (Triticum aestivum L.) to moderate and severe drought stress: a cue of wheat domestication. *Plant signaling & behavior*, 16 (1), p.1839710. [Accessed 7 May 2025].

Guo, H. et al. (2016). Up-regulation of abscisic acid signaling pathway facilitates aphid xylem absorption and osmoregulation under drought stress. *Journal of experimental botany*, 67 (3), pp.681–693. [Accessed 10 September 2025].

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.

Guo, W., Wang, D. and Lisch, D. (2021). RNA-directed DNA methylation prevents rapid and heritable reversal of transposon silencing under heat stress in Zea mays. *PLoS genetics*, 17 (6), p.e1009326. [Accessed 14 September 2025].

Gupta, C. and Salgotra, R. K. (2022). Epigenetics and its role in effecting agronomical traits. *Frontiers in plant science*, 13, p.925688. [Accessed 16 July 2025].

Gurven, M. D. and Davison, R. J. (2019). Periodic catastrophes over human evolutionary history are necessary to explain the forager population paradox. *Proceedings of the National Academy of Sciences of the United States of America*, 116 (26), pp.12758–12766.

Habibabadi, V. N. et al. (2025). Impact of drought stress memory and variations in type and degree on root characteristic, yield stability and drought tolerance in Orchardgrass. *BMC plant biology*, 25 (1), p.1149.

Haghpanah, M. et al. (2024). Drought tolerance in plants: Physiological and molecular responses. *Plants*, 13 (21), p.2962. [Accessed 20 September 2025].

Haider, S. and Farrona, S. (2024). Decoding histone 3 lysine methylation: Insights into seed germination and flowering. *Current opinion in plant biology*, 81 (102598), p.102598. [Accessed 15 September 2025].

Hakata, M. et al. (2017). Overexpression of TIFY genes promotes plant growth in rice through jasmonate signaling. *Bioscience, biotechnology, and biochemistry*, 81 (5), pp.906–913.

Hammond-Kosack, M. C. U. et al. (2021). Exploring the diversity of promoter and 5’UTR sequences in ancestral, historic and modern wheat. *Plant biotechnology journal*, 19 (12), pp.2469–2487. [Accessed 27 September 2025].

Hanin, M. et al. (2011). Plant dehydrins and stress tolerance: versatile proteins for complex mechanisms: versatile proteins for complex mechanisms. *Plant signaling & behavior*, 6 (10), pp.1503–1509. [Accessed 27 September 2025].

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.

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.

Hara, M. et al. (2009). DNA binding of citrus dehydrin promoted by zinc ion. *Plant, cell & environment*, 32 (5), pp.532–541.

Harkness, C. et al. (2020). Adverse weather conditions for UK wheat production under climate change. *Agricultural and forest meteorology*, 282–283 (107862), p.107862. [Accessed 1 May 2025].

Hasan, M. M. et al. (2024). Ethylene-mediated stomatal responses to dehydration and rehydration in seed plants. *Journal of experimental botany*, 75 (21), pp.6719–6732. [Accessed 10 September 2025].

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.

Hawkesford, M. J. et al. (2013). Prospects of doubling global wheat yields. *Food and energy security*, 2 (1), pp.34–48.

He, F. et al. (2022a). Genomic variants affecting homoeologous gene expression dosage contribute to agronomic trait variation in allopolyploid wheat. *Nature communications*, 13 (1), p.826. [Accessed 8 September 2025].

He, J. et al. (2024a). Drought priming promotes nitrogen use efficiency in wheat (*Triticum aestivum* L.) under drought stress during grain filling. *Journal of agronomy and crop science*, 210 (5), p.e12753. [Accessed 19 August 2025].

He, J.-F. et al. (2012). Water stress during grain development affects starch synthesis, composition and physicochemical properties in triticale. *Journal of cereal science*, 56 (3), pp.552–560. [Accessed 6 September 2025].

He, L. et al. (2022b). DNA methylation-free Arabidopsis reveals crucial roles of DNA methylation in regulating gene expression and development. *Nature communications*, 13 (1), p.1335. [Accessed 11 September 2025].

He, Y. et al. (2024b). Climate change enhances stability of wheat-flowering-date. *The Science of the total environment*, 917 (170305), p.170305. [Accessed 1 May 2025].

Hedden, P. (2003). The genes of the Green Revolution. *Trends in genetics: TIG*, 19 (1), pp.5–9. [Accessed 23 April 2025].

Heinrich, M. et al. (2013). High levels of jasmonic acid antagonize the biosynthesis of gibberellins and inhibit the growth of Nicotiana attenuata stems. *The Plant journal: for cell and molecular biology*, 73 (4), pp.591–606. [Accessed 28 February 2025].

Heinz, S. et al. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Molecular cell*, 38 (4), pp.576–589.

Heitz, T. et al. (2012). Cytochromes P450 CYP94C1 and CYP94B3 catalyze two successive oxidation steps of plant hormone Jasmonoyl-isoleucine for catabolic turnover. *The journal of biological chemistry*, 287 (9), pp.6296–6306. [Accessed 9 September 2024].

Henderson, A. N. et al. (2020). Phenotypic and physiological responses to salt exposure in Sorghum reveal diversity among domesticated landraces. *American journal of botany*, 107 (7), pp.983–992. [Accessed 9 May 2025].

Heslop-Harrison, J. S. P., Schwarzacher, T. and Liu, Q. (2023). Polyploidy: its consequences and enabling role in plant diversification and evolution. *Annals of botany*, 131 (1), pp.1–10. [Accessed 15 September 2025].

Hickman, R. et al. (2017). Architecture and dynamics of the jasmonic acid gene regulatory network. *The plant cell*, 29 (9), pp.2086–2105. [Accessed 9 September 2025].

Hidvégi, N., Gulyás, A. and Dobránszki, J. (2022). Ultrasound, as a hypomethylating agent, remodels DNA methylation and alters mRNA transcription in winter wheat (Triticum aestivum L.) seedlings. *Physiologia plantarum*, 174 (5), p.e13777. [Accessed 29 November 2024].

Hilker, M. and Schmülling, T. (2019). Stress priming, memory, and signalling in plants. *Plant, cell & environment*, 42 (3), pp.753–761.

Himanen, S. V. and Sistonen, L. (2019). New insights into transcriptional reprogramming during cellular stress. *Journal of cell science*, 132 (21), p.jcs238402. [Accessed 16 July 2025].

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.

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.

Holden, S. and Shiferaw, B. (2004). Land degradation, drought and food security in a less-favoured area in the Ethiopian highlands: a bio-economic model with market imperfections. *Agricultural economics (Amsterdam, Netherlands)*, 30 (1), pp.31–49. [Accessed 5 September 2025].

Horvath, R. et al. (2019). Single-cell expression noise and gene-body methylation in Arabidopsis thaliana. *Heredity*, 123 (2), pp.81–91. [Accessed 27 November 2024].

Hoseini, M. et al. (2024). Agro-physiological and DNA methylation responses to salinity stress in wheat (Triticum aestivum L.), Aegilops cylindrica host, and their introgressed lines. *Plants*, 13 (19), p.2673. [Accessed 12 September 2025].

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.

Hristov, J. et al. (2020). *Analysis of climate change impacts on EU agriculture by 2050: JRC PESETA IV project : Task 3*.

Hu, J., Xu, T. and Kang, H. (2024). Crosstalk between RNA m6A modification and epigenetic factors in plant gene regulation. *Plant communications*, 5 (10), p.101037. [Accessed 11 September 2025].

Huang, H. et al. (2019). Mechanisms of ROS Regulation of Plant Development and Stress Responses. *Frontiers in plant science*, 10, p.800.

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.

Huang, X. et al. (2018). ViewBS: a powerful toolkit for visualization of high-throughput bisulfite sequencing data. *Bioinformatics (Oxford, England)*, 34 (4), pp.708–709. [Accessed 15 November 2024].

Huh, I. et al. (2013). DNA methylation and transcriptional noise. *Epigenetics & chromatin*, 6 (1), p.9. [Accessed 24 July 2025].

Huh, I. et al. (2019). Detecting differential DNA methylation from sequencing of bisulfite converted DNA of diverse species. *Briefings in bioinformatics*, 20 (1), pp.33–46. [Accessed 11 September 2024].

Hunter, M. C. et al. (2017). Agriculture in 2050: Recalibrating targets for sustainable intensification. *Bioscience*, 67 (4), pp.386–391. [Accessed 2 December 2024].

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.

Ijaz, S., Bashir, A. and Malik, K. A. (2025). Expression of Agrobacterium Isopentenyl transferase (IPT) gene in wheat improves drought tolerance. *Transgenic research*, 34 (1), p.7. [Accessed 16 September 2025].

Ilyas, N. et al. (2017). Influence of salicylic acid and jasmonic acid on wheat under drought stress. *Communications in soil science and plant analysis*, pp.1–9. [Accessed 28 February 2025].

Iqbal, M. and Ashraf, M. (2013). Gibberellic acid mediated induction of salt tolerance in wheat plants: Growth, ionic partitioning, photosynthesis, yield and hormonal homeostasis. *Environmental and experimental botany*, 86, pp.76–85. [Accessed 10 September 2025].

Ito, H. et al. (2016). A stress-activated transposon in Arabidopsis induces transgenerational abscisic acid insensitivity. *Scientific reports*, 6 (1), p.23181. [Accessed 25 July 2025].

Ito, T. et al. (2015). Genome-wide negative feedback drives transgenerational DNA methylation dynamics in Arabidopsis. *PLoS Genetics*, 11 (4), p.e1005154. [Accessed 5 February 2026].

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.

Jaggard, K. W., Qi, A. and Ober, E. S. (2010). Possible changes to arable crop yields by 2050. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 365 (1554), pp.2835–2851. [Accessed 28 February 2025].

Janicka, M. et al. (2023). Jasmonic acid effect on Cucumis sativus L. growth is related to inhibition of plasma membrane proton pump and the uptake and assimilation of nitrates. *Cells (Basel, Switzerland)*, 12 (18), p.2263. [Accessed 10 September 2025].

Javed, A. et al. (2022). Grain yield, chlorophyll and protein contents of elite wheat genotypes under drought stress. *Journal of King Saud University. Science*, 34 (7), p.102279. [Accessed 28 February 2025].

Jay, F., Brioudes, F. and Voinnet, O. (2023). A contemporary reassessment of the enhanced transient expression system based on the tombusviral silencing suppressor protein P19. *The Plant journal: for cell and molecular biology*, 113 (1), pp.186–204. [Accessed 5 August 2025].

Jean Finnegan, E. et al. (2005). The downregulation of FLOWERING LOCUS C (FLC) expression in plants with low levels of DNA methylation and by vernalization occurs by distinct mechanisms: Epigenetic regulation of FLC expression. *The Plant journal: for cell and molecular biology*, 44 (3), pp.420–432. [Accessed 27 September 2025].

Jeger, M. J. (2022). The impact of climate change on disease in wild plant populations and communities. *Plant pathology*, 71 (1), pp.111–130. [Accessed 10 September 2025].

Jia, J. et al. (2025). Transposable elements drive the subgenomic divergence of homoeologous genes to shape wheat domestication and improvement. *Plant communications*, 6 (6), p.101341. [Accessed 22 September 2025].

Jiang, J. et al. (2022). Tetraploidy in Citrus wilsonii enhances drought tolerance via synergistic regulation of photosynthesis, phosphorylation, and hormonal changes. *Frontiers in plant science*, 13, p.875011. [Accessed 13 August 2025].

Jiang, T. et al. (2025). Understanding the impacts of extreme temperature and humidity compounds on winter wheat traits in China. *Agricultural and forest meteorology*, 362 (110354), p.110354.

Jimenez-Aleman, G. H. et al. (2019). Omega hydroxylated JA-Ile is an endogenous bioactive jasmonate that signals through the canonical jasmonate signaling pathway. *Biochimica et biophysica acta. Molecular and cell biology of lipids*, 1864 (12), p.158520. [Accessed 12 February 2025].

Joly-Lopez, Z. and Bureau, T. E. (2018). Exaptation of transposable element coding sequences. *Current opinion in genetics & development*, 49, pp.34–42. [Accessed 5 August 2025].

Jones, P. et al. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics* , 30 (9), pp.1236–1240.

de Jong, T. J. and Lin, T. (2017). How to quantify plant tolerance to loss of biomass? *Ecology and evolution*, 7 (9), pp.3080–3086. [Accessed 9 May 2025].

Ju, F. et al. (2023). Weighted gene co-expression network analysis revealed the key pathways and hub genes of potassium regulating cotton root adaptation to salt stress. *Frontiers in plant science*, 14, p.1132877. [Accessed 10 March 2025].

Jung, I. et al. (2016). Clustering and evolutionary analysis of small RNAs identify regulatory siRNA clusters induced under drought stress in rice. *BMC Systems Biology*, 10 (Suppl 4), p.115. [Accessed 28 January 2026].

Kakiyama, S. et al. (2019). Long DCL4-substrate dsRNAs efficiently induce RNA interference in plant cells. *Scientific reports*, 9 (1), p.6920. [Accessed 11 September 2025].

Kallavus, T. et al. (2024). Laboratory sprayer for dsRNA application: Design and bioassay validation. *MethodsX*, 12 (102734), p.102734. [Accessed 28 September 2025].

Kaluscha, S. et al. (2022). Evidence that direct inhibition of transcription factor binding is the prevailing mode of gene and repeat repression by DNA methylation. *Nature genetics*, 54 (12), pp.1895–1906. [Accessed 17 June 2025].

Kang, J. et al. (2023). Physiological mechanisms underlying reduced photosynthesis in wheat leaves grown in the field under conditions of nitrogen and water deficiency. *The crop journal*, 11 (2), pp.638–650. [Accessed 6 September 2025].

Kapazoglou, A. et al. (2013). The study of a barley epigenetic regulator, HvDME,in seed development and under drought. *BMC plant biology*, 13 (1), pp.172–172.

Kaplow, I. M. et al. (2015). A pooling-based approach to mapping genetic variants associated with DNA methylation. *Genome research*, 25 (6), pp.907–917.

Kassambara, A. (2023). *rstatix: Pipe-Friendly Framework for Basic Statistical Tests*. [Online]. Available at: https://rpkgs.datanovia.com/rstatix/.

Katoh, K. and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution*, 30 (4), pp.772–780. [Accessed 28 July 2025].

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), p.14867. [Accessed 15 September 2025].

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). [Online]. Available at: doi:10.1007/s10535-018-0786-3.

Kay, M. et al. (2025). *mjskay/ARTool: ARTool 0.11.2*. Zenodo. [Online]. Available at: doi:10.5281/ZENODO.594511.

