Elucidating the function of the transcriptional co-repressor JAZ6 in plant immunity

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Abstract

The defence of plants against pathogen infection is a topical issue critical for maintaining food security in the face of climate change. Plants have evolved complex mechanisms to regulate gene expression, enabling transcriptional reprogramming in the face of threats like herbivores and infectious pathogens. Plant hormones are crucial signalling components which contribute to transcriptional reprogramming, and for stresses like herbivores and necrotrophic pathogens the hormone jasmonic acid plays a central role. Jasmonic acid perception occurs via the F-box protein CORONATINE INSENSITIVE 1 (COI1), which enables the targeted degradation of JASMONATE ZIM DOMAIN (JAZ) proteins, the focus of this work.

In Arabidopsis, JAZ6 was previously shown to affect disease susceptibility to *Botrytis cinerea*, and how that susceptibility oscillates over the day. This work demonstrates that JAZ6 specifically represses defences against the necrotrophic pathogen *B. cinerea* at dusk, including plant defensins which are critical elements of plant defences against pathogens. JAZ6 is also found to regulate defence against the necrotrophic pathogen *Sclerotinia sclerotiorum* and the biotrophic pathogen *Hylaoperonospora arabidopsidis*. An unexpected finding was that JAZ6 has a novel role in promoting flowering.

This work also characterises the protein-protein binding of JAZ6 and PHD FINGER DOMAIN CONTAINING PROTEIN (PFP), a regulator of flowering time. PFP is also found to regulate defence against *B. cinerea* and *H. arabidopsidis*. Transcriptomic analysis suggests that JAZ6 and PFP genetically interact additively, possibly acting independently of each other.

Finally, orthologues of the Arabidopsis *JAZ* genes were identified in the crop plant lettuce. The expression of both Arabidopsis and lettuce *JAZ* genes is regulated by biotic and abiotic stress, possibly due to similar cis-regulatory elements in their promoters. This is indicative of possible conserved functions of *JAZ* genes across species.

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COVID-19 Impact Statement

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Author's declaration

I declare that this thesis is a presentation of original work, and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

1: Introduction

1.1 Plant pathology

1.1.1 Defining plant pathology

Plant pathology is the scientific study of diseases which plants suffer from, and the attempts to improve plant survival despite disease. Plant diseases caused by microorganisms limit the growth and supply of food, by attacking plants both before and after harvest. While the academic goal of plant pathology is to understand plant diseases, its practical goal is to protect the global supply of food from insecurity (Agrios, 2005).

Plants constitute the majority of the Earth's biomass on land. Thus, land-based ecosystems depend on plants to convert sunlight into stored chemical energy. This stored energy makes plants the target of herbivory and disease. Although plant pathology is primarily concerned with the impacts on plant health by microbial pathogens, plant herbivores often serve as vectors of plant diseases (Wu *et al.*, 2017). Consequently, plant pathology has developed as an integrative science combining entomological approaches with microbiology and plant science, along with many other branches of science to account for the impact of environmental effects on the spread of plant disease, such as weather and climate which are crucial factors.

The control of plant diseases and other pests in the 20th century depended on the development and application of chemical treatments, generally known as pesticides. However these toxic chemicals are notorious for affecting non-target organisms (Kabasenche and Skinner, 2014; Carreck, 2017). The long-term detrimental effects of these pesticides on both human health and the environment are controversial. Unfortunately such concerns have led to political pressure limiting the use of pesticides (Kabasenche and Skinner, 2014; Carreck, 2017; Manghi *et al.*, 2021), while at the same time plant pathogens are rapidly evolving resistance to them (Rupp *et al.*, 2017). Therefore developing stable, environmentally friendly methods of controlling disease is a major goal for modern plant pathology (Mullins, 2015), despite political pressure continuing to limit the genetic engineering of disease resistant crops (Smyth *et al.*, 2021).

Plant disease can be defined as the disruption of plant physiological functions by external stress (Scheffer, 1997). Plants have evolved many different types of specialised cells for specific physiological functions, however external stress such as an infection can disrupt the activity of these cells. Initially, plant infection will not be visible as it will be restricted to a few microscopic cells. However, as the disease worsens more parts of the plant are affected and changes will become visible symptoms. Plant diseases are generally classified according to the effects they have on plant physiology and the severity of the disease. Where symptoms first develop is a guide to the infection site within the plant, and subsequently which physiological processes are disrupted. For example, root infections may cause root rot and limit the supply of water and nutrients to the plant.

Plant pathogens are the transmissible parasitic microorganisms causing plant disease. Once identified as the causative agent, they can be used more specifically to classify the plant disease than depending on the observed general symptoms. Linking plant disease symptoms to a specific pathogen is important for subsequent control measures, and in order to differentiate the causative agent from abiotic environmental stress, like heat or light, which can also cause disease-like symptoms (H. Zhang *et al.*, 2022).

Plant pathogens can be found from across the Tree of Life. Fungi, oomycetes, eubacteria, molliculites, viruses, nematodes, protozoa, green algae, and even other plants can cause plant disease (Holliday, 2001). It is difficult to definitely say which of these is most significant as disease surveillance is limited, and arguably only present on a world scale for fungal wheat rusts and oomycete potato blight (Ristaino *et al.*, 2021). Some important infectious agents are considered in greater detail in a later Section (1.2) but are referred to briefly here to emphasize the enormity of plant pathology.

These pathogens cause disease by taking advantage of stored chemical energy in plants to fuel their own growth in a parasitic manner. They achieve this by disrupting plant cell function through the production of virulence factors (Choquer *et al.*, 2007; Hann, Gimenez-Ibanez and Rathjen, 2010). The virulence factors vary between organisms and may be

inorganic chemicals, proteins, or genetic material (Choquer *et al.*, 2007; Hann, Gimenez-Ibanez and Rathjen, 2010).

However, not all microorganisms living inside and around plants are pathogens. Within the endophytic community of microorganisms there are many commensal or mutualistic partners of the plants they inhabit (Oulhen, Schulz and Carrier, 2016). Well-known examples are nitrogen-fixing rhizobia bacteria in legumes (peas and clover), and arbuscular mycorrhizal fungi which aid plants in acquiring nutrients (Antoine *et al.*, 2021). Some pathogens even have endophytic non-pathogenic life phases (Kan *et al.*, 2014; Oulhen, Schulz and Carrier, 2016), during which they do not parasitise the host plant. Therefore, plants have evolved complex systems to both differentiate between virulent and non-virulent microbes, and subsequently to defend themselves from infection.

Due to the diversity of organisms causing plant diseases, plants do not have a single solution to protect themselves against microbial infections. Nevertheless, understanding the mechanisms of microbial virulence and the counter-defences which plants have evolved will help design better controls for the future and help protect food security.

The molecular basis of susceptibility to plant pathogens is determined by genes present and active in the host plant and also the pathogen (Loegering and Ellingboe, 1987). Detecting the pathogen and initiating a defence response by activating defence gene expression is key to resisting pathogen attack (Jones and Dangl, 2006; Seyfferth and Tsuda, 2014a). Amongst the genes with established roles in plant defence are the *JASMONATE ZIM DOMAIN (JAZ)* genes (Thines *et al.*, 2007), which are transcriptional co-repressors acting in response to the plant hormone jasmonic acid (JA) to regulate defence gene expression.

This thesis focuses on the role of the gene *JASMONATE ZIM DOMAIN 6 (JAZ6)* in plant defence against diseases caused by the fungal and oomycete plant pathogens *Botrytis cinerea (B. cinerea)* and *Hylaoperonospora arabidopsidis (H. arabidopsidis),* respectively. The importance of these systems will be explained in the following sections.

1.1.2 The history of plant pathology

The development of agriculture and plant husbandry were critical to the development of human civilisation. Due to dependency on subsistence farming for much of the population, early agricultural practices could be devastated by plant disease causing severe famine (Carefoot and Sprott, 1967). Greek philosophers noted the use of chemical treatments to protect against plant diseases (Smith and Secoy, 1975), but these diseases were attributed to divine beings punishing the populace through weather manipulation (Theophrastus, 1916).

Concurrently with Louis Pasteur's experiments establishing the 'germ theory of disease' in the 1860s (Pasteur, 1881), Anton deBary conducted a simple experiment demonstrating potato blight was caused by a microorganism, the oomycete *Phytophthora infestans* (deBary, 1876). He planted two sets of potatoes, one of which was inoculated with potato blight spores from infected tissue. The inoculated tubers produced blighted, sickly plants while the non-inoculated tubers produced healthy plants. He also showed that this blight did not arise spontaneously but appeared in Spring from potatoes in storage which were partially infected (de Bary, 1876).

deBary's contributions to plant pathology continued with the characterisation of the fungal plant pathogens causing various plant diseases, including downy mildews, smut, and rust (Large, 1962). During his studies of wheat rust, he demonstrated that wheat stem rust requires two host plants to complete its lifecycle (Drews, 2013), wheat and barberry. He also worked on *Sclerotinia sclerotiorum* (Large, 1962), a very closely related fungus to *Botrytis cinerea* (Amselem *et al.*, 2011). He demonstrated that *S. sclerotiorum* attacks plants using substances we now know to be extra-cellular enzymes, which diffuse through plant tissue ahead of the pathogen. The 'juice' from such diseased tissue induced rotting in healthy tissue (Large, 1962), unless it was boiled to denature the enzymes. Subsequently multiple cytolytic and pectic enzymes were discovered as causative agents in plant fungal infection in the early 20th century, including those produced by *B. cinerea* (Brown and Harvey, 1927).