Kelly, G. et al. (2019). Guard-cell hexokinase increases water-use efficiency under normal and drought conditions. *Frontiers in plant science*, 10, p.1499. [Accessed 20 June 2025].

van Kempen, M. et al. (2024). Fast and accurate protein structure search with Foldseek. *Nature biotechnology*, 42 (2), pp.243–246. [Accessed 19 September 2025].

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.

Kernaleguen, M. et al. (2018). Whole-genome bisulfite sequencing for the analysis of genome-wide DNA methylation and hydroxymethylation patterns at single-nucleotide resolution. *Methods in molecular biology (Clifton, N.J.)*, 1767, pp.311–349. [Accessed 21 September 2025].

Kettlewell, P., Byrne, R. and Jeffery, S. (2023). Wheat area expansion into northern higher latitudes and global food security. *Agriculture, ecosystems & environment*, 351 (108499), p.108499. [Accessed 28 February 2025].

Khalvandi, M. et al. (2021). Salicylic acid alleviated the effect of drought stress on photosynthetic characteristics and leaf protein pattern in winter wheat. *Heliyon*, 7 (1), p.e05908. [Accessed 10 September 2025].

Khan, M. A. et al. (2023). Multivariate and association analyses of quantitative attributes reveal drought tolerance potential of wheat (*Triticum aestivum* L.) genotypes. *Communications in soil science and plant analysis*, 54 (2), pp.178–195. [Accessed 28 February 2025].

Kharbikar, L. L. et al. (2025). An in - silico perspective on the role of methylation-related genes in wheat - Fusarium graminearum interaction. *3 biotech*, 15 (1), p.12.

Khodadadi, E. et al. (2021). Current Advances in DNA Methylation Analysis Methods. *BioMed research international*, 2021, p.8827516.

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.

Khoury, C. K. et al. (2022). Crop genetic erosion: understanding and responding to loss of crop diversity. *The new phytologist*, 233 (1), pp.84–118. [Accessed 24 April 2025].

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. [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.

Kim, Y.-C., Kim, G. W. and Lee, J. H. (2023). Transcriptome analysis of drought response in a domestic wheat cultivar (Triticum aestivum L. cv. Keumkang). *Plant biotechnology reports*, 17 (5), pp.715–724. [Accessed 27 November 2024].

King, K. C., Seppälä, O. and Neiman, M. (2012). Is more better? Polyploidy and parasite resistance. *Biology letters*, 8 (4), pp.598–600. [Accessed 13 August 2025].

Kissoudis, C. et al. (2016). Ethylene and abscisic acid signaling pathways differentially influence tomato resistance to combined powdery mildew and salt stress. *Frontiers in plant science*, 7, p.2009. [Accessed 10 September 2025].

Kong, L. et al. (2020). Insight into the Role of Epigenetic Processes in Abiotic and Biotic Stress Response in Wheat and Barley. *International journal of molecular sciences*, 21 (4). [Online]. Available at: doi:10.3390/ijms21041480.

Korpetis, E. et al. (2023). Bread wheat landraces adaptability to low-input agriculture. *Plants*, 12 (13), p.2561. [Accessed 4 March 2025].

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.

Kottler, E. J., VanWallendael, A. and Franks, S. J. (2018). Experimental treatment with a hypomethylating agent alters life history traits and fitness in Brassica rapa. *Journal of botany*, 2018 (1), pp.1–10. [Accessed 16 September 2025].

Kou, H. P. et al. (2011). Heritable alteration in DNA methylation induced by nitrogen-deficiency stress accompanies enhanced tolerance by progenies to the stress in rice (Oryza sativa L.). *Journal of plant physiology*, 168 (14), pp.1685–1693. [Accessed 14 September 2025].

Kou, S. et al. (2022). Genome-wide bisulphite sequencing uncovered the contribution of DNA methylation to rice short-term drought memory formation. *Journal of plant growth regulation*, 41 (7), pp.2903–2917. [Accessed 11 August 2025].

Krasileva, K. V. et al. (2017). Uncovering hidden variation in polyploid wheat. *Proceedings of the National Academy of Sciences of the United States of America*, 114 (6), pp.E913–E921. [Accessed 29 August 2025].

Kress, W. J. et al. (2022). Green plant genomes: What we know in an era of rapidly expanding opportunities. *Proceedings of the National Academy of Sciences of the United States of America*, 119 (4), p.e2115640118. [Accessed 8 September 2025].

Kribelbauer, J. F. et al. (2020). Toward a mechanistic understanding of DNA methylation readout by transcription factors. *Journal of molecular biology*, 432 (6), pp.1801–1815. [Accessed 17 June 2025].

Krishnamurthy, P. et al. (2020). Regulation of a cytochrome P450 gene CYP94B1 by WRKY33 transcription factor controls apoplastic barrier formation in roots to confer salt tolerance. *Plant physiology*, 184 (4), pp.2199–2215. [Accessed 11 March 2025].

Krueger, F. et al. (2023). *FelixKrueger/TrimGalore: v0.6.10*. Zenodo. [Online]. Available at: doi:10.5281/ZENODO.7598955 [Accessed 3 December 2024].

Krueger, F. and Andrews, S. R. (2011). Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics* , 27 (11), pp.1571–1572.

Kuhla, K., Puma, M. J. and Otto, C. (2024). International cooperation was key to stabilize wheat prices after the Russian Invasion of Ukraine. *Communications earth & environment*, 5 (1), pp.1–15. [Accessed 15 September 2025].

Kulkarni, M. et al. (2017). Drought response in wheat: Key genes and regulatory mechanisms controlling root system architecture and transpiration efficiency. *Frontiers in chemistry*, 5, p.106. [Accessed 28 February 2025].

Kumar, K. P. et al. (2019). 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.

Kumar, S. et al. (2021). Understanding 3D genome organization and its effect on transcriptional gene regulation under environmental stress in plant: A chromatin perspective. *Frontiers in cell and developmental biology*, 9, p.774719. [Accessed 15 September 2025].

Kumar, V., Thakur, J. K. and Prasad, M. (2021). Histone acetylation dynamics regulating plant development and stress responses. *Cellular and molecular life sciences: CMLS*, 78 (10), pp.4467–4486. [Accessed 10 September 2025].

Kuromori, T., Seo, M. and Shinozaki, K. (2018). ABA transport and plant water stress responses. *Trends in plant science*, 23 (6), pp.513–522. [Accessed 9 September 2025].

Kurotani, K.-I. et al. (2015). Elevated levels of CYP94 family gene expression alleviate the jasmonate response and enhance salt tolerance in rice. *Plant & cell physiology*, 56 (4), pp.779–789. [Accessed 9 September 2024].

Kushawaha, A. K. et al. (2021). Priming by high temperature stress induces MicroRNA regulated heat shock modules indicating their involvement in thermopriming response in rice. *Life (Basel, Switzerland)*, 11 (4), p.291. [Accessed 14 September 2025].

Lam, D. (2023). Has the world survived the population bomb? A 10-year update. *Population and environment*, 45 (2), p.10. [Accessed 23 April 2025].

Lamers, J., van der Meer, T. and Testerink, C. (2020). How plants sense and respond to stressful environments. *Plant physiology*, 182 (4), pp.1624–1635. [Accessed 15 September 2025].

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. [Accessed 8 June 2022].

Lan, Z., Yan hao, X. and Jian bo, W. (2009). DNA-methylation changes induced by salt stress in wheat Triticum aestivum. *African journal of biotechnology*, 8 (22), pp.6201–6207.

Langfelder, P. and Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. *BMC bioinformatics*, 9, p.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: https://www.ncbi.nlm.nih.gov/pubmed/23050260.

Langmead, B. and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature methods*, 9 (4), pp.357–359.

Laugerotte, J., Baumann, U. and Sourdille, P. (2022). Genetic control of compatibility in crosses between wheat and its wild or cultivated relatives. *Plant biotechnology journal*, 20 (5), pp.812–832. [Accessed 16 September 2025].

Lawson, T. and Leakey, A. D. B. (2024). Stomata: custodians of leaf gaseous exchange. *Journal of experimental botany*, 75 (21), pp.6677–6682. [Accessed 6 September 2025].

Le, T. N.-Y. et al. (2025). Key RNA elements influencing DCL1 cleavage in plant microRNA biogenesis. *Nature plants*, 11 (8), pp.1528–1543. [Accessed 11 September 2025].

Le, T.-N. et al. (2014). DNA demethylases target promoter transposable elements to positively regulate stress responsive genes in Arabidopsis. *Genome biology*, 15 (9), p.458. [Accessed 22 September 2025].

Leach, L. J. et al. (2014). Patterns of homoeologous gene expression shown by RNA sequencing in hexaploid bread wheat. *BMC genomics*, 15 (1), p.276.

Lee, B.-R. et al. (2008). Kinetics of drought-induced pathogenesis-related proteins and its physiological significance in white clover leaves. *Physiologia plantarum*, 132 (3), pp.329–337. [Accessed 14 July 2025].

Lee, S. et al. (2023). DDM1-mediated gene body DNA methylation is associated with inducible activation of defense-related genes in Arabidopsis. *Genome biology*, 24 (1), p.106. [Accessed 11 September 2025].

Lee, S. and Park, C.-M. (2012). Regulation of reactive oxygen species generation under drought conditions in Arabidopsis. *Plant signaling & behavior*, 7 (6), pp.599–601. [Accessed 8 September 2025].

Leng, G. and Hall, J. (2019). Crop yield sensitivity of global major agricultural countries to droughts and the projected changes in the future. *The Science of the total environment*, 654, pp.811–821. [Accessed 28 February 2025].

Levy, A. A. and Feldman, M. (2002). The impact of polyploidy on grass genome evolution. *Plant physiology*, 130 (4), pp.1587–1593. [Accessed 17 April 2025].

Levy, A. A. and Feldman, M. (2022). Evolution and origin of bread wheat. *The plant cell*, 34 (7), pp.2549–2567. [Accessed 15 April 2025].

Li, B. et al. (2023a). N6-methyladenosine RNA modification regulates cotton drought response in a Ca2+ and ABA-dependent manner. *Plant biotechnology journal*, 21 (6), pp.1270–1285. [Accessed 11 September 2025].

Li, D. et al. (2021a). Genome-wide identification and transcriptional characterization of DNA methyltransferases conferring temperature-sensitive male sterility in wheat. *BMC genomics*, 22 (1), p.310. [Accessed 12 September 2025].

Li, G. et al. (2013). Salicylic acid increases the contents of glutathione and ascorbate and temporally regulates the related gene expression in salt-stressed wheat seedlings. *Gene*, 529 (2), pp.321–325. [Accessed 10 September 2025].

Li, J. et al. (2022a). Evaluation of UAV-derived multimodal remote sensing data for biomass prediction and drought tolerance assessment in bioenergy sorghum. *The crop journal*, 10 (5), pp.1363–1375. [Accessed 9 May 2025].

Li, J. et al. (2024a). The RNA-binding domain of DCL3 is required for long-distance RNAi signaling. *aBIOTECH*, 5 (1), pp.17–28. [Accessed 11 September 2025].

Li, L. et al. (2024b). Drought priming enhances wheat grain starch and protein quality under drought stress during grain filling. *Journal of integrative agriculture*, 24 (8), pp.2888–2901. [Accessed 19 August 2025].

Li, M. et al. (2021b). Multiple responses contribute to the enhanced drought tolerance of the autotetraploid Ziziphus jujuba Mill. var. spinosa. *Cell & bioscience*, 11 (1), p.119. [Accessed 8 September 2025].

Li, M. et al. (2023b). DNA methylation role in subgenome expression dominance of Juglans regia and its wild relative J. mandshurica. *Plant physiology*, 193 (2), pp.1313–1329. [Accessed 22 September 2025].

Li, P. et al. (2021c). Wheat breeding highlights drought tolerance while ignores the advantages of drought avoidance: A meta-analysis. *European journal of agronomy: the journal of the European Society for Agronomy*, 122 (126196), p.126196. [Accessed 29 April 2025].

Li, P. F., Ma, B. L. and Xiong, Y. C. (2018). Modern hexaploid wheat differs from diploid and tetraploid ancestors in the importance of stress tolerance versus stress avoidance. *Crop & pasture science*, 69 (3), p.265. [Accessed 7 May 2025].

Li, Q. et al. (2023c). 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.

Li, Q. et al. (2024c). Climate change leads to an expansion of global drought-sensitive area. *Journal of hydrology*, 632 (130874), p.130874. [Accessed 7 May 2025].

Li, R. et al. (2020). Whole genome bisulfite sequencing methylome analysis of mulberry (Morus alba) reveals epigenome modifications in response to drought stress. *Scientific reports*, 10 (1), p.8013. [Accessed 31 July 2025].

Li, X. et al. (2012). Single-base resolution maps of cultivated and wild rice methylomes and regulatory roles of DNA methylation in plant gene expression. *BMC genomics*, 13 (1), p.300. [Accessed 31 July 2025].

Li, X. et al. (2016). Combined linkage and association mapping reveals QTL and candidate genes for plant and ear height in maize. *Frontiers in plant science*, 7, p.833. [Accessed 16 September 2025].

Li, X. et al. (2017). The biphasic root growth response to abscisic acid in Arabidopsis involves interaction with ethylene and auxin signalling pathways. *Frontiers in plant science*, 8, p.1493. [Accessed 10 September 2025].

Li, X. et al. (2024d). Polyploidization: A biological force that enhances stress resistance. *International journal of molecular sciences*, 25 (4), p.1957. [Accessed 8 September 2025].

Li, Y. et al. (2022b). Changes in wheat traits under future climate change and their contributions to yield changes in conventional vs. conservational tillage systems. *The Science of the total environment*, 815 (152947), p.152947. [Accessed 1 May 2025].

Li, Y. et al. (2024e). TEENA: an integrated web server for transposable element enrichment analysis in various model and non-model organisms. *Nucleic acids research*, 52 (W1), pp.W126–W131. [Accessed 11 November 2024].

Li, Z. et al. (2022c). Insight into the vacuolar compartmentalization process and the effect glutathione regulation to this process in the hyperaccumulator plant Solanum nigrum L. *BioMed research international*, 2022, p.4359645. [Accessed 15 September 2025].

Li, Z. et al. (2024f). Overexpression of NB-LRR gene AtRPM1(D505V) improved drought and salt resistance and decreased cold tolerance in transgenic rice. *Agronomy (Basel, Switzerland)*, 14 (5), p.1050. [Accessed 27 January 2025].

Liang, C. et al. (2023). Beyond loading: Functions of plant ARGONAUTE proteins. *International journal of molecular sciences*, 24 (22), p.16054. [Accessed 5 August 2025].

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.

Liao, Z. et al. (2023). Coordination of growth and drought responses by GA-ABA signaling in rice. *The new phytologist*, 240 (3), pp.1149–1161. [Accessed 10 September 2025].

Lichtenthaler, H. K. (1998). The stress concept in plants: an introduction. *Annals of the New York Academy of Sciences*, 851 (1 STRESS OF LIF), pp.187–198. [Accessed 9 September 2025].

Lima, M. (2014). Climate change and the population collapse during the ‘Great Famine’ in pre-industrial Europe. *Ecology and evolution*, 4 (3), pp.284–291. [Accessed 25 April 2025].

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.

Liu, C. et al. (2018). Arabidopsis ARGONAUTE 1 binds chromatin to promote gene transcription in response to hormones and stresses. *Developmental Cell*, 44 (3), pp.348-361.e7. [Accessed 28 January 2026].

Liu, H. et al. (2017a). Effects of jasmonic acid signalling on the wheat microbiome differ between body sites. *Scientific reports*, 7, p.41766. [Accessed 10 September 2025].

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.

Liu, H. et al. (2022a). Signaling transduction of ABA, ROS, and Ca2+ in plant stomatal closure in response to drought. *International journal of molecular sciences*, 23 (23), p.14824. [Accessed 27 January 2025].

Liu, H., Able, A. J. and Able, J. A. (2022). Priming crops for the future: rewiring stress memory. *Trends in plant science*, 27 (7), pp.699–716.

Liu, J. et al. (2022b). Shaping polyploid wheat for success: Origins, domestication, and the genetic improvement of agronomic traits. *Journal of integrative plant biology*, 64 (2), pp.536–563. [Accessed 13 August 2025].

Liu, J. and Chang, C. (2021). Concerto on chromatin: Interplays of different epigenetic mechanisms in plant development and environmental adaptation. *Plants*, 10 (12), p.2766. [Accessed 4 August 2025].

Liu, J. and He, Z. (2020). Small DNA methylation, big player in plant abiotic stress responses and memory. *Frontiers in plant science*, 11, p.595603. [Accessed 16 July 2025].

Liu, N. et al. (2015). N(6)-methyladenosine-dependent RNA structural switches regulate RNA-protein interactions. *Nature*, 518 (7540), pp.560–564. [Accessed 11 September 2025].

Liu, R.-X. et al. (2020a). Quantitative trait loci analysis for root traits in synthetic hexaploid wheat under drought stress conditions. *Journal of integrative agriculture*, 19 (8), pp.1947–1960. [Accessed 16 September 2025].

Liu, T. et al. (2025a). VRN1 regulates heading and plant height in wheat by activating gibberellin biosynthesis. *Plant communications*, 6 (7), p.101325. [Accessed 22 September 2025].

Liu, W. et al. (2022c). The mechanisms of siRNA selection by plant Argonaute proteins triggering DNA methylation. *Nucleic acids research*, 50 (22), pp.12997–13010. [Accessed 5 August 2025].

Liu, X. et al. (2020b). miR168 targets Argonaute1A mediated miRNAs regulation pathways in response to potassium deficiency stress in tomato. *BMC Plant Biology*, 20 (1), p.477. [Accessed 3 March 2026].

Liu, X. et al. (2022d). Global agricultural water scarcity assessment incorporating blue and green water availability under future climate change. *Earth's future*, 10 (4), p.e2021EF002567. [Accessed 7 May 2025].

Liu, X. et al. (2024a). MdTPR16, an apple tetratricopeptide repeat (TPR)-like superfamily gene, positively regulates drought stress in apple. *Plant physiology and biochemistry*, 210 (108572), p.108572.

Liu, X. et al. (2025b). An elite allele TaDT1-AhapI enhances drought tolerance via mediating autophagic pathways in wheat. *Nature communications*, 16 (1), p.6563. [Accessed 16 September 2025].

Liu, X. and Hou, X. (2018). Antagonistic regulation of ABA and GA in metabolism and signaling pathways. *Frontiers in plant science*, 9, p.251. [Accessed 10 September 2025].

Liu, Y. et al. (2017b). Multifunctional roles of plant dehydrins in response to environmental stresses. *Frontiers in plant science*, 8, p.1018. [Accessed 19 September 2025].

Liu, Y. et al. (2024b). Genetic diversity and population structure of wheat landraces in Southern Winter Wheat Region of China. *BMC genomics*, 25 (1), p.664.

Liu, Y. et al. (2025c). The action of Arabidopsis DICER-LIKE 2 in plant growth inhibition. *The plant cell*, 37 (8), p.koaf206. [Accessed 11 September 2025].

Liu, Z., Lv, A. and Li, T. (2024). Intensified drought threatens future food security in major food-producing countries. *Atmosphere*, 16 (1), p.34.

Long, J. et al. (2023). Contribution of the histone variant H2A.Z to expression of responsive genes in plants. *Seminars in cell & developmental biology*, 135, pp.85–92. [Accessed 21 September 2025].

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. [Accessed 17 April 2025].

Lorrai, R. et al. (2018). Abscisic acid inhibits hypocotyl elongation acting on gibberellins, DELLA proteins and auxin. *AoB plants*, 10 (5), p.ly061. [Accessed 10 September 2025].

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.

Lu, W. et al. (2020). Linkage-linkage disequilibrium dissection of the epigenetic quantitative trait loci (epiQTLs) underlying growth and wood properties in Populus. *The new phytologist*, 225 (3), pp.1218–1233. [Accessed 16 September 2025].

Lu, X. et al. (2017). Single-base resolution methylomes of upland cotton (Gossypium hirsutum L.) reveal epigenome modifications in response to drought stress. *BMC genomics*, 18 (1), p.297. [Accessed 12 September 2025].

Lucibelli, F., Valoroso, M. C. and Aceto, S. (2022). Plant DNA Methylation: An Epigenetic Mark in Development, Environmental Interactions, and Evolution. *International journal of molecular sciences*, 23 (15). [Online]. Available at: doi:10.3390/ijms23158299.

Luo, D. et al. (2023). Physiological and DNA methylation analysis provides epigenetic insights into kenaf cadmium tolerance heterosis. *Plant science: an international journal of experimental plant biology*, 331, p.111663. [Accessed 23 July 2025].

Luo, G.-Z. et al. (2014). Unique features of the m6A methylome in Arabidopsis thaliana. *Nature communications*, 5 (1), p.5630. [Accessed 11 September 2025].

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.

Lv, Y. et al. (2019). Maize transposable elements contribute to long non-coding RNAs that are regulatory hubs for abiotic stress response. *BMC genomics*, 20 (1), p.864. [Accessed 5 August 2025].

Ma, B. et al. (2024a). RtNAC055 promotes drought tolerance via a stomatal closure pathway linked to methyl jasmonate/hydrogen peroxide signaling in Reaumuria trigyna. *Horticulture research*, 11 (2), p.uhae001. [Accessed 9 September 2025].

Ma, F. et al. (2020). Novel crosstalk between ethylene- and jasmonic acid-pathway responses to a piercing-sucking insect in rice. *The new phytologist*, 225 (1), pp.474–487. [Accessed 13 February 2025].

Ma, Y. et al. (2009). Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science (New York, N.Y.)*, 324 (5930), pp.1064–1068. [Accessed 9 September 2025].

Ma, Y. et al. (2024b). Mild drought conditions at the tillering stage promote dry matter accumulation and increase grain weight in drip-irrigated spring wheat (Triticum aestivum L.). *Frontiers in plant science*, 15, p.1509325. [Accessed 3 September 2025].

Madrid-Espinoza, J. et al. (2018). Genome-wide identification and transcriptional regulation of aquaporin genes in bread wheat (Triticum aestivum L.) under water stress. *Genes*, 9 (10), p.497. [Accessed 27 September 2025].

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.

Maksimov, I. et al. (2014). Salicylic acid increases the defense reaction against bunt and smut pathogens in wheat calli. *Journal of plant interactions*, 9 (1), pp.306–314. [Accessed 10 September 2025].

Mal, S. and Panchal, S. (2024). Drought and salt stress mitigation in crop plants using stress-tolerant auxin-producing endophytic bacteria: a futuristic approach towards sustainable agriculture. *Frontiers in plant science*, 15, p.1422504. [Accessed 10 September 2025].

Manzi, M. et al. (2015). Root ABA accumulation in long-term water-stressed plants is sustained by hormone transport from aerial organs. *Plant & cell physiology*, 56 (12), pp.2457–2466. [Accessed 9 September 2025].

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.

Mao, H. et al. (2022). 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. [Accessed 16 September 2025].

Marquis, V. et al. (2022). Broad-spectrum stress tolerance conferred by suppressing jasmonate signaling attenuation in Arabidopsis JASMONIC ACID OXIDASE mutants. *The Plant journal: for cell and molecular biology*, 109 (4), pp.856–872.

Maurel, C. et al. (2008). Plant aquaporins: membrane channels with multiple integrated functions. *Annual review of plant biology*, 59, pp.595–624.

Maurya, M. K. et al. (2022). Impact of climate change on diseases of crops and their management—A review. *Journal of Agricultural Science and Technology B*, 12 (1). [Online]. Available at: doi:10.17265/2161-6264/2022.01.001.

McCue, A. D. et al. (2012). Gene expression and stress response mediated by the epigenetic regulation of a transposable element small RNA. *PLoS genetics*, 8 (2), p.e1002474. [Accessed 4 August 2025].

McCue, A. D., Nuthikattu, S. and Slotkin, R. K. (2013). Genome-wide identification of genes regulated in trans by transposable element small interfering RNAs. *RNA biology*, 10 (8), pp.1379–1395. [Accessed 4 August 2025].

Medrano, M. et al. (2020). Comparative genetic and epigenetic diversity in pairs of sympatric, closely related plants with contrasting distribution ranges in south-eastern Iberian mountains. *AoB plants*, 12 (3). [Online]. Available at: doi:10.1093/aobpla/plaa013.

Mehrabad Pour-Benab, S., Fabriki-Ourang, S. and Mehrabi, A.-A. (2019). Expression of dehydrin and antioxidant genes and enzymatic antioxidant defense under drought stress in wild relatives of wheat. *Biotechnology, biotechnological equipment*, 33 (1), pp.1063–1073. [Accessed 27 September 2025].

Meng, F. et al. (2016). Physiological and proteomic responses to salt stress in chloroplasts of diploid and tetraploid black locust (Robinia pseudoacacia L.). *Scientific reports*, 6 (1), p.23098. [Accessed 13 August 2025].

Met Office. (2023). *UK Climate Projections: Headline Findings August 2022*. Department for Environment Food & Rural Affairs. [Online]. Available at: https://www.metoffice.gov.uk/research/approach/collaboration/ukcp/summaries/index.

Mette, M. F. et al. (2000). Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *The EMBO journal*, 19 (19), pp.5194–5201. [Accessed 11 September 2025].

Miao, R. et al. (2024). Histone variant H2A.Z is required for plant salt response by regulating gene transcription. *Plant, cell & environment*, 47 (7), pp.2693–2709. [Accessed 21 September 2025].

Miller, C. N. et al. (2016). Elucidation of the genetic basis of variation for stem strength characteristics in bread wheat by Associative Transcriptomics. *BMC genomics*, 17 (1), p.500. [Accessed 16 September 2025].

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.

Mishra, V. et al. (2022). HPCA1 and HSL3: two plasma membrane proteins that probably cooperate to modulate H2O2 signalling under drought conditions. *Plant growth regulation*, 98 (1), pp.1–3. [Accessed 27 January 2025].

Miura, A. et al. (2001). Mobilization of transposons by a mutation abolishing full DNA methylation in Arabidopsis. *Nature*, 411 (6834), pp.212–214.

Mohr, P. G. and Cahill, D. M. (2007). Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in Arabidopsis infected with Pseudomonas syringae pv. tomato. *Functional & integrative genomics*, 7 (3), pp.181–191. [Accessed 14 July 2025].

Mokhtari, N., Majidi, M. M. and Mirlohi, A. (2022). Potentials of synthetic hexaploid wheats to improve drought tolerance. *Scientific reports*, 12 (1), p.20482. [Accessed 16 September 2025].

Mokhtari, N., Majidi, M. M. and Mirlohi, A. (2024). Physiological and antioxidant responses of synthetic hexaploid wheat germplasm under drought. *BMC plant biology*, 24 (1), p.747. [Accessed 16 September 2025].

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.

Molotoks, A., Smith, P. and Dawson, T. P. (2021). Impacts of land use, population, and climate change on global food security. *Food and energy security*, 10 (1), p.e261. [Accessed 25 April 2025].

Mordaunt, C. E. et al. (2022). Comethyl: a network-based methylome approach to investigate the multivariate nature of health and disease. *Briefings in bioinformatics*, 23 (2). [Online]. Available at: doi:10.1093/bib/bbab554.

Morgan, D., DeMeo, D. L. and Glass, K. (2024). Using methylation data to improve transcription factor binding prediction. *Epigenetics: official journal of the DNA Methylation Society*, 19 (1), p.2309826. [Accessed 17 June 2025].

Morgounov, A. et al. (2018). High-yielding winter synthetic hexaploid wheats resistant to multiple diseases and pests. *Plant genetic resources: characterization and utilization*, 16 (3), pp.273–278.

Mouat, J. S. et al. (2023). Networks of placental DNA methylation correlate with maternal serum PCB concentrations and child neurodevelopment. *Environmental research*, 220, p.115227.

Mu, Q. et al. (2022). Physiological response of winter wheat (Triticum aestivum L.) during vegetative growth to gradual, persistent and intermittent drought. *Agricultural water management*, 274 (107911), p.107911. [Accessed 6 September 2025].

Muchlas, M. et al. (2023). Mild drought priming-induced salicylic acid involves in subsequent drought tolerance by modulating glutathione redox in antagonism with abscisic acid in Brassica napus. *Plant growth regulation*. [Online]. Available at: doi:10.1007/s10725-023-01070-5.

Muhammad Aslam, M. et al. (2022). Mechanisms of abscisic acid-mediated drought stress responses in plants. *International journal of molecular sciences*, 23 (3), p.1084. [Accessed 9 September 2025].

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.

Munemasa, S. et al. (2015). Mechanisms of abscisic acid-mediated control of stomatal aperture. *Current opinion in plant biology*, 28, pp.154–162. [Accessed 6 September 2025].

Munné-Bosch, S. et al. (2001). Diterpenes and antioxidative protection in drought-stressed Salvia officinalis plants. *Journal of plant physiology*, 158 (11), pp.1431–1437.

Muyle, A. M. et al. (2022). Gene Body Methylation in Plants: Mechanisms, Functions, and Important Implications for Understanding Evolutionary Processes. *Genome biology and evolution*, 14 (4). [Online]. Available at: doi:10.1093/gbe/evac038.