In 1942 Flor showed that in rust disease of flax, single genes determined the pathogenicity of the rust, and these corresponded to single genes for resistance in flax (Loegering and Ellingboe, 1987). This advanced the classic gene-for-gene concept of plant disease and demonstrated that disease outcomes were dependent on the genetic makeup of both the host and the pathogen. This concept was further expanded in the latter part of the century with the cloning of avirulence and resistance (R) genes from pathogens and plant hosts respectively (Keen, 2000).

The observation of growth defects in plants infected with various diseases led to investigations of hormone involvement in plant disease in the mid-20th century. In 1939 Gibberellin was identified as the growth promoting hormone produced during infection of rice with the fungus *Gibberella fujikuroi* and named after the fungus (Hedden and Sponsel, 2015). In subsequent decades multiple fungal and bacterial plant pathogens were shown to induce hormone production, including auxin and cytokinin (Costacurta and Vanderleyden, 1995). But perhaps the most influential of these discoveries for the development of plant science was the investigation of *Agrobacterium tumefaciens* induced crown gall disease (Van Larebeke *et al.*, 1974). It was demonstrated that the transformation-inducing (Ti) plasmid of the bacteria includes DNA which is integrated into the host plant genome by non-homologous end joining, and then expressed (Tinland, Hohn and Puchta, 1994; Nester, 2014). This DNA was termed the transforming DNA (T-DNA) and encodes for both auxin and cytokinin producing enzymes (Nester, 2014). The subsequent human manipulation of this 'infection' mechanism is a cornerstone of the development of molecular plant pathology for investigating gene function, including the experiments in this thesis which all involved T-DNA transformed plants in one form or another.

1.1.3 The development of molecular plant pathology

With the modern synthesis of genetics and heritability from the mid-20th century, there was increasing interest in the genetic basis for virulence of pathogens and their resistance in plant hosts. For example, T-DNA from *A. tumefaciens* was manipulated to show that genes other than auxin and cytokinin enzymes could be introduced into plants, from other plants, viruses, animals, or even synthetic artificial genes (Jaynes *et al.*, 1986; Tinland, Hohn and Puchta, 1994). This made it possible to use directed plant engineering to disrupt the genetic

makeup of their plant host and thereby alter their physiology and disease outcomes (Dong and Ronald, 2019). Several other methods were developed for introducing novel genetic material into plants, more than just infiltration with *A. tumefaciens* (termed agroinfiltration). These included viruses and bombardment with DNA adhered to gold particles in 'gene guns' (Mourgues, Brisset and Chevreau, 1998), as well as dipping Arabidopsis flowers into *A. tumefaciens* cultures (Clough and Bent, 1998).

'Model' organisms are a key part of biological research as results can be translated to other related organisms (Flavell, 2009). *Arabidopsis thaliana* (Arabidopsis) is not cultivated for agriculture or horticulture. However it was adopted as a model organism in the 1980's due to its small genome size; rising interest in flowering plants; key discussions at scientific conferences; and support from funding agencies (Koornneef and Meinke, 2010). Before this period Arabidopsis was unpopular as a model for plant-pathogen interactions and knowledge about plant disease was diffuse among a variety of crop plants and their diseases (Nishimura and Dangl, 2010). However, during the 1980's and early 1990's Arabidopsis was demonstrated to be a host for the pathogens cauliflower mosaic virus (CMV), *Xanthomonas campestris* pv. *campestris, Pseudomonas syringae*, and *H. arabidopsidis* (Nishimura and Dangl, 2010). The range of Arabidopsis homozygous genotypes collected from nature (termed ecotypes or accessions) allowed for genetic screening for resistance to infection, which led to the discovery of resistance (R) genes in Arabidopsis encoding for ribosomal protein S2 (*RPS2)* and a peripheral membrane protein (*RPM1)* following the earlier gene-for-gene model proposed by Flor (Nishimura and Dangl, 2010). RPS2 and RPM1 were defined as NB-LRR proteins due to the nucleotide binding properties and leucine rich repeats (Staskawicz *et al.*, 1995).

Nicotiana benthamiana (a close relative of tobacco) was used as a model organism for plant viral infections in the 1970's (Goodin *et al.*, 2008), especially for viruses which did not infect Arabidopsis. The wider use of *N. benthamiana* as a model coincided with the development of technology to express DNA from viral vectors; viral induced gene silencing or VIGS; and agroinfiltration (Goodin *et al.*, 2008). These techniques were powerful tools for functional characterisation of genes, and notably these techniques worked considerably better in *N.*

benthamiana than other plants like Arabidopsis (Goodin *et al.*, 2008). This prompted the use of *N. benthamiana* alongside the use of Arabidopsis in many studies, including this thesis. Notably for this work, *N. benthamiana* has become a platform for plant protein-protein interaction studies such as bimolecular fluorescence complementation or BiFC (Miller *et al.*, 2015), and *N. benthamiana* leaves yield large amounts of protein for further protein interaction studies (Goodin *et al.*, 2008).

Genome sequencing marked another milestone for plant pathology around the turn of the millennium. These huge datasets were initially available for the model plant *A. thaliana*, then extended to other plant hosts and their pathogens by many sequencing initiatives (Aragona *et al.*, 2021; Y. Sun *et al.*, 2022). The Arabidopsis genome has five chromosomes and a total size of approximately 135-megabases. Knowledge of the Arabidopsis genome was put to use almost immediately in plant pathology, as it allowed for the wider discovery of the gene family of the R genes *RPS2* and *RPM1*, the NB-LRRs (Meyers *et al.*, 2003). One approach possible with the genome of Arabidopsis was the development of a mutant library, where random mutagenesis via T-DNA insertion by *Agrobacterium* could be mapped to individual genes (O'Malley and Ecker, 2010). This has resulted in libraries of thousands of gene mutants for use in reverse genetics experiments (O'Malley, Barragan and Ecker, 2015). Subsequently, with multiple plant genomes available comparative genomics approaches became possible (Chaney *et al.*, 2016), further facilitating the translation of knowledge from the model plant Arabidopsis to a variety of important crop plants. Genetic data has also advanced the understanding of plant pathogens, refining the phylogenetic relationships of fungi and oomycetes so that they sit in distinct groups (Baldauf and Palmer, 1993; Wainright *et al.*, 1993).

In recent decades gene expression profiling has also become significantly easier and cheaper, with microarrays and improvements in sequencing technology (Zhu, 2003; Lister, Gregory and Ecker, 2009). This has enabled transcriptomic profiling of gene expression during infection and other environmental stresses (De Vos *et al.*, 2005; Windram *et al.*, 2012), identifying the dynamics of stress responses. These transcriptomic datasets allow for the construction of gene regulatory networks (Karlebach and Shamir, 2008; Penfold *et al.*,

2015), which identify possible causative regulatory relationships between genes. Gene expression profiling can also be applied to the comparison of different plant species by comparative genomics, in gene co-expression networks of similar genes in different species (Ruprecht *et al.*, 2017).

The recent development of the CRISPR toolset for genetic engineering has enabled the precise and specific modification of plant genomes (Gao *et al.*, 2016). CRISPR was discovered in DNA from *E. coli* bacteria in 1987 (Gostimskaya, 2022). However, it was a breakthrough by Francisco Mojica in recognising similar sequences in the archaea *Haloferax mediterranei* which advanced the understanding of the CRISPR loci as part of an immune system of bacteria and archaea (Gostimskaya, 2022). In recent years, the CRISPR mechanism has been adapted for genetic engineering in a wide range of organisms (Gostimskaya, 2022). In the CRISPR mechanism used in genetic engineering, RNA molecules termed guide RNA are detected by CRISPR-Cas9, which targets genomic DNA corresponding to the guide RNA. CRISPR also allows for 'tagging' proteins expressed in native contexts (Miki *et al.*, 2018), or single nucleotide polymorphisms. However, a disadvantage of CRISPR are off target effects, where non-target regions of the genome are modified (Q. Zhang *et al.*, 2018). Recent examples of CRISPR used in crop plants include the biochemical engineering of *Camelina sativa* to enhance production of omega-3 long-chain polyunsaturated fatty acids (Han, Haslam, *et al.*, 2022), and engineering resistance to potato virus Y and *Phytopthhora infestans* in potato (Tiwari *et al.*, 2022).