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.

Naderi, S. et al. (2024). Physio-biochemical and DNA methylation analysis of the defense response network of wheat to drought stress. *Plant physiology and biochemistry*, 209 (108516), p.108516. [Accessed 2 December 2024].

Nardino, M. et al. (2022). Understanding drought response mechanisms in wheat and multi-trait selection. *PloS one*, 17 (4), p.e0266368.

Nazar, R. et al. (2015). Salicylic acid supplementation improves photosynthesis and growth in mustard through changes in proline accumulation and ethylene formation under drought stress. *Suid-Afrikaanse tydskrif vir plantkunde [South African journal of botany]*, 98, pp.84–94. [Accessed 10 September 2025].

Nazari, M., Mirgol, B. and Salehi, H. (2021). Climate change impact assessment and adaptation strategies for rainfed wheat in contrasting climatic regions of Iran. *Frontiers in agronomy*, 3, p.806146. [Accessed 29 April 2025].

Ndecky, S. et al. (2023). Jasmonate signaling controls negative and positive effectors of salt stress tolerance in rice. *Journal of experimental botany*, 74 (10), pp.3220–3239. [Accessed 21 September 2025].

Ndecky, S. et al. (2025). Rice JASMONIC ACID OXIDASES control resting jasmonate metabolism to promote growth and repress basal immune responses. *Plant physiology*, 198 (1), p.kiaf161. [Accessed 21 September 2025].

Nejadsadeghi, L. et al. (2014). Comparative analysis of physio-biochemical responses to cold stress in tetraploid and hexaploid wheat. *Cell biochemistry and biophysics*, 70 (1), pp.399–408. [Accessed 7 May 2025].

Newton, A. C. et al. (2010). Cereal landraces for sustainable agriculture. A review. *Agronomy for sustainable development*, 30 (2), pp.237–269.

Nielsen, C. P. S. et al. (2024). Evidence for an RNAi-independent role of Arabidopsis DICER-LIKE2 in growth inhibition and basal antiviral resistance. *The plant cell*, 36 (6), pp.2289–2309. [Accessed 11 September 2025].

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.

Niu, Y. et al. (2023). Comparative transcriptome analysis reveals the key genes and pathways involved in drought stress response of two wheat (Triticum aestivum L) varieties. *Genomics*, 115 (5), p.110688. [Accessed 26 November 2024].

NOAA National Centers for Environmental Information. (2022a). *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. (2022b). *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].

Noir, S. et al. (2013). Jasmonate controls leaf growth by repressing cell proliferation and the onset of endoreduplication while maintaining a potential stand-by mode. *Plant physiology*, 161 (4), pp.1930–1951. [Accessed 10 September 2025].

Noman, A., Aqeel, M. and Lou, Y. (2019). PRRs and NB-LRRs: From signal perception to activation of plant innate immunity. *International journal of molecular sciences*, 20 (8), p.1882. [Accessed 27 January 2025].

Nyaga, D. M. et al. (2024). Benchmarking nanopore sequencing and rapid genomics feasibility: validation at a quaternary hospital in New Zealand. *npj genomic medicine*, 9 (1), p.57. [Accessed 12 September 2025].

Nyaupane, S. et al. (2024). Drought stress effect, tolerance, and management in wheat – a review. *Cogent food & agriculture*, 10 (1). [Online]. Available at: doi:10.1080/23311932.2023.2296094 [Accessed 28 February 2025].

Ochieng, G. et al. (2021). Novel sources of drought tolerance from landraces and wild sorghum relatives. *Crop science*, 61 (1), pp.104–118. [Accessed 4 March 2025].

OECD. (2025). *Global drought outlook*. OECD Publishing.

O'Hara, N. B., Rest, J. S. and Franks, S. J. (2016). Increased susceptibility to fungal disease accompanies adaptation to drought in Brassica rapa: BRIEF COMMUNICATION. *Evolution; international journal of organic evolution*, 70 (1), pp.241–248. [Accessed 10 September 2025].

Oishy, M. N. et al. (2025). Unravelling the effects of climate change on the soil-plant-atmosphere interactions: A critical review. *Soil & Environmental Health*, 3 (1), p.100130. [Accessed 7 May 2025].

de Ollas, C., Arbona, V. and Gómez-Cadenas, A. (2015). Jasmonic acid interacts with abscisic acid to regulate plant responses to water stress conditions. *Plant signaling & behavior*, 10 (12), p.e1078953.

Olova, N. et al. (2018). Comparison of whole-genome bisulfite sequencing library preparation strategies identifies sources of biases affecting DNA methylation data. *Genome biology*, 19 (1), p.33. [Accessed 21 September 2025].

O'Malley, R. C. et al. (2016). Cistrome and epicistrome features shape the regulatory DNA landscape. *Cell*, 165 (5), pp.1280–1292. [Accessed 23 July 2025].

Omere, E. A., Nwaoguala, C. N. C. and Emede, T. O. (2023). Polyploidy and its relevance in crop improvement. *Nigerian journal of biotechnology*, 39 (2), pp.9–19. [Accessed 8 September 2025].

Ono, K. et al. (2019). Jasmonate production through chlorophyll a degradation by Stay-Green in Arabidopsis thaliana. *Journal of plant physiology*, 238, pp.53–62. [Accessed 4 March 2025].

Ortigosa, A. et al. (2020). The JA-pathway MYC transcription factors regulate photomorphogenic responses by targeting HY5 gene expression. *The Plant journal: for cell and molecular biology*, 102 (1), pp.138–152. [Accessed 4 September 2024].

Osabe, K. et al. (2014). Genetic and DNA methylation changes in cotton (Gossypium) genotypes and tissues. *PloS one*, 9 (1), p.e86049. [Accessed 4 September 2025].

Ou, X. et al. (2012). Transgenerational inheritance of modified DNA methylation patterns and enhanced tolerance induced by heavy metal stress in rice (Oryza sativa L.). *PloS one*, 7 (9), p.e41143. [Accessed 14 September 2025].

Panda, K. et al. (2023). The plant response to high CO2 levels is heritable and orchestrated by DNA methylation. *The new phytologist*, 238 (6), pp.2427–2439. [Accessed 14 September 2025].

Panda, K. and Slotkin, R. K. (2020). Long-read cDNA sequencing enables a 'gene-like' transcript annotation of transposable elements. *The plant cell*, 32 (9), pp.2687–2698. [Accessed 22 September 2025].

Pandian, B. A. et al. (2020). Role of cytochrome P450 enzymes in plant stress response. *Antioxidants (Basel, Switzerland)*, 9 (5), p.454. [Accessed 9 September 2024].

Pankasem, N. et al. (2024). Warming triggers stomatal opening by enhancement of photosynthesis and ensuing guard cell CO2 sensing, whereas higher temperatures induce a photosynthesis-uncoupled response. *The new phytologist*, 244 (5), pp.1847–1863. [Accessed 9 September 2025].

Papikian, A. et al. (2019). Site-specific manipulation of Arabidopsis loci using CRISPR-Cas9 SunTag systems. *Nature communications*, 10 (1), p.729. [Accessed 16 September 2025].

Pardo-Diaz, J. et al. (2021). Robust gene coexpression networks using signed distance correlation. *Bioinformatics (Oxford, England)*, 37 (14), pp.1982–1989. [Accessed 21 September 2025].

Parent, J.-S. et al. (2015). Respective contributions of Arabidopsis DCL2 and DCL4 to RNA silencing. *The Plant journal: for cell and molecular biology*, 81 (2), pp.223–232. [Accessed 11 September 2025].

Park, J. M. and Lazarovits, G. (2014). Involvement of hexokinase1 in plant growth promotion as mediated by Burkholderia phytofirmans. *Canadian journal of microbiology*, 60 (6), pp.343–354. [Accessed 20 June 2025].

Parry, M. A. J. et al. (2002). Rubisco activity: effects of drought stress. *Annals of botany*, 89 Spec No (7), pp.833–839. [Accessed 6 September 2025].

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.

Patro, R. et al. (2017). Salmon provides fast and bias-aware quantification of transcript expression. *Nature methods*, 14 (4), pp.417–419.

Peng, J. et al. (1999). 'Green revolution' genes encode mutant gibberellin response modulators. *Nature*, 400 (6741), pp.256–261. [Accessed 23 April 2025].

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. [Accessed 17 April 2025].

Pereyra, S. et al. (2025). Cost-effective promoter methylation analysis via target long-read bisulfite sequencing: a case study in severe preterm birth. *BMC medical genomics*, 18 (1), p.122. [Accessed 12 September 2025].

Pérez-Díaz, J. et al. (2021). Prunus Hexokinase 3 genes alter primary C-metabolism and promote drought and salt stress tolerance in Arabidopsis transgenic plants. *Scientific reports*, 11 (1), p.7098. [Accessed 20 June 2025].

Pfeifer, M. et al. (2014). Genome interplay in the grain transcriptome of hexaploid bread wheat. *Science (New York, N.Y.)*, 345 (6194), p.1250091. [Accessed 28 February 2025].

Pfleger, A. et al. (2011). The effect of extremely diluted agitated gibberellic acid (10e-30) on wheat stalk growth--a two researcher pilot study. *Complementary therapies in medicine*, 19 (3), pp.164–169. [Accessed 10 September 2025].

Pfluger, J. and Wagner, D. (2007). Histone modifications and dynamic regulation of genome accessibility in plants. *Current opinion in plant biology*, 10 (6), pp.645–652. [Accessed 15 September 2025].

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. [Accessed 23 April 2025].

Pisupati, R. et al. (2023). On the causes of gene-body methylation variation in Arabidopsis thaliana. *PLoS genetics*, 19 (5), p.e1010728. [Accessed 22 September 2025].

Plumpton, H. and Wentworth, J. (2019). *Climate change and agriculture*. Parliamentary Office of Science and Technology. [Online]. Available at: doi:10.58248/pn600.

Polavarapu B, K. K. et al. (2022). Abscisic acid and its role in the modulation of plant growth, development, and yield stability. *Trends in plant science*, 27 (12), pp.1283–1295. [Accessed 28 February 2025].

Ponferrada-Marín, M. I., Roldán-Arjona, T. and Ariza, R. R. (2009). ROS1 5-methylcytosine DNA glycosylase is a slow-turnover catalyst that initiates DNA demethylation in a distributive fashion. *Nucleic acids research*, 37 (13), pp.4264–4274. [Accessed 5 August 2025].

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.

del Pozo, J. C. and Ramirez-Parra, E. (2014). Deciphering the molecular bases for drought tolerance in Arabidopsis autotetraploids: Polyploidy increases drought tolerance. *Plant, cell & environment*, 37 (12), pp.2722–2737. [Accessed 13 August 2025].

Pratap, S. and Markonis, Y. (2022). The response of the hydrological cycle to temperature changes in recent and distant climatic history. *Progress in earth and planetary science*, 9 (1), pp.1–37. [Accessed 7 May 2025].

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.

Puy, J. et al. (2018). Improved demethylation in ecological epigenetic experiments: Testing a simple and harmless foliar demethylation application. *Methods in Ecology and Evolution*, 9 (3), pp.744–753. [Accessed 16 September 2025].

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.

Qi, P.-F. et al. (2019). Functional analysis of FgNahG clarifies the contribution of salicylic acid to wheat (Triticum aestivum) resistance against Fusarium head blight. *Toxins*, 11 (2), p.59. [Accessed 10 September 2025].

Qiao, H. et al. (2021). TaWRKY13-A serves as a mediator of jasmonic acid-related leaf senescence by modulating jasmonic acid biosynthesis. *Frontiers in plant science*, 12, p.717233. [Accessed 9 September 2025].

Qiao, M. et al. (2024). Impacts of drought on photosynthesis in major food crops and the related mechanisms of plant responses to drought. *Plants*, 13 (13), p.1808. [Accessed 6 September 2025].

Qin, C. et al. (2017). Roles of dicer-like proteins 2 and 4 in intra- and intercellular antiviral silencing. *Plant physiology*, 174 (2), pp.1067–1081. [Accessed 11 September 2025].

Quinlan, A. R. and Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics (Oxford, England)*, 26 (6), pp.841–842. [Accessed 3 December 2024].

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, M. M. et al. (2021). Improving Wheat Yield Prediction Using Secondary Traits and High-Density Phenotyping Under Heat-Stressed Environments. *Frontiers in plant science*, 12, p.633651.

Rajkumar, M. S. et al. (2020). DNA methylation reprogramming during seed development and its functional relevance in seed size/weight determination in chickpea. *Communications biology*, 3 (1), p.340. [Accessed 16 July 2025].

Ramachandra Reddy, A., Chaitanya, K. V. and Vivekanandan, M. (2004). Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *Journal of plant physiology*, 161 (11), pp.1189–1202. [Accessed 6 September 2025].

Ramanna, M. S. and Jacobsen, E. (2003). Relevance of sexual polyploidization for crop improvement – A review. *Euphytica; Netherlands journal of plant breeding*, 133 (1), pp.3–8. [Accessed 8 September 2025].

Rathan, N. D. et al. (2022). Genome-wide association study identifies loci and candidate genes for grain micronutrients and quality traits in wheat (Triticum aestivum L.). *Scientific reports*, 12 (1), p.7037. [Accessed 16 September 2025].

Ray, D. K. et al. (2012). Recent patterns of crop yield growth and stagnation. *Nature communications*, 3 (1), p.1293. [Accessed 24 April 2025].

Ray, D. K. et al. (2013). Yield Trends Are Insufficient to Double Global Crop Production by 2050. *PloS one*, 8 (6), p.e66428.

Rehman, M. et al. (2023). The multifaceted role of jasmonic acid in plant stress mitigation: An overview. *Plants*, 12 (23), p.3982. [Accessed 29 April 2025].

Reid, X. J., Zhong, Y. and Mackay, J. P. (2024). How does CHD4 slide nucleosomes? *Biochemical Society transactions*, 52 (5), pp.1995–2008. [Accessed 10 September 2025].

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.

Reinhart, B. J. et al. (2002). MicroRNAs in plants. *Genes & development*, 16 (13), pp.1616–1626. [Accessed 5 August 2025].

Ren, J. et al. (2022). Comparative physiological and coexpression network analyses reveal the potential drought tolerance mechanism of peanut. *BMC plant biology*, 22 (1), p.460. [Accessed 10 March 2025].

Renkow, M. and Byerlee, D. (2010). The impacts of CGIAR research: A review of recent evidence. *Food policy*, 35 (5), pp.391–402.

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. [Accessed 16 September 2025].