1.1.4 Impacts of plant disease

Plant diseases have a significant impact on worldwide food production as they are a severe threat to food security (Strange and Scott, 2005). Estimates on the precise losses vary significantly according to crop and disease (Oerke, 2006). Furthermore, the underlying data can be patchy and miss key segments of food production like smallholder farmers (Boa, 2016). Nevertheless, for the five major crops wheat, rice, maize, potato, and soybean losses due to pathogens and pests can be estimated at around 21.5, 30.0, 22.6, 17.2, and 21.4% respectively (Savary *et al.*, 2019). As an example of the significant financial impact of plant disease, in 2019 the U.S. state of Georgia experienced losses of 13.3% or $832 million to plant diseases (Little, 2021), including control costs. Losses in 2018 were similar at 13.5% or

$844 million (Little, 2020). Plant diseases can have significant social and political effects (Zadoks, 2017), and revolts have historically occurred in 1789 and 1846 following severe plant disease epidemics (Zadoks, 2017).

In the future climate change will exacerbate the effects of plant diseases as global warming will increase the spread of plant pathogens (Chaloner, Gurr and Bebber, 2021). For example, because increased temperatures may enable pathogens to expand to different geographic areas, as well as increasing their virulence and reproductive rates (Agrios, 2005; Son and Park, 2022). High CO_2 concentrations may increase resistance to pathogens by stimulating stomatal closure (Son and Park, 2022), however high temperatures may decrease resistance to pathogens by decreasing Effector Triggered Immunity (Desaint *et al.*, 2021), a mechanism of plant defence which will be further explained in Section 1.3. Due to these contrasting effects, there is a need for further research to clarify the precise effects of global warming on plant diseases.

Historically, the potato blight oomycete *Phytophthora infestans* has had a dramatic effect on food cultivation causing the Irish potato famine of the mid-19th century (deBary, 1876). The impacts of the famine were extensive, as people died or fled Ireland (Woodham-Smith, 1962). More recently, sudden oak death caused by a related oomycete pathogen *Phytophthora ramorum* has been a significant problem worldwide (Grünwald, LeBoldus and Hamelin, 2019). Named for the infection of oak trees in California (Rizzo, Garbelotto and Hansen, 2005), sudden oak death devastated this landscape with the resulting dead standing wood. In subsequent heatwaves, the interaction between sudden oak death and wildfires led to even greater ecological damage (Cobb, Meentemeyer and Rizzo, 2016). Such synergistic effects between the effects of plant pathogens and environmental stresses will grow more likely with further climate change (Cobb, 2022). Already, *P. ramorum* has spread to the UK and is now threatening larch tree populations (Grünwald, LeBoldus and Hamelin, 2019).

Olive oil production in Europe is currently under threat from the bacterium *Xylella fastidiosa*, which causes olive quick decline syndrome (Rapicavoli *et al.*, 2018). Previously it was identified as the causative agent of Pierce's disease of grapevine, thought to be a threat to wine production in California (Rapicavoli *et al.*, 2018). Recently it has appeared in Europe and has since swept through the olive tree population thanks to insect vectors (Strona, Carstens and Beck, 2017). Controversy over the destruction of infected plants and insecticide treatments has led to legal action against the scientists involved (Abbott, 2015), leading to months of inaction and significantly delaying control measures (Abbott, 2018).

Hylaoperonospora arabidopsidis (H. arabidopsidis) is an obligate biotroph oomycete pathogen which specifically infects the model plant host Arabidopsis (Coates and Beynon, 2010). It was one of the first 'wild' pathogens for Arabidopsis discovered and the development of the Arabidopsis-*H. arabidopsidis* pathosystem was significant in the development of Arabidopsis as a model for plant pathogen interactions (Nishimura and Dangl, 2010). Other oomycetes cause downy mildew in commercially important crops (Salcedo *et al.*, 2021), and severe downy mildew epidemics have historically caused great shifts in cultivation for plants like hops and maize (Edwardson, 1952; Frederiksen and Renfro, 1977). Therefore, laboratory studies using the Arabidopsis-*H. arabidopsidis* pathosystem can be applied to better understand the immune system of important crops such as soybean (Anderson *et al.*, 2012).

Fungal plant pathogens are serious threats to agriculture and horticulture worldwide (Scheffer, 1997). *Botrytis cinerea* (*B. cinerea*) is the second most important fungal plant pathogen (Dean *et al.*, 2012), second only to the rice blast disease fungus *Magnaporthe oryzae*. This is due to its large host range, resistance to fungicides, and the extent of post-harvest losses (Dean *et al.*, 2012). *B. cinerea* is a fungal pathogen found worldwide (Rosslenbroich and Stuebler, 2000), capable of infecting many widely cultivated fruit and vegetable species including grapes and strawberries (Williamson *et al.*, 2007). As a pathogen with a broad host range of over 500 plant species *B. cinerea* causes over $10 billion in crop losses per year (Williamson *et al.*, 2007; Hua *et al.*, 2018). Added to this is the cost of fungicides and other methods to control *B. cinerea* surpasses one billion euros per year

(Dean *et al.*, 2012). *B. cinerea* infestation can cause serious damage not just to growing plants pre-harvest, but to harvested fruit from seemingly unaffected plants (Hua *et al.*, 2018). It is notable as the most important post-harvest fungal pathogen (Droby and Lichter, 2007), causing fruit, vegetable, and ornamental losses after they have been harvested. *B. cinerea* is a costly growing threat to modern agriculture. But as its fungicide resistance continues to evolve and spread (Rupp *et al.*, 2017), fungicide use is increasingly restricted by law (Manghi *et al.*, 2021). Ironically however*, B. cinerea* is also useful as a beneficial part of wine production (Magyar, 2011). In regions of France and Hungary with a moist climate *B. cinerea* is traditionally cultivated on wine grapes as a desired fungal infection called noble rot (Magyar, 2011). These botrytized wines have a sweet taste, due to redox and hormonal changes in grapes, which are associated with the noble rot infection because it increases the sugar content of the grape (Pogány *et al.*, 2022).

Sclerotinia sclerotiorum (*S. sclerotiorum*) is a fungal pathogen with a broad host range of over 400 host species (Bolton, Thomma and Nelson, 2006), which is closely related to *B. cinerea* (Amselem *et al.*, 2011). *S. sclerotiorum* causes over $200 million of damage in the U.S. annually (Bolton, Thomma and Nelson, 2006). Among the diseases it causes is lettuce drop (Wu and Subbarao, 2006), which can result in the loss of entire lettuce crops. Yearly losses of soybean to sclerotinia stem rot caused by *S. sclerotiorum* in the U.S. regularly exceed 270 million kg (Peltier *et al.*, 2012), and in 2009 reached 1.61 billion kg which is equivalent to $560 million (Peltier *et al.*, 2012). In Argentina sclerotinia blight of peanut caused by *S. sclerotiorum* and similar Sclerotiniaceae species was found on up to 45% of plants and caused loses up to 870 kg/ha (Marinelli *et al.*, 1998).

1.1.5 Preventing and treating plant diseases

Plant diseases can be treated in several ways. For abiotic (non-infectious) plant diseases, nutrient supplements, water, shade, or light may help relieve disease symptoms. However, for plant pathogens the contagious nature of disease presents additional obstacles to treatment.

A simple approach to treating infected plants is to cull them, as well as nearby plants which may be cryptically infected and therefore not showing any symptoms (Ndeffo Mbah and

Gilligan, 2010). This approach has been used to combat citrus canker in Florida and sudden olive decline syndrome in Italy (Brown, 2001; Abbott, 2018). Unfortunately, in Italy negative public reactions limited the effectiveness of this approach and led to criminal proceedings against the scientists involved (Abbott, 2015). Subsequently disease monitoring was severely limited.

Historically, from Ancient Greece to the modern day, treatments of plant pathogens have involved the use of chemical applications (Smith and Secoy, 1975; Rosslenbroich and Stuebler, 2000). The use of chemicals as pesticides has resulted in the strong selection pressure for the evolution of pesticide resistance in pathogens like *B. cinerea*. This fungal pathogen has recently evolved resistance to many of the most commonly used fungicides in Europe (Rupp *et al.*, 2017).

However, in contrast to the above approach, chemicals may also be applied to plants to 'prime' them for infection and boost their defences against pathogens (Westman *et al.*, 2019). One example would be the application of β-aminobutyric acid on tomato to increase resistance to *B. cinerea* infection (Luna *et al.*, 2016), which induces long-term changes to the epigenome and stress-related gene expression (Catoni *et al.*, 2022). Another example is the synthetic compound 2,6 dichloro-isonicotinic acid (INA), which induces resistance to *P. syringae* in the common bean *Phaseolus vulgaris* (Martínez-Aguilar, Hernández-Chávez and Alvarez-Venegas, 2021). The stress-free offspring of the INA primed plants exhibited enrichment of epigenetic histone modifications H3K4me3 and H3K36me3, as well as low nucleosome occupancy at the disease resistance related *PvPR1* gene (Martínez-Aguilar, Hernández-Chávez and Alvarez-Venegas, 2021).

An alternative approach to chemical treatment is biological control. There are several approaches to biological control depending on the pathogen involved. For example pathogenic bacteria like *Xylella fastidiosa* can themselves be infected and killed with viral bacteriophage (Das *et al.*, 2015). Bacteria can be introduced to prevent critical numbers of fungal pathogens accumulating. This has been used as a viable treatment for both *B. cinerea* and *Sclerotinia sclerotiorum* infections (Essghaier *et al.*, 2009; Chen *et al.*, 2016).

Finally, plant breeding can produce plants resistant to infection. Single dominant resistance genes (R genes) have served as the basis for increased pathogen resistance in many crops, which are 'deployed' as they are grown in the field. These include *Bs2* transformed into tomato for resistance against bacterial spot caused by *Xanthomonas campestris* (Adhikari *et al.*, 2020). However, similar to fungicide treatment, the deployment of single R genes applies strong selection pressure and consequently pathogens can quickly overcome the resistance (Palloix, Ayme and Moury, 2009). One example is the evolution in three years of the fungal pathogen *Leptosphaeria maculans* to overcome R gene resistance in *Brassica napus* (Sprague *et al.*, 2006). To combat pathogen evolution numerous strategies are used, including the stacking of multiple R genes in a single deployment as used for resistance against *Phytopthora infestans* in potato by stacking *Rpi-sto1* and *Rpi-vnt1.1* (Jo *et al.*, 2014).

Disease resistant crops may be developed by various means. A notable example is resistance provided by multiple genes each with small individual effects. This is termed quantitative resistance (Corwin and Kliebenstein, 2017). Other approaches determine genes to target in quantitative resistance which involves genome wide association studies (GWAS) or associative transcriptomics quantitative trait loci (QTL) which identify genes responsible for specific phenotypic traits (Zhao *et al.*, 2011; Harper *et al.*, 2012). The loci found by these techniques typically include many genes of which a subset will affect the trait of interest, possibly only by acting in concert with other genes. Genes in crop plants linked to QTLs for pathogen resistance typically include orthologues of Arabidopsis genes known to be involved in defence, such as plant hormone signalling genes (Corwin and Kliebenstein, 2017), and can offer resistance to multiple diseases simultaneously (Wiesner-Hanks and Nelson, 2016). In lettuce 5 QTLs have been identified for resistance to either *B. cinerea* or *S. sclerotiorum* (Pink *et al.*, 2022), though no QTLs were found for resistance to both pathogens.

Reverse genetics approaches can also be used to find targets for quantitative resistance. Comparative genomics can identify homologous genes with conserved roles in defence, as demonstrated with elicitor peptide 1 (encoded by *PROPEP1)* which acts as an endogenous

signal to activate defence in both Arabidopsis and maize (Huffaker, Pearce and Ryan, 2006; Huffaker, Dafoe and Schmelz, 2011). Elicitors are molecules which can induce defence responses which may originate in plants or plant pathogens, and act by interacting with specific receptors to trigger defence signalling (Angelova, Georgiev and Roos, 2006). In this way, comparative genomics utilises our understanding of defence in model plants like Arabidopsis for an improved understanding of defence in crop plants. Together forward and reverse genetic approaches provide targets for traditional plant breeding as well as modern genetic engineering to improve disease resistance.

1.1.6 Plant pathology in lettuce

Lettuce is a commercial crop worth £200M per year in the UK (Department for Environment, Food & Rural Affairs, 2021). Lettuce has been cultivated for thousands of years, and was considered an aphrodisiac and soporific by the ancient Egyptians and Greeks (Harlan, 1986). The genome and transcriptome of lettuce were assembled recently (Reyes-Chin-Wo *et al.*, 2017). It has a diploid genome (2n = 2x = 18 chromosomes) and an estimated genome size of 2.5 Gb. This is considerably larger than that of Arabidopsis (135 Mb). Combining transcriptomics with comparative genomic approaches has led to the identification of orthologous and paralogous genes in Arabidopsis and lettuce (Reyes-Chin-Wo *et al.*, 2017), which can subsequently be used to investigate evidence of conserved function (Timms *et al.*, 2006). Examples of conserved function of orthologous genes in Arabidopsis and lettuce has been demonstrated for control of flowering time by the *FLOWERING LOCUS T (FT)* transcription factor (Chen *et al.*, 2018), and plant growth by *GROWTH-REGULATING FACTOR (GRF)* transcription factors (B. Zhang *et al.*, 2021).

Profitable cultivation of lettuce is threatened by losses due to the closely related necrotrophic pathogens *B. cinerea* and *S. sclerotiorum* (Young *et al.*, 2004; Amselem *et al.*, 2011). In particular, from lettuce drop caused by *S. sclerotiorum* (Wu and Subbarao, 2006). Unfortunately, as previously stated, both pathogens are rapidly evolving resistance to fungicides (Rupp *et al.*, 2017; Hou *et al.*, 2018). Biological control agents have shown some effectiveness against disease (Essghaier *et al.*, 2009; Chen *et al.*, 2016), nevertheless developing lettuce resistance to these pathogens is a subject of considerable commercial interest.

Given the importance for food security, achieving a greater understanding of plant defence systems is very important to reduce losses due to plant pathogens. An important defence system in plants is the JASMONATE ZIM DOMAIN (JAZ) proteins, and therefore these are the focus of this thesis.

1.2 Plant pathogens

1.2.1 Eukaryotic plant pathogens

Plants are infected by a range of microorganisms. Some of the most significant plant pathogens are the eukaryotic microbes fungi and oomycetes, which are the main focus of this thesis. Despite their morphological similarities, fungi and oomycetes are distinct, phylogenetically distant groups of Eukaryotes (Baldauf and Palmer, 1993; Wainright *et al.*, 1993). However it took the development of molecular phylogenetic analysis before they could be distinguished and classified correctly (Dick, 1997). While *B. cinerea* and *S. sclerotiorum* are fungi, *H. arabidopsidis* is an oomycete.

Both fungi and oomycetes contain necrotrophic and biotrophic plant pathogens. While necrotrophic pathogens kill host plant cells to obtain nutrients for growth, biotrophic pathogens parasitise living plant host cells (Glazebrook, 2005). Historically *B. cinerea* and *S. sclerotiorum* have been considered classic necrotrophs, while *H. arabidopsidis* is a biotroph. However, there is a continuum of pathogenicity between these extremes, as exemplified by the bacterial pathogen *Pseudomonas syringae* which is described as a hemibiotroph because it exhibits both necrotrophy and biotrophy (Pfeilmeier, Caly and Malone, 2016). Pathogens can also behave differently at different times, such as *B. cinerea* which can live endophytically without causing disease symptoms (Shaw *et al.*, 2016), as well as exhibiting a short biotrophic phase during infection (Veloso and van Kan, 2018).

1.2.2 Plant disease development

For a pathogen to infect a plant, they must first overcome the physical barriers which protect plant cells from external threats. Consequently, the first stage of plant disease development is the penetration of these physical defences. This is followed by the second stage where the pathogen must overcome the host's innate immunity and other defence

mechanisms. In order to achieve this, pathogens have evolved various mechanisms to overcome or bypass these barriers as will be outlined below.

Microbial entry into plants can be via insect hosts as seen for viruses vectored by aphids (Wang and Blanc, 2021), or direct invasion through stomata as seen for *P. syringae* (Melotto *et al.*, 2006). Fungi can also enter through the formation of appressoria infection structures, which can physically penetrate through plant cells or secrete enzymes to degrade the plant cell walls (Laluk and Mengiste, 2010). As broad range necrotrophs, *S. sclerotiorum* and *B. cinerea* use an arsenal of enzymes to degrade and weaken the plant cuticle and cell walls (Bellincampi, Cervone and Lionetti, 2014).

Once inside the plant, pathogens use virulence factors to disable the plant's defence systems. These virulence factors can take the form of chemicals, proteins, or genetic material. Work in the 1990's using molecular biology clarified pathogen mechanisms for suppressing resistance of plant hosts (Keen, 2000). These included type III secretion systems (T3SS) bacteria use to inject plant cells with virulence factors (Mudgett, 2005).

One bacterial virulence factor is the chemical coronatine, which targets JAZ proteins for degradation. It is produced by the pathogenic bacterium *P. syringae* (Geng *et al.*, 2014). Coronatine mimics jasmonic acid in plants, acting through CORONATINE INSENSITIVE 1 (COI1), the protein which binds to and ubiquitinates JAZ proteins via jasmonic acid or coronatine (Feys *et al.*, 1994; Yan *et al.*, 2009). This allows for activation of jasmonic acid type responses, which inhibit salicylic acid production and responses (Zheng *et al.*, 2012). Thus, *P. syringae* uses coronatine to take advantage of the antagonism between salicylic and jasmonic acid to control plant defence. As *P. syringae* is a hemibiotroph, the suppression of salicylic acid responses enables greater growth of the pathogen, while activation of jasmonic acid responses does not appear to affect it aversely.