Riache, M. et al. (2023). Genetic analyses of stay green for tolerance to water stress and nitrogen deficiency in Algerian Saharan maize populations. *Euphytica; Netherlands journal of plant breeding*, 219 (6), pp.1–18. [Accessed 4 March 2025].

Riboni, M. et al. (2016). ABA-dependent control of GIGANTEA signalling enables drought escape via up-regulation of FLOWERING LOCUS T in Arabidopsis thaliana. *Journal of experimental botany*, 67 (22), pp.6309–6322. [Accessed 10 September 2025].

Riemann, M. et al. (2015). Exploring jasmonates in the hormonal network of drought and salinity responses. *Frontiers in plant science*, 6, p.1077. [Accessed 9 September 2025].

Riemondy, K. A. et al. (2017). valr: Reproducible genome interval analysis in R. *F1000Research*, 6, p.1025.

Rimoldi, M. et al. (2024). DNA methylation patterns of transcription factor binding regions characterize their functional and evolutionary contexts. *Genome biology*, 25 (1), p.146. [Accessed 17 June 2025].

Rippke, U. et al. (2016). Timescales of transformational climate change adaptation in sub-Saharan African agriculture. *Nature climate change*, 6 (6). [Online]. Available at: doi:10.1038/nclimate2947.

Rokotyan, N., Stukova, O., Kolmakova D. & Ovsyannikov, D. (2022). *Cosmograph: GPU-accelerated Force Graph Layout and Rendering*. [Online]. Available at: https://cosmograph.app/.

Ronchi, A. et al. (2025). Arabidopsis thaliana roots exposed to extracellular self-DNA: Evidence of epigenetic effects. *Epigenomes*, 9 (2), p.13. [Accessed 22 September 2025].

Rosado, A. et al. (2006). The Arabidopsis tetratricopeptide repeat-containing protein TTL1 is required for osmotic stress responses and abscisic acid sensitivity. *Plant physiology*, 142 (3), pp.1113–1126.

Rose, G. et al. (2016). Impact of progressive global warming on the global-scale yield of maize and soybean. *Climatic Change*, 134 (3), pp.417–428. [Accessed 1 May 2025].

Rosyara, U. et al. (2019). Genetic contribution of synthetic hexaploid wheat to CIMMYT's spring bread wheat breeding germplasm. *Scientific reports*, 9 (1), p.12355. [Accessed 16 September 2025].

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), p.5314. [Accessed 16 September 2025].

Roy, A. et al. (2024). Towards strategic interventions for global food security in 2050. *The Science of the total environment*, 954 (176811), p.176811. [Accessed 25 April 2025].

Ru, C. et al. (2025). Intermittent drought priming improves photosynthesis, water status, antioxidant capacity, and tolerance to drought stress in winter wheat. *Journal of plant growth regulation*, pp.1–20. [Accessed 19 August 2025].

Ruan, J. et al. (2019). Jasmonic acid signaling pathway in plants. *International journal of molecular sciences*, 20 (10), p.2479. [Accessed 10 September 2024].

Ruiz, M. et al. (2020). Synthetic polyploidy in grafted crops. *Frontiers in plant science*, 11, p.540894. [Accessed 8 September 2025].

Sabot, F. et al. (2005). Updating of transposable element annotations from large wheat genomic sequences reveals diverse activities and gene associations. *Molecular genetics and genomics: MGG*, 274 (2), pp.119–130. [Accessed 17 April 2025].

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.

Sadok, W. and Schoppach, R. (2019). Potential involvement of root auxins in drought tolerance by modulating nocturnal and daytime water use in wheat. *Annals of botany*, 124 (6), pp.969–978. [Accessed 10 September 2025].

Safari, M. and Majidi, M. M. (2025). Role of genetic diversity and salicylic acid in drought stress memory of tall fescue. *Scientific reports*, 15 (1), p.7932.

Sajedi, S. et al. (2023). Integrating DNA methylation and gene expression data in a single gene network using the iNETgrate package. *Scientific reports*, 13 (1), p.21721.

Salgotra, R. K. and Chauhan, B. S. (2023). Genetic diversity, conservation, and utilization of plant genetic resources. *Genes*, 14 (1), p.174. [Accessed 10 March 2025].

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.

Sammons, R. D. et al. (2011). Polynucleotide molecules for gene regulation in plants. *US Patent*. [Online]. Available at: https://patentimages.storage.googleapis.com/fa/1e/dc/557fb7faca7c5c/US20110296556A1.pdf [Accessed 23 September 2025].

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.

Saputro, T. B. et al. (2023). OsBTBZ1 Confers Salt Stress Tolerance in Arabidopsis thaliana. *International journal of molecular sciences*, 24 (19), p.14483. [Accessed 9 September 2025].

Sarwar, M. et al. (2023). Silver nanoparticles protect tillering in drought-stressed wheat by improving leaf water relations and physiological functioning. *Functional plant biology: FPB*, 50 (11), pp.901–914.

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.

Savchenko, T. et al. (2014). Functional convergence of oxylipin and abscisic acid pathways controls stomatal closure in response to drought. *Plant physiology*, 164 (3), pp.1151–1160. [Accessed 9 September 2025].

Schaart, J. G., van de Wiel, C. C. M. and Smulders, M. J. M. (2021). Genome editing of polyploid crops: prospects, achievements and bottlenecks. *Transgenic research*, 30 (4), pp.337–351. [Accessed 15 September 2025].

Schapire, A. L., Valpuesta, V. and Botella, M. A. (2006). TPR proteins in plant hormone signaling. *Plant signaling & behavior*, 1 (5), pp.229–230. [Accessed 19 September 2025].

Schauberger, B. et al. (2018). Yield trends, variability and stagnation analysis of major crops in France over more than a century. *Scientific reports*, 8 (1), p.16865. [Accessed 24 April 2025].

Schewe, J. et al. (2014). Multimodel assessment of water scarcity under climate change. *Proceedings of the National Academy of Sciences of the United States of America*, 111 (9), pp.3245–3250. [Accessed 7 May 2025].

Schindelin, J. et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nature methods*, 9 (7), pp.676–682. [Accessed 20 August 2025].

Schippers, J. H. M. et al. (2015). Living to die and dying to live: The survival strategy behind leaf senescence. *Plant physiology*, 169 (2), pp.914–930.

Schlagenhauf, E. and Wicker, T. (2019). *The TREP platform: A curated database of transposable elements*. [Online]. TREP. Available at: https://trep-db.uzh.ch [Accessed 21 May 2025].

Schmidt, J. et al. (2019). Novel Alleles for Combined Drought and Heat Stress Tolerance in Wheat. *Frontiers in plant science*, 10, p.1800.

Schmitz, R. J. et al. (2007). DICER-LIKE 1 and DICER-LIKE 3 redundantly act to promote flowering via repression of FLOWERING LOCUS C in Arabidopsis thaliana. *Genetics*, 176 (2), pp.1359–1362. [Accessed 11 September 2025].

Schneider, J. et al. (2021). Assessing the suitability of elite lines for hybrid seed production and as testers in wide crosses with wheat genetic resources. *Frontiers in plant science*, 12, p.689825. [Accessed 16 September 2025].

Schrey, A. W. et al. (2012). Epigenetic variation may compensate for decreased genetic variation with introductions: A case study using house sparrows (Passer domesticus) on two continents. *Genetics research international*, 2012, p.979751.

Schuppler, U. et al. (1998). Effect of water stress on cell division and cell-division-cycle 2-like cell-cycle kinase activity in wheat leaves. *Plant physiology*, 117 (2), pp.667–678. [Accessed 6 September 2025].

Secco, D. et al. (2015). Stress induced gene expression drives transient DNA methylation changes at adjacent repetitive elements. *eLife*, 4. [Online]. Available at: doi:10.7554/eLife.09343 [Accessed 22 September 2025].

Sehar, Z. et al. (2021). Ethylene reduces glucose sensitivity and reverses photosynthetic repression through optimization of glutathione production in salt-stressed wheat (Triticum aestivum L.). *Scientific reports*, 11 (1), p.12650. [Accessed 10 September 2025].

Sehgal, A. et al. (2018). Drought or/and heat-stress effects on seed filling in food crops: Impacts on functional biochemistry, seed yields, and nutritional quality. *Frontiers in plant science*, 9, p.1705. [Accessed 6 September 2025].

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.

Senapati, N., Halford, N. G. and Semenov, M. A. (2021). Vulnerability of European wheat to extreme heat and drought around flowering under future climate. *Environmental Research Letters*, 16 (2), p.024052.

Seo, J.-S. et al. (2011). OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice: OsbHLH148 interacting with OsJAZ proteins confers drought tolerance in rice. *The Plant journal: for cell and molecular biology*, 65 (6), pp.907–921. [Accessed 9 September 2025].

Seo, K.-W. et al. (2025). Abrupt sea level rise and Earth's gradual pole shift reveal permanent hydrological regime changes in the 21st century. *Science (New York, N.Y.)*, 387 (6741), pp.1408–1413. [Accessed 7 May 2025].

Serdeczny, O. et al. (2017). Climate change impacts in Sub-Saharan Africa: from physical changes to their social repercussions. *Regional Environmental Change*, 17 (6). [Online]. Available at: doi:10.1007/s10113-015-0910-2.

Serrano-Mislata, A. et al. (2025). Growth arrest is a DNA damage protection strategy in Arabidopsis. *Nature communications*, 16 (1), p.5635.

Sewelam, N., Kazan, K. and Schenk, P. M. (2016). Global plant stress signaling: Reactive oxygen species at the Cross-Road. *Frontiers in plant science*, 7, p.187. [Accessed 9 September 2025].

Shahbazi, F., Shahbazi, S. and Zare, D. (2025). Losses in agricultural produce: Causes and effects on food security. *Food and energy security*, 14 (3), p.e70086. [Accessed 5 September 2025].

Shahzad, Z. et al. (2025). Gene body methylation regulates gene expression and mediates phenotypic diversity in natural Arabidopsis populations. *Nature plants*, pp.1–16. [Accessed 22 September 2025].

Shannon, P. et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome research*, 13 (11), pp.2498–2504.

Sharma, P. et al. (2025). Novel and conserved drought-responsive microRNAs expression analysis in root tissues of wheat (Triticum asetivum L.) at reproductive stage. *Frontiers in plant science*, 16, p.1581542. [Accessed 22 September 2025].

Sharma, R. et al. (2017). Epiallelic changes in known stress-responsive genes under extreme drought conditions in Brassica juncea (L.) Czern. *Plant cell reports*, 36 (1), pp.203–217.

Sharma, V. et al. (2022). Response of wheat genotypes to drought stress stimulated by PEG. *Stresses*, 2 (1), pp.26–51. [Accessed 2 December 2024].

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. [Accessed 5 September 2025].

Shazadi, K., Christopher, J. T. and Chenu, K. (2024). Does late water deficit induce root growth or senescence in wheat? *Frontiers in plant science*, 15, p.1351436. [Accessed 5 September 2025].

Sheldon, C. C. et al. (2000). The molecular basis of vernalization: The central role of FLOWERING LOCUS C (FLC). *Proceedings of the National Academy of Sciences of the United States of America*, 97 (7), pp.3753–3758. [Accessed 27 September 2025].

Sherstneva, O. et al. (2024). Prediction of biomass accumulation and tolerance of wheat seedlings to drought and elevated temperatures using hyperspectral imaging. *Frontiers in plant science*, 15, p.1344826. [Accessed 9 May 2025].

Shi, M. et al. (2023). Role of methylation in vernalization and photoperiod pathway: a potential flowering regulator? *Horticulture research*, 10 (10), p.uhad174. [Accessed 23 September 2025].

Shiferaw, B. et al. (2013). Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security. *Food Security*, 5 (3), pp.291–317.

Shu, K. et al. (2016). ABSCISIC ACID-INSENSITIVE 4 negatively regulates flowering through directly promoting Arabidopsis FLOWERING LOCUS C transcription. *Journal of experimental botany*, 67 (1), pp.195–205. [Accessed 10 September 2025].

Sigurpalsdottir, B. D. et al. (2024). A comparison of methods for detecting DNA methylation from long-read sequencing of human genomes. *Genome biology*, 25 (1), p.69. [Accessed 21 September 2025].

Silva, G. C. B. et al. (2024). Identification of DEMETER-like DNA demethylase gene family in citrus and their role in drought stress-adaptive responses. *Computational biology and chemistry*, 112 (108128), p.108128. [Accessed 31 August 2025].

Silva, J. V. et al. (2023). Pathways to wheat self-sufficiency in Africa. *Global food security*, 37 (100684), p.100684. [Accessed 25 April 2025].

Simpson, J. T. et al. (2017). Detecting DNA cytosine methylation using nanopore sequencing. *Nature methods*, 14 (4), pp.407–410.

Şimşek, Ö. et al. (2024). Advanced biotechnological interventions in mitigating drought stress in plants. *Plants*, 13 (5), p.717. [Accessed 5 September 2025].

Singh, A. K. et al. (2011). Silencing genes encoding omega-3 fatty acid desaturase alters seed size and accumulation of Bean pod mottle virus in soybean. *Molecular plant-microbe interactions: MPMI*, 24 (4), pp.506–515. [Accessed 10 September 2024].

Singh, K. et al. (2022a). BS-Seq reveals major role of differential CHH methylation during leaf rust resistance in wheat (Triticum aestivum L.). *Molecular genetics and genomics: MGG*, 297 (3), pp.731–749. [Accessed 26 November 2024].

Singh, P. et al. (2022b). Specific roles of lipoxygenases in development and responses to stress in plants. *Plants*, 11 (7), p.979. [Accessed 10 September 2024].

Singh, P. and Mukhopadhyay, K. (2021). Comprehensive molecular dissection of TIFY Transcription factors reveal their dynamic responses to biotic and abiotic stress in wheat (Triticum aestivum L.). *Scientific reports*, 11 (1), p.9739. [Accessed 9 September 2024].

Sirhindi, G. et al. (2016). Jasmonic acid modulates the physio-biochemical attributes, antioxidant enzyme activity, and gene expression in Glycine max under nickel toxicity. *Frontiers in plant science*, 7, p.591. [Accessed 9 September 2025].

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.

Slater, L. J. et al. (2022). Resilience of UK crop yields to compound climate change. *Earth system dynamics*, 13 (3), pp.1377–1396. [Accessed 1 May 2025].

Slawin, C., Ajayi, O. and Mahalingam, R. (2024). Association mapping unravels the genetic basis for drought related traits in different developmental stages of barley. *Scientific reports*, 14 (1), p.25121.

Smirnova, E. et al. (2017). Jasmonic acid oxidase 2 hydroxylates jasmonic acid and represses basal defense and resistance responses against Botrytis cinerea infection. *Molecular plant*, 10 (9), pp.1159–1173.