The oomycete pathogen *H. arabidopsidis* is known to produce multiple effector proteins to control plant defence. The RxL21 effector, for example, binds to the transcriptional corepressor TOPLESS, interfering with its ability to repress gene transcription (Harvey *et al.*,

2020). This alters the expression of auxin-related genes, rendering plants susceptible by exploiting the growth-defence trade-off.

On the other hand, necrotrophic fungi such as *B. cinerea* and *S. sclerotiorum* are known to use intracellular effector proteins to target host cell walls. Several virulence factors appear to consist of extracellular enzymes which degrade plant cell walls (Laluk and Mengiste, 2010). This may reflect the broad host range of several hundred plant species for these necrotrophic pathogens (Bolton, Thomma and Nelson, 2006; Williamson *et al.*, 2007), as a broad host range could preclude targeting specific and variable host genes in defence. However, *B. cinerea* also secretes an exopolysaccharide (EPS) chemical virulence factor into plant cells (El Oirdi *et al.*, 2011), which increases salicylic acid levels to make tomato plants more susceptible to *B. cinerea* infection.

1.3 Plant defence against pathogens

1.3.1 Detecting plant pathogens

Aside from physical barriers against invasion, plants possess an array of inducible defences against pathogen attack. These are summarised in Figure 1.1. It was shown in the 1980's that components of oomycete pathogen cell walls were capable of eliciting plant defence responses (Keen, 2000). This observation developed into an understanding of innate immunity in plants, which is based on the identification of cell wall derivatives from bacteria, fungi, and oomycetes (Schwessinger and Ronald, 2012). When plants are attacked by pathogens they can sense the molecular derivatives of cell walls by pathogen associated molecular pattern triggered immunity (PTI) (Jones and Dangl, 2006). In PTI, fragments of plant and pathogen cell walls and proteins like bacterial flagellin are detected by plant cell surface receptors (Chinchilla *et al.*, 2007) known as transmembrane pattern recognition receptor proteins (PRRs). This triggers a MAPK kinase cascade, amplifying the signal, which is transduced into transcriptional reprogramming (Spoel and Dong, 2012).

Innate immunity can also be triggered by endogenous plant internal elicitors (Ryan, Huffaker and Yamaguchi, 2007). The expression of the genes encoding these internal elicitors is induced by cellular damage resulting from biotic stress. While systemin peptides act to activate defence against herbivore attack (Orozco-Cardenas and Ryan, 1999; Ryan and

Pearce, 2003), the elicitor peptide family of peptides activate pathogen defences by interacting with PEPR receptors (Huffaker, Pearce and Ryan, 2006; Huffaker, Dafoe and Schmelz, 2011). Pathogen virulence factors may disable PTI by using virulence factors which disrupt signal transduction resulting in effector triggered susceptibility. Therefore, plants have further defence mechanisms.

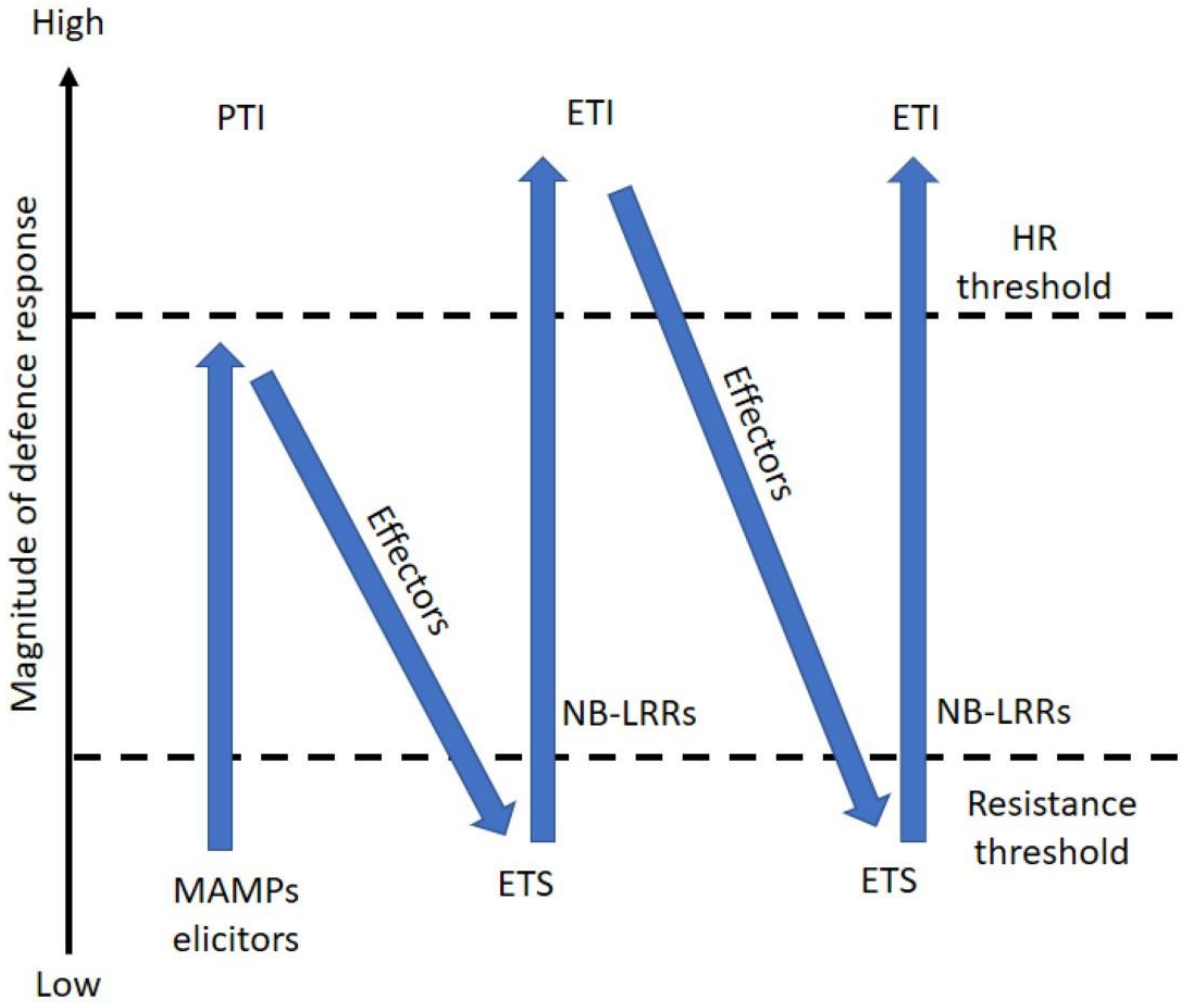


Figure 1.1: Zig-zag model of plant defence.

Plant defence consists of multiple layers of defence. Initially, detection of microbe associated molecular patters (MAMPs) or elicitors causes pathogen associated molecular pattern triggered immunity (PTI), as transcriptional reprogramming activates defences against pathogens. Effector protein virulence factors can disable PTI by binding to and disrupting key parts of the defence response, resulting in effector triggered susceptibility (ETS). However, R genes such as NB-LRRs in the plant can detect pathogen effectors and trigger a greater immune response, a hypersensitive response (HR), in what is called effector triggered immunity (ETI). Further effectors may target ETI, with further layers of R genes to detect them. Adapted from Jones and Dangl, 2006.

If the plant can find and recognise the pathogen effectors, it can activate effector triggered immunity (ETI) (Jones and Dangl, 2006). In ETI, pathogen effector proteins injected into plant cells as virulence factors may be detected by NB-LRR proteins resulting in a hypersensitive response (Khan, Subramaniam and Desveaux, 2016). PTI and ETI mechanisms act as a multi-layered zig-zag defence system (Jones and Dangl, 2006), counteracting pathogen virulence factors which attempt to disrupt pathogen detection. As with PTI, ETI leads to a MAPK kinase cascade of signal amplification, which in turn leads to transcriptional reprogramming and the activation of numerous defence genes (Spoel and Dong, 2012). Effectors can also target ETI defence mechanisms, and these effectors can be detected resulting in further zig-zag layers of plant immunity.

1.3.2 Hormones in plant defence

Plant hormones are essential for defence against pathogens. Jasmonic acid and salicylic acid are two key hormones of relevance for defence, as the antagonism between them underlies the antagonism between responses to necrotrophic and biotrophic pathogens (Takahashi *et al.*, 2004). In general, jasmonic acid responses are essential for resistance to necrotrophic pathogens, while salicylic acid is essential for resistance to biotrophic pathogens.

The plant hormone salicylic acid was identified as a causal agent in flowering (Cleland and Ajami, 1974; Khurana and Cleland, 1992). However, it is now recognised as a crucial component of host defence responses. Specifically it is necessary for systemic acquired resistance (SAR) which refers to induced defence against pathogens in distal parts of the plant to those inoculated, which can be induced by viruses, non-pathogenic rhizobacteria, and mycorrhizal fungi (Chen, Silva and Klessig, 1993a; Lawton *et al.*, 1995; van Loon, Bakker and Pieterse, 1998; Pieterse *et al.*, 2014). Salicylic acid and methyl salicylic acid are two of the signalling molecules which travel through the plant phloem in SAR signal generation and transmission (Vlot *et al.*, 2021).