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.

Snyder, C. W. (2016). Evolution of global temperature over the past two million years. *Nature*, 538 (7624), pp.226–228. [Accessed 2 May 2025].

Soneson, C., Love, M. I. and Robinson, M. D. (2015). Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. *F1000Research*, 4 (1521), p.1521. [Accessed 2 September 2022].

Song, P., Cai, Z. and Jia, G. (2024). Principles, functions, and biological implications of m6A in plants. *RNA (New York, N.Y.)*, 30 (5), pp.491–499. [Accessed 11 September 2025].

Song, X. et al. (2025). Inheritance of acquired adaptive cold tolerance in rice through DNA methylation. *Cell*, 188 (16), pp.4213-4224.e12. [Accessed 23 February 2026].

Song, Y. et al. (2016). Stable methylation of a non-coding RNA gene regulates gene expression in response to abiotic stress in *Populus simonii*. *Journal of experimental botany*, 67 (5). [Online]. Available at: doi:10.1093/jxb/erv543.

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.

de Sousa, T. et al. (2021). The 10,000-year success story of wheat! *Foods (Basel, Switzerland)*, 10 (9), p.2124. [Accessed 15 April 2025].

Stelpflug, S. C. et al. (2014). Consistent and heritable alterations of DNA methylation are induced by tissue culture in maize. *Genetics*, 198 (1), pp.209–218. [Accessed 14 September 2025].

Stephens, M. (2017). False discovery rates: a new deal. *Biostatistics* , 18 (2), pp.275–294.

Stroud, H. et al. (2013). Comprehensive analysis of silencing mutants reveals complex regulation of the Arabidopsis methylome. *Cell*, 152 (1–2), pp.352–364. [Accessed 5 February 2026].

Stroud, H. et al. (2014). Non-CG methylation patterns shape the epigenetic landscape in Arabidopsis. *Nature structural & molecular biology*, 21 (1), pp.64–72. [Accessed 29 July 2025].

Su, Z. et al. (2013). Flower development under drought stress: morphological and transcriptomic analyses reveal acute responses and long-term acclimation in Arabidopsis. *The plant cell*, 25 (10), pp.3785–3807. [Accessed 22 September 2025].

Su, Z. Y. et al. (2021). Comparing transcriptional responses to Fusarium crown rot in wheat and barley identified an important relationship between disease resistance and drought tolerance. *BMC plant biology*, 21 (1), p.73. [Accessed 29 April 2025].

Sun, F. et al. (2013). Widespread, abundant, and diverse TE-associated siRNAs in developing wheat grain. *Gene*, 522 (1), pp.1–7. [Accessed 13 August 2025].

Sun, M. et al. (2022a). DNA Methylation in Plant Responses and Adaption to Abiotic Stresses. *International journal of molecular sciences*, 23 (13). [Online]. Available at: doi:10.3390/ijms23136910.

Sun, R.-Z. et al. (2021a). DNA methylation-mediated modulation of rapid desiccation tolerance acquisition and dehydration stress memory in the resurrection plant Boea hygrometrica. *PLoS genetics*, 17 (4), p.e1009549.

Sun, Y. et al. (2020). Food Insecurity Is Associated With Cardiovascular and All-Cause Mortality Among Adults in the United States. *Journal of the American Heart Association*, 9 (19), pp.14629–14629.

Sun, Z. et al. (2021b). 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.

Sun, Z. et al. (2022b). DNA methylation in transposable elements buffers the connection between three-dimensional chromatin organization and gene transcription upon rice genome duplication. *Journal of advanced research*, 42, pp.41–53. [Accessed 4 August 2025].

Sura, W. et al. (2017). Dual role of the histone variant H2A.Z in transcriptional regulation of stress-response genes. *The plant cell*, 29 (4), pp.791–807. [Accessed 21 September 2025].

Suter, L. and Widmer, A. (2013). Environmental Heat and Salt Stress Induce Transgenerational Phenotypic Changes in Arabidopsis thaliana. Blazquez, M. A. (Ed). *PLoS ONE*, 8 (4), p.e60364. [Accessed 14 September 2025].

Suzuki, M. et al. (2018). Whole-genome bisulfite sequencing with improved accuracy and cost. *Genome research*, 28 (9), pp.1364–1371. [Accessed 12 September 2025].

Szabados, L. and Savouré, A. (2010). Proline: a multifunctional amino acid. *Trends in plant science*, 15 (2), pp.89–97. [Accessed 8 September 2025].

Szklarczyk, D. et al. (2023). The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. *Nucleic acids research*, 51 (D1), pp.D638–D646. [Accessed 21 September 2025].

Tabassum, T. et al. (2017). Seed priming and transgenerational drought memory improves tolerance against salt stress in bread wheat. *Plant physiology and biochemistry*, 118, pp.362–369. [Accessed 19 August 2025].

Takahashi, F. et al. (2020). Drought stress responses and resistance in plants: From cellular responses to long-distance intercellular communication. *Frontiers in plant science*, 11, p.556972. [Accessed 9 September 2025].

Takuno, S. and Gaut, B. S. (2013). Gene body methylation is conserved between plant orthologs and is of evolutionary consequence. *Proceedings of the National Academy of Sciences of the United States of America*, 110 (5), pp.1797–1802. [Accessed 11 September 2025].

Takuno, S., Seymour, D. K. and Gaut, B. S. (2017). The evolutionary dynamics of orthologs that shift in gene body methylation between Arabidopsis species. *Molecular biology and evolution*, 34 (6), pp.1479–1491. [Accessed 31 July 2025].

Talarico, E. et al. (2024). Unravelling the epigenetic code: DNA methylation in plants and its role in stress response. *Epigenomes*, 8 (3), p.30. [Accessed 16 July 2025].

Tan, B. et al. (2025). Transcriptome profiling of spike development reveals key genes and pathways associated with early heading in wheat-psathyrstachys huashanica 7Ns chromosome addition line. *Plants*, 14 (13), p.2077. [Accessed 27 September 2025].

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. [Accessed 10 March 2025].

Taniguchi, S. et al. (2014). Isolation of jasmonate-induced sesquiterpene synthase of rice: product of which has an antifungal activity against Magnaporthe oryzae. *Journal of plant physiology*, 171 (8), pp.625–632. [Accessed 14 July 2025].

Tanksley, S. and McCouch, S. (1997). Seed Banks and Molecular Maps: Unlocking Genetic Potential from the Wild. *Science*, 277 (5329), pp.1063–1066.

Tanurdzic, M. et al. (2008). Epigenomic consequences of immortalized plant cell suspension culture. *PLoS biology*, 6 (12), pp.2880–2895. [Accessed 22 September 2025].

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.

Taub, D. R., Miller, B. and Allen, H. (2008). Effects of elevated CO2 on the protein concentration of food crops: a meta-analysis: ELEVATED CO2 AND CROP PROTEIN CONCENTRATIONS. *Global change biology*, 14 (3), pp.565–575. [Accessed 1 May 2025].

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.

Thaler, J. S., Owen, B. and Higgins, V. J. (2004). The role of the jasmonate response in plant susceptibility to diverse pathogens with a range of lifestyles. *Plant physiology*, 135 (1), pp.530–538. [Accessed 10 September 2025].

Thomas, A. (2020). *Improving Crop Yields in Sub-Saharan Africa: What Does the East African Data Say? WP No. 20/95*. [Online]. Available at: https://ssrn.com/abstract=3642661.

Thriveni, V. et al. (2024). Impact of polyploidy on crop improvement and plant breeding strategies: A review. *Journal of advances in biology & biotechnology*, 27 (11), pp.714–725. [Accessed 8 September 2025].

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.

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.

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.

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.

Todorova, D. et al. (2022). The physiological responses of wheat and maize seedlings grown under water deficit are modulated by pre-application of auxin-type plant growth regulators. *Plants*, 11 (23), p.3251. [Accessed 10 September 2025].

Tomlin, E. S. et al. (2000). Changes in volatile terpene and diterpene resin acid composition of resistant and susceptible white spruce leaders exposed to simulated white pine weevil damage. *Tree physiology*, 20 (16), pp.1087–1095. [Accessed 5 September 2024].

Tossi, V. E. et al. (2022). Impact of polyploidy on plant tolerance to abiotic and biotic stresses. *Frontiers in plant science*, 13, p.869423. [Accessed 8 September 2025].

Trapeznikov, V. K., Ivanov, I. I. and Kudoyarova, G. R. (2003). Effect of heterogeneous distribution of nutrients on root growth, ABA content and drought resistance of wheat plants. *Plant and soil*, 252 (2), pp.207–214. [Accessed 9 September 2025].

Tremblay, B. L. et al. (2019). Network analysis of the potential role of DNA methylation in the relationship between plasma carotenoids and lipid profile. *Nutrients*, 11 (6), p.1265. [Accessed 22 September 2025].

Tresas, T. et al. (2025). A brief overview of the epigenetic regulatory mechanisms in plants. *International journal of molecular sciences*, 26 (10), p.4700. [Accessed 10 September 2025].

Tripathy, B. C. and Oelmüller, R. (2012). Reactive oxygen species generation and signaling in plants. *Plant signaling & behavior*, 7 (12), pp.1621–1633. [Accessed 8 September 2025].

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.

Turnbull, C., Lillemo, M. and Hvoslef-Eide, T. A. K. (2021). Global regulation of genetically modified crops amid the gene edited crop boom - A review. *Frontiers in plant science*, 12, p.630396. [Accessed 16 September 2025].

Ulu, F., Unel, N. M. and Baloglu, M. C. (2025). Genome-wide analysis of miRNAs and their target genes in wheat cultivars with different ploidy levels under drought stress. *Planta*, 262 (2), p.38. [Accessed 22 September 2025].

UNESCO World Water Assessment Programme. (2018). *The United Nations World Water Development Report 2018: Nature-based Solutions for Water*. Paris.

Untergasser, A. et al. (2012). Primer3--new capabilities and interfaces. *Nucleic acids research*, 40 (15), p.e115. [Accessed 8 September 2025].

Uslu, V. V. et al. (2022). High-pressure sprayed siRNAs influence the efficiency but not the profile of transitive silencing. *The Plant journal: for cell and molecular biology*, 109 (5), pp.1199–1212.

Uslu, V. V. et al. (2025). Foliar mRNA spray induces protein synthesis in monocot crop and dicot model plant species. *bioRxiv*. [Online]. Available at: doi:10.1101/2025.08.07.668951.

Uyehara, A. N. et al. (2023). Cytokinin promotes jasmonic acid accumulation in the control of maize leaf growth. *Plants*, 12 (16), p.3014. [Accessed 10 September 2025].

Van de Peer, Y. et al. (2021). Polyploidy: an evolutionary and ecological force in stressful times. *The plant cell*, 33 (1), pp.11–26. [Accessed 7 May 2025].

Van Dooren, T. J. M. et al. (2020). Mild drought in the vegetative stage induces phenotypic, gene expression, and DNA methylation plasticity in Arabidopsis but no transgenerational effects. *Journal of experimental botany*, 71 (12), pp.3588–3602. [Accessed 14 September 2025].

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. [Accessed 12 May 2023].

Vanden Broeck, A. et al. (2018). Variability in DNA methylation and generational plasticity in the Lombardy poplar, a single genotype worldwide distributed since the eighteenth century. *Frontiers in plant science*, 9, p.1635. [Accessed 22 September 2025].

Varotto, S. et al. (2022). Exploitation of epigenetic variation of crop wild relatives for crop improvement and agrobiodiversity preservation. *Theoretical and applied genetics*, 135 (11), pp.3987–4003. [Accessed 16 September 2025].

Vatov, E., Zentgraf, U. and Ludewig, U. (2022). Moderate DNA methylation changes associated with nitrogen remobilization and leaf senescence in Arabidopsis. *Journal of experimental botany*, 73 (14), pp.4733–4752. [Accessed 17 June 2025].

Vaucheret, H. and Voinnet, O. (2024). The plant siRNA landscape. *The Plant Cell*, 36 (2), pp.246–275. [Accessed 28 January 2026].

Vaughn, M. W. et al. (2007). Epigenetic natural variation in Arabidopsis thaliana. *PLoS biology*, 5 (7), p.e174. [Accessed 22 November 2024].

Venetsky, A. et al. (2015). Structure and extent of DNA methylation-based epigenetic variation in wild emmer wheat (T. turgidum ssp. dicoccoides) populations. *BMC plant biology*, 15 (1), p.200.

Venske, E. et al. (2019). Bread wheat: a role model for plant domestication and breeding. *Hereditas*, 156 (1), p.16. [Accessed 15 April 2025].

Verbeke, S. et al. (2022). Osmotic adjustment in wheat (Triticum aestivum L.) during pre- and post-anthesis drought. *Frontiers in plant science*, 13, p.775652. [Accessed 28 February 2025].

Verhoeven, K. J. F. et al. (2010). Stress-induced DNA methylation changes and their heritability in asexual dandelions. *The New phytologist*, 185 (4). [Online]. Available at: doi:10.1111/j.1469-8137.2009.03121.x.

Verma, S. et al. (2022). Auxin response factors in plant adaptation to drought and salinity stress. *Physiologia plantarum*, 174 (3), p.e13714. [Accessed 10 September 2025].

Vialet-Chabrand, S. and Lawson, T. (2020). Thermography methods to assess stomatal behaviour in a dynamic environment. *Journal of experimental botany*, 71 (7), pp.2329–2338.

Vijayaraghavareddy, P. et al. (2022). Production and scavenging of reactive oxygen species confer to differential sensitivity of rice and wheat to drought stress. *Crop and Environment*, 1 (1), pp.15–23. [Accessed 5 March 2025].

Vining, K. J. et al. (2012). Dynamic DNA cytosine methylation in the Populus trichocarpa genome: tissue-level variation and relationship to gene expression. *BMC genomics*, 13 (1), p.27.

Voinnet, O. (2009). Origin, biogenesis, and activity of plant microRNAs. *Cell*, 136 (4), pp.669–687. [Accessed 11 September 2025].

Voss-Fels, K. P. et al. (2017). Linkage drag constrains the roots of modern wheat. *Plant, cell & environment*, 40 (5), pp.717–725. [Accessed 16 September 2025].

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.

Walkowiak, S. et al. (2020). Multiple wheat genomes reveal global variation in modern breeding. *Nature*, 588 (7837), pp.277–283.

Wall, S. et al. (2022). Stomata on the abaxial and adaxial leaf surfaces contribute differently to leaf gas exchange and photosynthesis in wheat. *The new phytologist*, 235 (5), pp.1743–1756. [Accessed 6 September 2025].