Jasmonic acid was first identified as a volatile component behind the smell of jasmine (Demole, Lederer and Mercier, 1962), before it was identified as having physiological roles

in senescence and plant growth inhibition (Ueda and Kato, 1980; Dathe *et al.*, 1981). Jasmonic acid promotes defence against herbivores (Kang *et al.*, 2006), and acts as a systemic signal of wounding resulting from herbivore attack (Wasternack *et al.*, 2006). For plant defence, jasmonic acid promotes transcription of *PLANT DEFENSIN 1.2a (PDF1.2a)* which encodes for an antimicrobial protein (Penninckx *et al.*, 1996).

Ethylene is also an important hormone in plant defence against pathogens. It acts synergistically with jasmonic acid to activate plant defences like the plant defensins (Penninckx *et al.*, 1998). Abscisic acid acts antagonistically with ethylene, inducing resistance to herbivore attack at the expense of susceptibility to necrotrophic pathogens (Anderson *et al.*, 2004; Verhage *et al.*, 2011). The relative levels of ethylene and abscisic acid determine if plants are resistant to pathogens or herbivores.

The key plant hormone auxin has also been linked to defence, with the induction of auxin biosynthesis by pathogens aiming to infect plants like *A. tumefaciens* and *H. arabidopsidis* (Nester, 2014; Harvey *et al.*, 2020).

1.3.3 Salicylic acid in plant defence

Salicylic acid is the name given to 2-hydroxybenzoic acid, a phenolic compound containing a benzene ring, which is industrially acetylated to produce the well-known drug aspirin (Jack, 1997). There are two main synthesis pathways for salicylic acid, the isochorismate pathway and the phenylalanine ammonia-lyase pathway (Dempsey and Klessig, 2017). The isochorismate pathway is of key importance in regulating salicylic acid levels, and is mediated by *ISOCHORISMATE SYNTHASE 1 (ICS1)* (Wildermuth *et al.*, 2001), which is required for plant defence and induced by salicylic acid in a positive feedback loop. This has been of use in the construction in GUS reporter lines which utilise *ICS1* expression to indicate both the presence and synthesis of salicylic acid (Hunter *et al.*, 2013).

Salicylic acid has long been linked to pathogen defence in plants. The multiple roles of salicylic acid in virus resistance are well established (Singh *et al.*, 2004), with systemic acquired resistance to viruses induced by salicylic acid (White, 1979). Systemic acquired resistance is a type of resistance in which a localised infection (or artificial treatment)

induces resistance in other parts of the plant by systemic signalling (Uknes *et al.*, 1992). This is predicated upon salicylic acid, and is not specific to a single type of pathogen (Lawton *et al.*, 1995). While salicylic acid and salicylic acid synthesis genes such as *ICS1* are known to be essential for systemic acquired resistance they are not the translocating signal themselves (Dempsey and Klessig, 2012).

The perception of salicylic acid occurs through the NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1, 3, and 4 (NPR1, NPR3, and NPR4) which all bind to salicylic acid (Fu *et al.*, 2012; Manohar *et al.*, 2014), as summarised in figure 1.2. NPR1 is a positive regulator of the transcriptional response to salicylic acid, with NPR3 and NPR4 negative regulators (Dempsey and Klessig, 2017; Kazan, 2018). There are two proposed mechanisms for sensing salicylic acid with these three genes (Seyfferth and Tsuda, 2014b). In the first mechanism salicylic acid interacts with NPR3 at high concentrations and NPR4 at low concentrations (Fu *et al.*, 2012), forming a 26S proteasome to degrade NPR1 in both cases. Therefore, it is only at intermediate concentrations that NPR1 can induce defence genes. In the second mechanism NPR binds to salicylic acid through the cysteine residues Cys(521/529) via copper (Wu *et al.*, 2012), inducing conformational changes which relieve repression of the transcriptional activation domain by an auto inhibitory N-terminal domain.

Salicylic acid induces changes in the redox state of the cytosol, causing constitutive monomerization of the oligomer NPR1 is held in, permitting NPR1 monomers to translocate to the nucleus to promote the transcription of defence genes (Mou, Fan and Dong, 2003). NPR1 is able to induce transcription of the defence gene *PATHOGENESIS-RELATED 1 (PR1)* (Seyfferth and Tsuda, 2014b), and upregulates several defence-related WRKY transcription factors (Wang, Amornsiripanitch and Dong, 2006). Further transcriptional reprogramming in both biotic and abiotic defence responses are mediated by interplay with reactive oxygen species (ROS) (Chen, Silva and Klessig, 1993b; Herrera-Vásquez, Salinas and Holuigue, 2015).

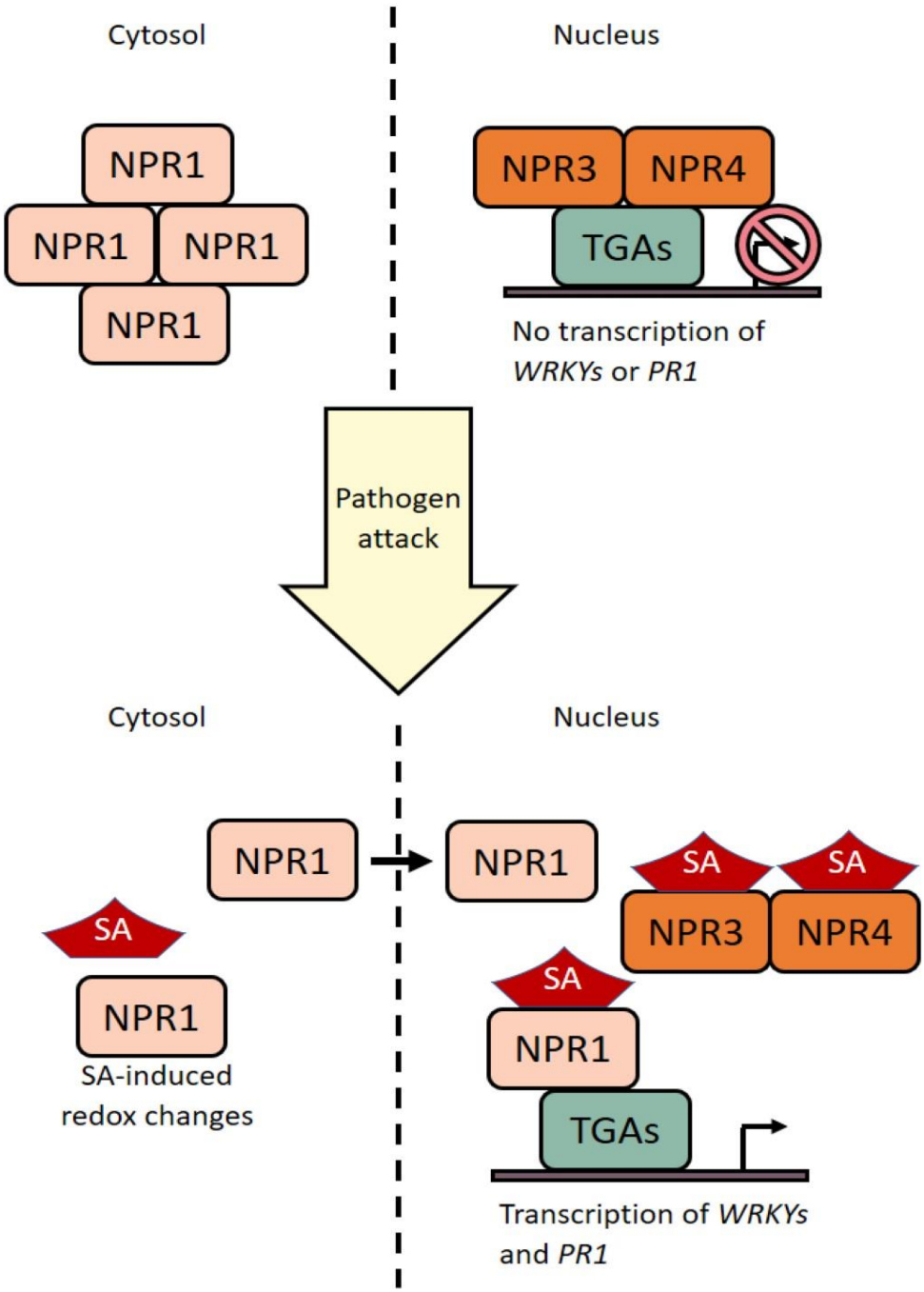


Figure 1.2: Salicylic acid perception.

Prior to infection, NPR1 is bound in tetramers in the cytosol, while NPR3 and NPR4 bind TGA transcription factors to prevent transcription of PR1 and WRKY transcription factors. During pathogen infection, salicylic acid (SA) levels increase. Firstly, this causes redox changes to NPR1 which enable it to break away from tetramers as a monomer and so move into the nucleus. Secondly, NPR3 and NPR4 are bound by SA and prevented from binding TGA transcription factors, while SA enables NPR1 to bind TGA transcription factors and so activate transcription of PR1 and WRKY transcription factors. Adapted from Kazan, 2018.

1.3.4 Jasmonic acid in plant defence

Jasmonic acid is a well-recognised plant hormone with established roles in plant defence against pathogens (Wasternack and Hause, 2013). Its production is stimulated by wounding and the detection of necrotrophic pathogens (Glazebrook, 2005; Wasternack *et al.*, 2006). It is synthesised from a trienoic fatty acid through octadecanoid pathways (Staswick and Tiryaki, 2004). Jasmonic acid is then conjugated with isoleucine to synthesise the bioactive form of jasmonic acid: (+)-7-iso-jasmonoyl-l-isoleucine (JA-Ile) by JAR1 (Suza and Staswick, 2008).

Bioactive jasmonic acid is then detected by a unique sensor system which is comprised of the F-box CORONATINE INSENSITIVE 1 (COI1), and JASMONATE ZIM DOMAIN (JAZ) proteins (Thines *et al.*, 2007), of which JAZ6 is the focus of this work. This is summarised in figure 1.3. JAZ proteins repress transcription factors such as MYC2 by binding to them to inhibit jasmonic acid responses (Zhang *et al.*, 2015). When jasmonic acid is present, due to wounding or pathogen attack, COI1 can bind JAZ proteins through the intermediary of jasmonic acid. COI1 then ubiquitinates JAZ proteins, targeting them for degradation (Thines *et al.*, 2007). The degradation of JAZ proteins frees transcription factors like MYC2 to bind to DNA and activate transcription of downstream jasmonic acid responses such as *VEGETATIVE STORAGE PROTEIN 2 (VSP2)* (Kazan and Manners, 2013), as MYC2 binds to the MEDIATOR 25 (MED25) subunit of the Mediator protein complex which recruits Polymerase II (Çevik *et al.*, 2012).

Jasmonic acid and ethylene synergistically activate the production of antimicrobial proteins called plant defensins (Penninckx *et al.*, 1998), most notably PLANT DEFENISIN 1.2a (PDF1.2a). These proteins are potent defences against microbial pathogens as they can fatally permeate through pathogen plasma membranes, causing cell death (Thomma, Cammue and Thevissen, 2002). This activation is through de-repression of EIN3 by JAZ proteins (Zhu *et al.*, 2011), which allows EIN3 to activate transcription of ERF transcription factors like ERF1 (Huang, Catinot and Zimmerli, 2016). ERF1 then activates transcription of PDF1.2a.

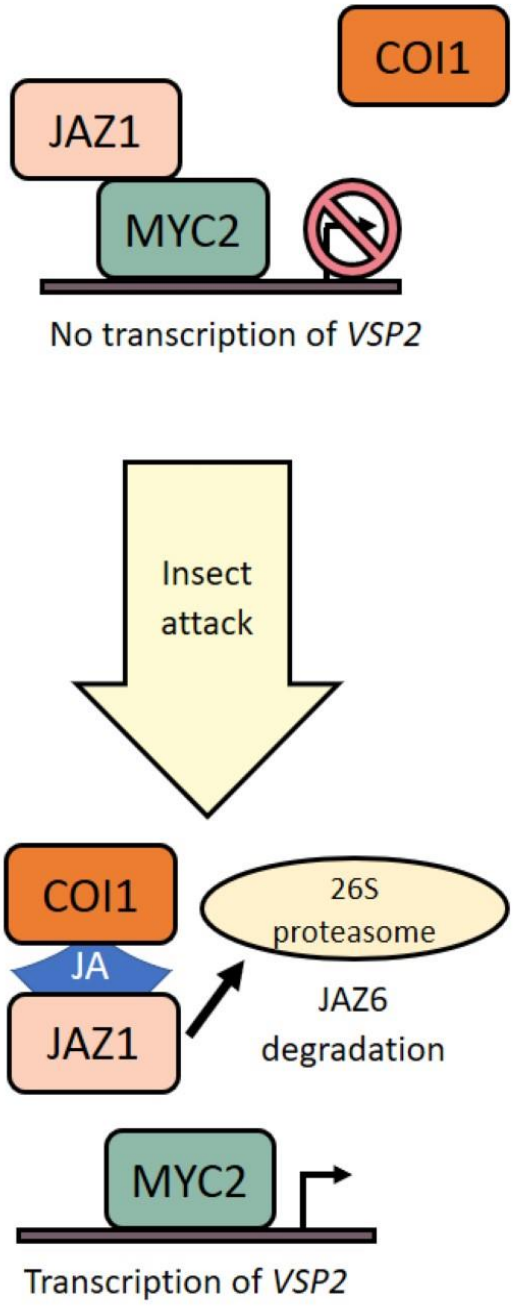


Figure 1.3: Jasmonic acid perception.

Prior to infection, JAZ proteins including JAZ1 bind transcription factors such as MYC2, preventing them from activating transcription of defence related genes such as *VSP2*, preventing the downstream activation of plant defensins. During pathogen infection, jasmonic acid (JA) levels increase. Firstly, this enables COI1 to bind to JAZ proteins, which causes the JAZ proteins to be ubiquitinated and so degraded by the 26S proteasome. This enables transcription factors such as MYC2 to activate transcription of defence related genes like *VSP2*, resulting in downstream activation of plant defensins. Adapted from Santner and Estelle, 2007.

1.3.5 Hormone crosstalk in necrotroph/herbivore defence

Within jasmonic acid responses, there is antagonistic crosstalk between abscisic acid and ethylene which underlies the responses to herbivorous insects and necrotrophic pathogens (Pieterse *et al.*, 2012), as detailed in figure 1.4.

Abscisic acid acts to promote defence against insect herbivores by promoting *MYC2* transcription (Abe *et al.*, 2003). Homologues of MYC2 in both Arabidopsis and tomato promotes the transcription of *VEGETATIVE STORAGE PROTEIN 2 (VSP2)* to increase plant resistance to herbivore attack (Boter *et al.*, 2004). MYC2 also promotes the transcription of *ANAC019* and *ANAC055* transcription factors (Zheng *et al.*, 2012), which similarly promote *VSP1* transcription (Bu *et al.*, 2008). ABA also enables the ABA receptor PYRABACTIN RESISTANCE 6 (PYL6) to bind to MYC2 (Aleman *et al.*, 2016). This has a complex effect on MYC2 activity, as it increases the binding affinity of MYC2 for some promoters such as *pJAZ8*, while decreasing the binding affinity of MYC2 for other promoters like *pJAZ6*. This complex interaction enables selective transcriptional activation of JAZ genes to tailor transcriptional responses to insect herbivores.

While both EIN3 and MYC2 are repressed by JAZ proteins, they also act antagonistically with each other (Song *et al.*, 2014). EIN3 and EIN3 LIKE 1 (EIL1) physically bind to the MYC2. While this inhibits transcriptional activity of EIN3 and EIL1, it also inhibits the transcriptional activity of MYC2. MYC2 promotes the transcription of *ANAC019* (Zheng *et al.*, 2012), which repress transcription of plant defensin PDF1.2a (Bu *et al.*, 2008).

Other than MYC2, ABA-INDUCIBLE BHLH-TYPE TRANSCRIPTION FACTOR/JA-ASSOCIATED MYC2-LIKE (JAM) transcription factors bind to JAZ proteins and act to promote abscisic acid responses and repress jasmonic acid signalling (Sasaki-Sekimoto *et al.*, 2013, 2014). They act similarly but independently of MYC2 as abscisic acid induced transcription factors, reducing responsiveness to jasmonic acid and regulating defence against herbivorous insects.