Wan, C. et al. (2022a). How Does the Environment Affect Wheat Yield and Protein Content Response to Drought? A Meta-Analysis. *Frontiers in plant science*, 13, p.896985.

Wan, H. et al. (2022b). Allopolyploidization increases genetic recombination in the ancestral diploid D genome during wheat evolution. *The crop journal*, 10 (3), pp.743–753. [Accessed 17 April 2025].

Wan, Q. et al. (2025). JA and ABA signaling pathways converge to protect plant regeneration in stress conditions. *Cell reports*, 44 (3), p.115423. [Accessed 10 September 2025].

Wang, B. et al. (2020a). Structural insights into target DNA recognition by R2R3-MYB transcription factors. *Nucleic acids research*, 48 (1), pp.460–471. [Accessed 23 July 2025].

Wang, F. et al. (2024). Plant responses to abiotic stress regulated by histone acetylation. *Frontiers in plant science*, 15, p.1404977. [Accessed 10 September 2025].

Wang, H. et al. (2021a). Response of wheat DREB transcription factor to osmotic stress based on DNA methylation. *International journal of molecular sciences*, 22 (14), p.7670. [Accessed 12 September 2025].

Wang, H. et al. (2023a). Advances in the research on plant WRKY transcription factors responsive to external stresses. *Current issues in molecular biology*, 45 (4), pp.2861–2880. [Accessed 11 March 2025].

Wang, J. et al. (2020b). Functions of jasmonic acid in plant regulation and response to abiotic stress. *International journal of molecular sciences*, 21 (4), p.1446. [Accessed 9 September 2025].

Wang, L. et al. (2022a). m6A RNA methylation impairs gene expression variability and reproductive thermotolerance in Arabidopsis. *Genome biology*, 23 (1), p.244. [Accessed 11 September 2025].

Wang, M.-Z. et al. (2020c). Correlations between genetic, epigenetic and phenotypic variation of an introduced clonal herb. *Heredity*, 124 (1), pp.146–155.

Wang, Q. et al. (2021b). Maize DNA methylation in response to drought stress is involved in target gene expression and alternative splicing. *International journal of molecular sciences*, 22 (15), p.8285. [Accessed 2 December 2024].

Wang, S. et al. (2023b). m6A mRNA modification promotes chilling tolerance and modulates gene translation efficiency in Arabidopsis. *Plant physiology*, 192 (2), pp.1466–1482. [Accessed 11 September 2025].

Wang, W. et al. (2016a). Genome-wide differences in DNA methylation changes in two contrasting rice genotypes in response to drought conditions. *Frontiers in plant science*, 7, p.1675. [Accessed 2 December 2024].

Wang, X. et al. (2016b). Heat Priming Induces Trans-generational Tolerance to High Temperature Stress in Wheat. *Frontiers in plant science*, 7. [Online]. Available at: doi:10.3389/fpls.2016.00501.

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.

Wang, X. and Yamaguchi, N. (2024). Cause or effect: Probing the roles of epigenetics in plant development and environmental responses. *Current opinion in plant biology*, 81 (102569), p.102569. [Accessed 16 July 2025].

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.

Wang, Y. et al. (2021d). Function and mechanism of jasmonic acid in plant responses to abiotic and biotic stresses. *International journal of molecular sciences*, 22 (16), p.8568. [Accessed 9 September 2025].

Wang, Y. et al. (2021e). Gene body methylation confers transcription robustness in mangroves during long-term stress adaptation. *Frontiers in plant science*, 12, p.733846. [Accessed 27 November 2024].

Wang, Z. et al. (2022b). Subgenome dominance and its evolutionary implications in crop domestication and breeding. *Horticulture research*, 9, p.uhac090. [Accessed 22 September 2025].

Wanke, D. and Üner Kolukisaoglu, H. (2010). An update on the ABCC transporter family in plants: many genes, many proteins, but how many functions? *Plant biology*, 12. [Online]. Available at: doi:10.1111/j.1438-8677.2010.00380.x.

Wasternack, C. (2014). Action of jasmonates in plant stress responses and development--applied aspects. *Biotechnology advances*, 32 (1), pp.31–39.

Weber, R. L. M. et al. (2014). Expression of an osmotin-like protein from Solanum nigrum confers drought tolerance in transgenic soybean. *BMC plant biology*, 14 (1), p.343. [Accessed 20 August 2025].

Wegren, S. K. (2011). Food security and Russia's 2010 drought. *Eurasian geography and economics*, 52 (1), pp.140–156.

Wei, H. et al. (2021). Phytohormones and their crosstalk in regulating stomatal development and patterning. *Journal of experimental botany*, 72 (7), pp.2356–2370. [Accessed 9 September 2025].

Wei, L. et al. (2014). Dicer-like 3 produces transposable element-associated 24-nt siRNAs that control agricultural traits in rice. *Proceedings of the National Academy of Sciences of the United States of America*, 111 (10), pp.3877–3882. [Accessed 5 August 2025].

Weng, X. et al. (2023). Transcriptome and DNA methylome divergence of inflorescence development between 2 ecotypes in Panicum hallii. *Plant physiology*, 192 (3), pp.2374–2393. [Accessed 31 July 2025].

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.

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.

Westman, S. M. et al. (2019). Defence priming in Arabidopsis – a Meta-Analysis. *Scientific reports*, 9 (1), pp.13309–13309.

Wibowo, A. et al. (2016). Hyperosmotic stress memory in Arabidopsis is mediated by distinct epigenetically labile sites in the genome and is restricted in the male germline by DNA glycosylase activity. *eLife*, 5. [Online]. Available at: doi:10.7554/eLife.13546 [Accessed 14 September 2025].

Wicker, T. et al. (2007). A unified classification system for eukaryotic transposable elements. *Nature reviews. Genetics*, 8 (12), pp.973–982. [Accessed 4 August 2025].

Wicker, T. et al. (2018). Impact of transposable elements on genome structure and evolution in bread wheat. *Genome biology*, 19 (1), p.103.

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer Science & Business Media.

Wik, M., Pingali, P. and Brocai, S. (2012). *Global agricultural performance: past trends and future prospects*. World Bank.

Wilkinson, S. W. et al. (2019). Surviving in a hostile world: Plant strategies to resist pests and diseases. *Annual review of phytopathology*, 57 (1), pp.505–529. [Accessed 4 August 2025].

Wilkinson, S. W. et al. (2023). Long-lasting memory of jasmonic acid-dependent immunity requires DNA demethylation and ARGONAUTE1. *Nature plants*, 9 (1), pp.81–95.

Wingen, L. U. et al. (2014). Establishing the A. E. Watkins landrace cultivar collection as a resource for systematic gene discovery in bread wheat. *Theoretical and applied genetics*, 127 (8), pp.1831–1842. [Accessed 16 September 2025].

WMO. (2021). *State of the global climate 2020*.

Wreczycka, K. et al. (2017). Strategies for analyzing bisulfite sequencing data. *Journal of biotechnology*, 261, pp.105–115. [Accessed 21 September 2025].

Wu, H. et al. (2015a). Detection of differentially methylated regions from whole-genome bisulfite sequencing data without replicates. *Nucleic acids research*, 43 (21), p.e141. [Accessed 5 December 2024].

Wu, J. et al. (2015b). Viral-inducible Argonaute18 confers broad-spectrum virus resistance in rice by sequestering a host microRNA. *eLife*, 4, p.e05733. [Accessed 28 January 2026].

Wu, J. et al. (2020). Roles of the Brassica napus DELLA protein BnaA6.RGA, in modulating drought tolerance by interacting with the ABA signaling component BnaA10.ABF2. *Frontiers in plant science*, 11, p.577. [Accessed 27 January 2025].

Wu, L. et al. (2025). Transcriptomic analysis of two Chinese wheat landraces with contrasting Fusarium head blight resistance reveals miRNA-mediated defense mechanisms. *Frontiers in plant science*, 16, p.1537605. [Accessed 22 September 2025].

Wu, T. et al. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation (Cambridge (Mass.))*, 2 (3), p.100141.

Wu, X. et al. (2022a). Evolution of osmosensing OSCA1 Ca2+ channel family coincident with plant transition from water to land. *The plant genome*, 15 (2), p.e20198. [Accessed 15 September 2025].

Wu, Y. et al. (2022b). ERF subfamily transcription factors and their function in plant responses to abiotic stresses. *Frontiers in plant science*, 13, p.1042084. [Accessed 23 July 2025].

Xia, Z. et al. (2013). The maize AAA-type protein SKD1 confers enhanced salt and drought stress tolerance in transgenic tobacco by interacting with Lyst-interacting protein 5. *PloS one*, 8 (7), p.e69787. [Accessed 27 January 2025].

Xiao, C. et al. (2021). Expression Pattern and Functional Analyses of Arabidopsis Guard Cell-Enriched GDSL Lipases. *Frontiers in plant science*, 12, p.748543.

Xie, B. and Dean, A. (2025). Chromatin-associated RNAs regulate gene expression and chromatin structure. *Non-coding RNA*, 11 (5), p.68. [Accessed 15 September 2025].

Xie, M. and Yu, B. (2015). SiRNA-directed DNA methylation in plants. *Current genomics*, 16 (1), pp.23–31. [Accessed 11 September 2025].

Xie, S. et al. (2019a). The TIFY Gene Family in Wheat and its Progenitors: Genome-wide Identification, Evolution and Expression Analysis. *Current genomics*, 20 (5), pp.371–388.

Xie, Z. et al. (2019b). The AP2/ERF transcription factor TINY modulates brassinosteroid-regulated plant growth and drought responses in Arabidopsis. *The plant cell*, 31 (8), pp.1788–1806.

Xiong, L. and Zhu, J.-K. (2003). Regulation of abscisic acid biosynthesis. *Plant physiology*, 133 (1), pp.29–36. [Accessed 9 September 2025].

Xiong, Z., Gaeta, R. T. and Pires, J. C. (2011). Homoeologous shuffling and chromosome compensation maintain genome balance in resynthesized allopolyploid Brassica napus. *Proceedings of the National Academy of Sciences of the United States of America*, 108 (19), pp.7908–7913. [Accessed 1 September 2025].

Xu, W., Thieme, M. and Roulin, A. C. (2024). Natural Diversity of Heat-Induced Transcription of Retrotransposons in Arabidopsis thaliana. *Genome biology and evolution*, 16 (11), p.evae242. [Accessed 25 July 2025].

Xu, Y.-H. et al. (2016). Jasmonic acid is a crucial signal transducer in heat shock induced sesquiterpene formation in Aquilaria sinensis. *Scientific reports*, 6 (1), p.21843. [Accessed 14 February 2025].

Xu, Z. et al. (2022). DNA methylation and RNA-sequencing analysis to identify genes related to spontaneous leaf spots in a wheat variety 'zhongkenuomai no.1'. *Agronomy (Basel, Switzerland)*, 12 (7), p.1519. [Accessed 2 December 2024].

Yabe, K. et al. (2024). H3K9 methylation regulates heterochromatin silencing through incoherent feedforward loops. *Science advances*, 10 (26), p.eadn4149. [Accessed 15 September 2025].

Yadav, S. B. K. and Bhatt, S. (2024). Heat stress and it's tolerance in wheat. *Cogent food & agriculture*, 10 (1). [Online]. Available at: doi:10.1080/23311932.2024.2413398 [Accessed 9 September 2025].

Yamaguchi, N. et al. (2021). H3K27me3 demethylases alter HSP22 and HSP17.6C expression in response to recurring heat in Arabidopsis. *Nature communications*, 12 (1), p.3480.

Yan, J. et al. (2023a). Ethylene regulates combined drought and low nitrogen tolerance in wheat: Proteomic analysis. *Agronomy (Basel, Switzerland)*, 13 (7), p.1950. [Accessed 10 September 2025].

Yan, J. et al. (2023b). Phylogeny of the plant receptor-like kinase (RLK) gene family and expression analysis of wheat RLK genes in response to biotic and abiotic stresses. *BMC genomics*, 24 (1), p.224. [Accessed 27 January 2025].

Yan, K. et al. (2015a). Contrasting photosynthesis and photoinhibition in tetraploid and its autodiploid honeysuckle (Lonicera japonica Thunb.) under salt stress. *Frontiers in plant science*, 6, p.227. [Accessed 13 August 2025].

Yan, K. et al. (2015b). Salt priming improved salt tolerance in sweet sorghum by enhancing osmotic resistance and reducing root Na+ uptake. *Acta physiologiae plantarum / Polish Academy of Sciences, Committee of Plant Physiology Genetics and Breeding*, 37 (10), p.203.

Yan, T. et al. (2025). Differentiation of genome-wide DNA methylation between japonica and indica rice. *The Plant journal: for cell and molecular biology*, 121 (2), p.e17218. [Accessed 11 September 2025].

Yang, C. et al. (2014). Evolution of physiological responses to salt stress in hexaploid wheat. *Proceedings of the National Academy of Sciences of the United States of America*, 111 (32), pp.11882–11887. [Accessed 7 May 2025].

Yang, J. et al. (2019). The crosstalks between jasmonic acid and other plant hormone signaling highlight the involvement of jasmonic acid as a core component in plant response to biotic and abiotic stresses. *Frontiers in plant science*, 10, p.1349. [Accessed 13 February 2025].

Yang, J. et al. (2023a). Impact of drought stress on spring wheat grain yield and quality. *Agrosystems, Geosciences & Environment*, 6 (1), p.e20351. [Accessed 6 September 2025].

Yang, J. and Zhang, J. (2006). Grain filling of cereals under soil drying: Tansley review. *The new phytologist*, 169 (2), pp.223–236. [Accessed 6 September 2025].

Yang, M. et al. (2023b). Drought priming mechanisms in wheat elucidated by in-situ determination of dynamic stomatal behavior. *Frontiers in plant science*, 14, p.1138494. [Accessed 3 September 2025].

Yang, Q. et al. (2024). Abscisic acid root-to-shoot translocation by transporter AtABCG25 mediates stomatal movements in Arabidopsis. *Plant physiology*, 195 (1), pp.671–684. [Accessed 9 September 2025].

Yang, T. et al. (2023c). Tomato CYP94C1 terminates jasmonate signaling during fruit ripening by inactivating bioactive jasmonoyl-L-isoleucine. *bioRxiv*, p.2023.05.17.541065. [Online]. Available at: doi:10.1101/2023.05.17.541065 [Accessed 9 September 2024].

Yang, X. et al. (2022a). Overexpression of an aquaporin gene EsPIP1;4 enhances abiotic stress tolerance and promotes flowering in Arabidopsis thaliana. *Plant physiology and biochemistry*, 193, pp.25–35. [Accessed 27 September 2025].

Yang, Y. et al. (2018). Dynamic transcriptomic m6A decoration: writers, erasers, readers and functions in RNA metabolism. *Cell research*, 28 (6), pp.616–624. [Accessed 11 September 2025].

Yang, Y. et al. (2021). Transcriptome profiling of developing leaf and shoot apices to reveal the molecular mechanism and co-expression genes responsible for the wheat heading date. *BMC genomics*, 22 (1), p.468. [Accessed 27 September 2025].

Yang, Y. et al. (2025). mRNA m6A regulates gene expression via H3K4me3 shift in 5' UTR. *Genome biology*, 26 (1), p.54. [Accessed 11 September 2025].

Yang, Z. et al. (2022b). DNA hypermethylation promotes the flowering of orchardgrass during vernalization. *Plant physiology*, 190 (2), pp.1490–1505. [Accessed 23 September 2025].

Yao, X. et al. (2022). Brassinosteroids enhance BES1-required thermomemory in Arabidopsis thaliana. *Plant, cell & environment*, 45 (12), pp.3492–3504. [Accessed 14 September 2025].

Ye, B.-B. et al. (2020). AP2/ERF transcription factors integrate age and wound signals for root regeneration. *The plant cell*, 32 (1), pp.226–241.

Ye, C. et al. (2021a). 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.

Ye, H. et al. (2021b). Genome-wide identification of wheat WRKY gene family reveals that TaWRKY75-A is referred to drought and salt resistances. *Frontiers in plant science*, 12. [Online]. Available at: doi:10.3389/fpls.2021.663118.

Yin, Y. et al. (2017). Impact of cytosine methylation on DNA binding specificities of human transcription factors. *Science (New York, N.Y.)*, 356 (6337). [Online]. Available at: doi:10.1126/science.aaj2239 [Accessed 17 June 2025].

Yong-Villalobos, L. et al. (2015). Methylome analysis reveals an important role for epigenetic changes in the regulation of the Arabidopsis response to phosphate starvation. *Proceedings of the National Academy of Sciences of the United States of America*, 112 (52), pp.E7293-302.

Yoo, C. Y. et al. (2010). The Arabidopsis GTL1 transcription factor regulates water use efficiency and drought tolerance by modulating stomatal density via transrepression of SDD1. *The plant cell*, 22 (12), pp.4128–4141. [Accessed 14 July 2025].

Yu, B. et al. (2020a). 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.

Yu, H. et al. (2024). PanDepth, an ultrafast and efficient genomic tool for coverage calculation. *Briefings in bioinformatics*, 25 (3), p.bbae197. [Accessed 3 December 2024].

Yu, S. et al. (2012). Identification of rice transcription factors associated with drought tolerance using the Ecotilling method. *PloS one*, 7 (2), p.e30765.

Yu, Y. et al. (2020b). Arabidopsis REM16 acts as a B3 domain transcription factor to promote flowering time via directly binding to the promoters of SOC1 and FT. *The Plant journal: for cell and molecular biology*, 103 (4), pp.1386–1398. [Accessed 22 September 2025].

Yu, Z. et al. (2019). The functional analysis of a wheat group 3 late embryogenesis abundant protein in Escherichia coli and Arabidopsis under abiotic stresses. *Plant signaling & behavior*, 14 (11), p.1667207. [Accessed 27 September 2025].

Yuan, F. et al. (2014). OSCA1 mediates osmotic-stress-evoked Ca2+ increases vital for osmosensing in Arabidopsis. *Nature*, 514 (7522), pp.367–371. [Accessed 15 September 2025].

Yuan, J. et al. (2020). Dynamic and reversible DNA methylation changes induced by genome separation and merger of polyploid wheat. *BMC biology*, 18 (1), p.171. [Accessed 23 May 2025].

Yung, W.-S. et al. (2022). Priming-induced alterations in histone modifications modulate transcriptional responses in soybean under salt stress. *The Plant journal: for cell and molecular biology*, 109 (6), pp.1575–1590.

Yung, W.-S. et al. (2024). DNA hypomethylation is one of the epigenetic mechanisms involved in salt-stress priming in soybean seedlings. *Plant, cell & environment*. [Online]. Available at: doi:10.1111/pce.15297.

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.

Zahid, H. J., Robinson, E. and Kelly, R. L. (2016). Agriculture, population growth, and statistical analysis of the radiocarbon record. *Proceedings of the National Academy of Sciences of the United States of America*, 113 (4), pp.931–935. [Accessed 25 April 2025].

Zahra, S. et al. (2021). EMS-based mutants are useful for enhancing drought tolerance in spring wheat. *Plant Biology*, bioRxiv. [Online]. Available at: https://www.biorxiv.org/content/10.1101/2021.01.05.425390v1.full.pdf.

Zaitchik, B. F. et al. (2023). Wetting and drying trends under climate change. *Nature Water*, 1 (6), pp.502–513. [Accessed 7 May 2025].

Zan, T. et al. (2020). Genome-wide identification and characterization of late embryogenesis abundant protein-encoding gene family in wheat: Evolution and expression profiles during development and stress. *Gene*, 736 (144422), p.144422. [Accessed 27 September 2025].

Zastąpiło, J. et al. (2024). Gene body methylation buffers noise in gene expression in plants. *Molecular Biology*, bioRxiv. [Online]. Available at: https://www.biorxiv.org/content/10.1101/2024.07.01.601483v1.full.pdf.

Zeibig, F., Kilian, B. and Frei, M. (2022). The grain quality of wheat wild relatives in the evolutionary context. *Theoretical and applied genetics*, 135 (11), pp.4029–4048. [Accessed 17 April 2025].

Zeng, G. et al. (2024). The ameliorative effects of exogenous methyl jasmonate on grapevines under drought stress: Reactive oxygen species, carbon and nitrogen metabolism. *Scientia horticulturae*, 335 (113354), p.113354. [Accessed 9 September 2025].

Zeven, A. C. (1998). Landraces: A review of definitions and classifications. *Euphytica/ Netherlands journal of plant breeding*, 104 (2), pp.127–139.

Zhang, B. et al. (2014). The mitochondrial outer membrane AAA ATPase AtOM66 affects cell death and pathogen resistance in Arabidopsis thaliana. *The Plant journal: for cell and molecular biology*, 80 (4), pp.709–727. [Accessed 27 January 2025].

Zhang, H. et al. (2018a). Molecular cloning and functional characterization of the dehydrin (IpDHN) gene from Ipomoea pes-caprae. *Frontiers in plant science*, 9, p.1454. [Accessed 19 September 2025].

Zhang, H. et al. (2021a). Genome-wide expression analysis of phospholipase A1 (PLA1) gene family suggests phospholipase A1-32 gene responding to abiotic stresses in cotton. *International journal of biological macromolecules*, 192, pp.1058–1074. [Accessed 10 September 2024].

Zhang, H., Lang, Z. and Zhu, J.-K. (2018). Dynamics and function of DNA methylation in plants. *Nature reviews. Molecular cell biology*, 19 (8), pp.489–506.

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.

Zhang, H.-R. et al. (2023a). Genome wide association analysis reveals multiple QTLs controlling root development in maize. *Journal of integrative agriculture*, 24 (5), pp.1656–1670. [Accessed 16 September 2025].

Zhang, H.-Y. et al. (2016). Global methylation patterns and their relationship with gene expression and small RNA in rice lines with different ploidy. *Frontiers in plant science*, 7, p.1002. [Accessed 22 September 2025].

Zhang, J. et al. (2018b). 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. (2024a). An unprecedented fall drought drives Dust Bowl-like losses associated with La Niña events in US wheat production. *Science advances*, 10 (31), p.eado6864. [Accessed 28 February 2025].

Zhang, L. et al. (2025a). Terpene synthase TgHPTPS2 from Torreya grandis modulates terpenoid profiles to balance ROS and confer drought tolerance in plants. *International journal of biological macromolecules*, 314 (144402), p.144402. [Accessed 10 July 2025].

Zhang, M. et al. (2022a). N6-methyladenosine RNA modification regulates photosynthesis during photodamage in plants. *Nature communications*, 13 (1), p.7441.

Zhang, Q. et al. (2025b). DNA methylation-activated LaCOMT1 expression promotes cluster root formation of white Lupin via a mechanism involving the melatonin synthesis. *Plant, Cell & Environment*, 48 (12), pp.8548–8560. [Accessed 4 February 2026].

Zhang, R. et al. (2024b). Genome-wide identification of hormone biosynthetic and metabolism genes in the 2OGD family of tobacco and JOX genes silencing enhances drought tolerance in plants. *International journal of biological macromolecules*, 280 (Pt 2), p.135731. [Accessed 13 February 2025].

Zhang, T. et al. (2022b). Climate change may outpace current wheat breeding yield improvements in North America. *Nature communications*, 13 (1), p.5591. [Accessed 28 February 2025].

Zhang, T. et al. (2025c). Regulation of stomatal responses to pathogen and drought stress by the F-box protein AtSKIP5. *Molecular plant pathology*, 26 (3), p.e70074. [Accessed 9 September 2025].

Zhang, T.-T. et al. (2024c). The AP2/ERF transcription factor MdDREB2A regulates nitrogen utilisation and sucrose transport under drought stress. *Plant, cell & environment*, 47 (5), pp.1668–1684.

Zhang, X. et al. (2021b). Structure-guided analysis of Arabidopsis JASMONATE-INDUCED OXYGENASE (JOX) 2 reveals key residues for recognition of jasmonic acid substrate by plant JOXs. *Molecular plant*, 14 (5), pp.820–828. [Accessed 13 February 2025].

Zhang, X. et al. (2025d). Stress priming mechanisms that enhance plant high temperature tolerance. *Environmental and experimental botany*, 237 (106208), p.106208. [Accessed 14 September 2025].

Zhang, Y. et al. (2020). Jasmonic acid promotes leaf senescence through MYC2-mediated repression of CATALASE2 expression in Arabidopsis. *Plant science: an international journal of experimental plant biology*, 299 (110604), p.110604. [Accessed 9 September 2025].

Zhang, Y. et al. (2023b). Plants' response to abiotic stress: Mechanisms and strategies. *International journal of molecular sciences*, 24 (13), p.10915. [Accessed 9 September 2025].

Zhang, Y. et al. (2023c). Wheat TaSnRK2.10 phosphorylates TaERD15 and TaENO1 and confers drought tolerance when overexpressed in rice. *Plant physiology*, 191 (2), pp.1344–1364. [Accessed 9 September 2025].

Zhang, Y. et al. (2025e). Crop root system architecture in drought response. *Yi chuan xue bao [Journal of genetics and genomics]*, 52 (1), pp.4–13. [Accessed 29 April 2025].

Zhang, Y.-Y. et al. (2013). Epigenetic variation creates potential for evolution of plant phenotypic plasticity. *The new phytologist*, 197 (1), pp.314–322. [Accessed 16 September 2025].

Zhao, J. et al. (2023). Centromere repositioning and shifts in wheat evolution. *Plant Communications*, 4 (4), p.100556.

Zhao, W. et al. (2020). Effects of water stress on photosynthesis, yield, and water use efficiency in winter wheat. *Water*, 12 (8), p.2127. [Accessed 5 September 2025].

Zhao, X. et al. (2024). Development and application of an RNA nanostructure to induce transient RNAi in difficult transgenic plants. *Biotechnology journal*, 19 (5), p.e2400024.

Zhao, Y. et al. (2016). ABA receptor PYL9 promotes drought resistance and leaf senescence. *Proceedings of the National Academy of Sciences of the United States of America*, 113 (7), pp.1949–1954. [Accessed 4 March 2025].

Zhao, Y. et al. (2021). The crosstalk between m6A RNA methylation and other epigenetic regulators: a novel perspective in epigenetic remodeling. *Theranostics*, 11 (9), pp.4549–4566. [Accessed 11 September 2025].

Zheng, X. et al. (2013). Transgenerational variations in DNA methylation induced by drought stress in two rice varieties with distinguished difference to drought resistance. *PloS one*, 8 (11), p.e80253. [Accessed 14 September 2025].

Zhong, X. et al. (2014). Molecular mechanism of action of plant DRM de novo DNA methyltransferases. *Cell*, 157 (5), pp.1050–1060. [Accessed 11 September 2025].

Zhou, H. et al. (2020). The function of histone lysine methylation related SET domain group proteins in plants. *Protein science: a publication of the Protein Society*, 29 (5), pp.1120–1137. [Accessed 15 September 2025].

Zhou, S. et al. (2012). Overexpression of the wheat aquaporin gene, TaAQP7, enhances drought tolerance in transgenic tobacco. *PloS one*, 7 (12), p.e52439. [Accessed 27 September 2025].

Zhu, C. et al. (2023). WGCNA based identification of hub genes associated with cold response and development in Apis mellifera metamorphic pupae. *Frontiers in physiology*, 14, p.1169301. [Accessed 20 June 2025].

Zhu, J.-K. (2016). Abiotic stress signaling and responses in plants. *Cell*, 167 (2), pp.313–324. [Accessed 15 September 2025].

Zhu, R., Xue, Y. and Qian, W. (2025). Molecular mechanisms and biological functions of active DNA demethylation in plants. *Epigenetics & chromatin*, 18 (1), p.41. [Accessed 11 September 2025].

Zhu, T. et al. (2021a). Optical maps refine the bread wheat Triticum aestivum cv. Chinese Spring genome assembly. *The Plant journal: for cell and molecular biology*, 107 (1), pp.303–314. [Accessed 2 December 2024].

Zhu, T. et al. (2021b). Warm temperature triggers JOX and ST2A-mediated jasmonate catabolism to promote plant growth. *Nature communications*, 12 (1), p.4804.

Zhu, X. et al. (2015). Jasmonic acid promotes degreening via MYC2/3/4- and ANAC019/055/072-mediated regulation of major chlorophyll catabolic genes. *The Plant journal: for cell and molecular biology*, 84 (3), pp.597–610. [Accessed 4 March 2025].

Zi, N. et al. (2024). DNA methylation participates in drought stress memory and response to drought in Medicago ruthenica. *Genes*, 15 (10), p.1286. [Accessed 4 September 2025].

Ziegler, D. J. et al. (2023). Genomic asymmetry of the Brassica napus seed: epigenetic contributions of DNA methylation and small RNAs to subgenome bias. *The Plant journal: for cell and molecular biology*, 115 (3), pp.690–708. [Accessed 22 September 2025].

Zilberman, D. et al. (2008). Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. *Nature*, 456 (7218), pp.125–129. [Accessed 21 September 2025].

Zulfiqar, B. et al. (2024). Abscisic acid improves drought resilience, growth, physio-biochemical and quality attributes in wheat (Triticum aestivum L.) at critical growth stages. *Scientific reports*, 14 (1), p.20411. [Accessed 9 September 2025].