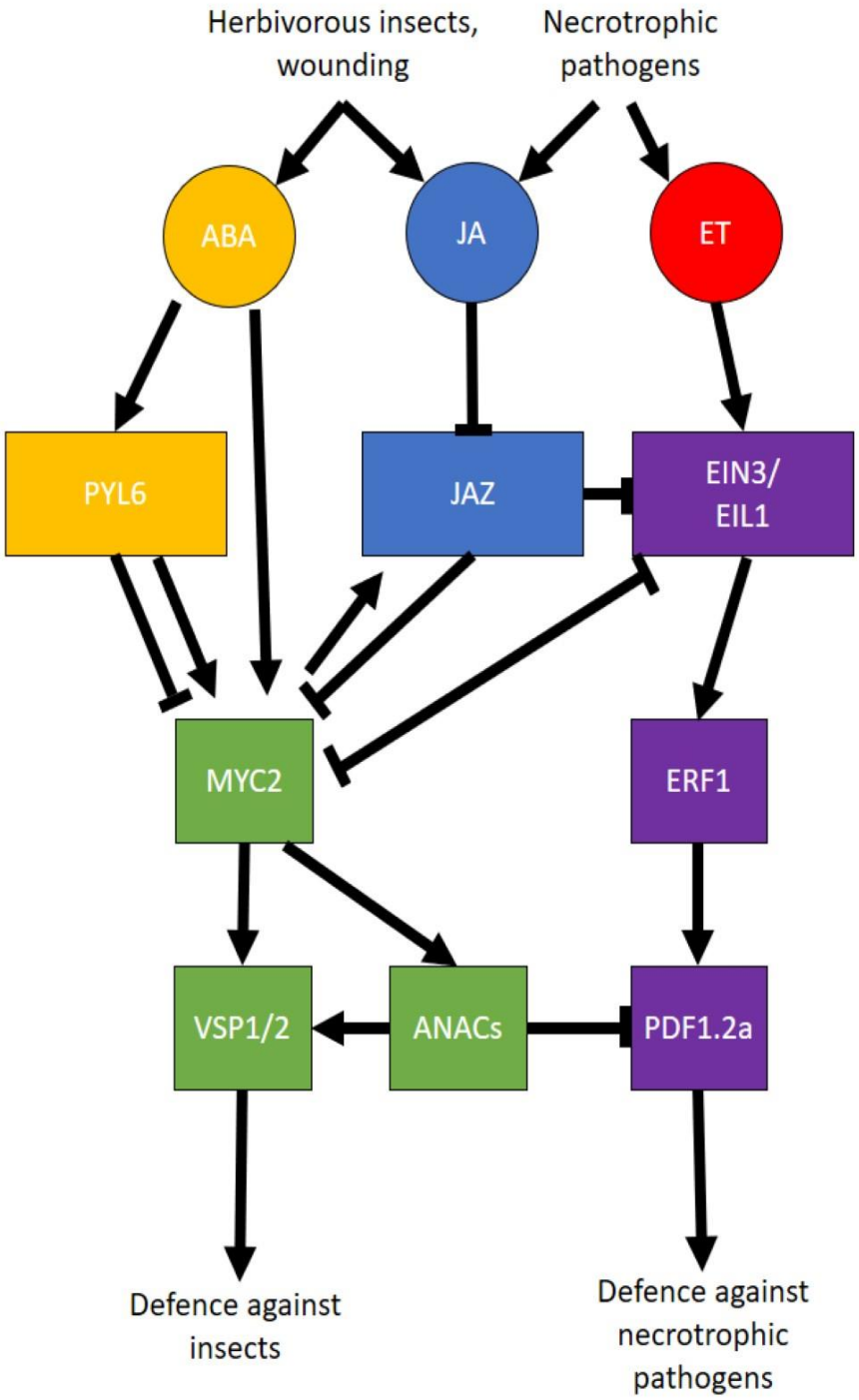


Figure 1.4: Abscisic acid and ethylene crosstalk in defence against herbivores and necrotrophic pathogens.

Herbivorous insect attack activates abscisic acid (ABA) and jasmonic acid (JA) responses, while necrotrophic pathogen infection activates ethylene (ET) and JA responses.

Adapted from Pieterse et al., 2012.

1.3.6 Hormone crosstalk in necrotroph/biotroph defence

Jasmonic acid and salicylic acid are commonly thought of as antagonistic (Pieterse *et al.*, 2009). Jasmonic acid promotes defence against necrotrophic pathogens and salicylic acid promotes defence against biotrophic pathogens, as detailed further in figure 1.5.

Experiments on the wound response of plants highlighted the antagonistic relationship between salicylic acid and jasmonic acid (Doherty, Selvendran and Bowles, 1988; Wasternack *et al.*, 2006), which has since been further elaborated. NPR1 acts as a transcriptional co-repressor of MYC2 when salicylic acid is present (Nomoto *et al.*, 2021), preventing activation of necrotrophic pathogen defences. EIN3 targets ORA59 for destruction in the presence of salicylic acid and jasmonic acid (He *et al.*, 2017), resulting in less activation of plant defensins than if only jasmonic acid was present. However, EIN3 also represses biotrophic pathogen defences by repressing *ICS1* which synthesises salicylic acid (Chen *et al.*, 2009). MYC2 promotes the transcription of *ANAC019, ANAC055,* and *ANAC072* transcription factors, which similarly repress salicylic acid synthesis by repressing *ICS1* (Zheng *et al.*, 2012).

However, the interaction between salicylic acid and jasmonic acid is not entirely antagonistic. Synergistic effects have been observed for combinatorial treatments of salicylic acid and jasmonic acid (Mur *et al.*, 2006). Specifically, in Arabidopsis combinatorial treatment resulted in greater expression of defence related genes *PR1* and *PDF1.2a*, compared with single hormone treatments. While in tobacco, combinatorial treatment led to greater amounts of programmed cell death. More recently, it has been shown that the salicylic acid receptors NPR3 and NPR4 degrade JAZ proteins when salicylic acid is present (L. Liu *et al.*, 2016), allowing for activation of both salicylic acid and jasmonic acid defences. Curiously, JAZ6 may be unique among JAZ proteins in that it does not require salicylic acid to bind NPR3, which may reflect a unique function in hormone crosstalk.

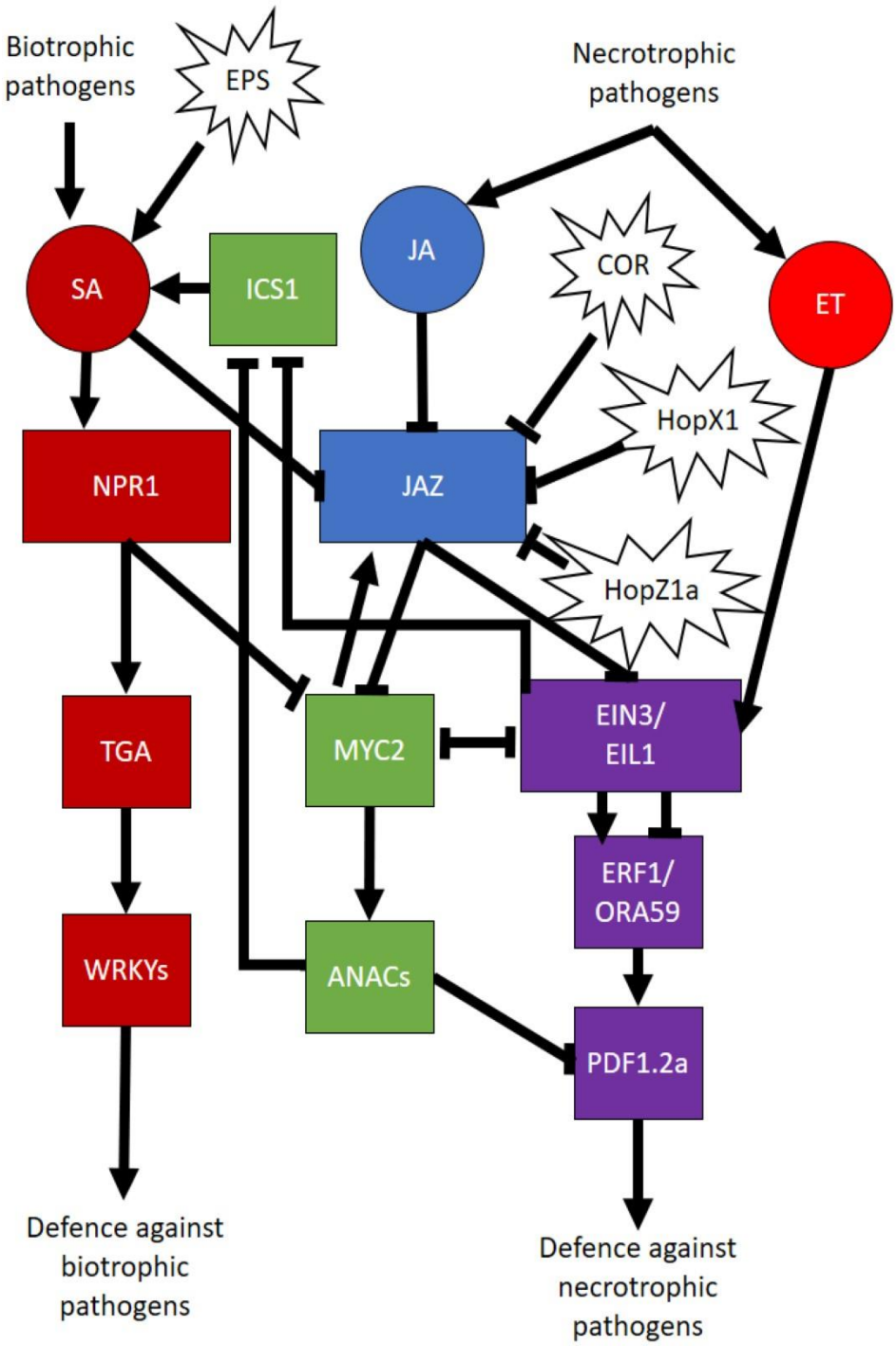


Figure 1.5: SA and JA crosstalk in defence against biotrophic and necrotrophic pathogens, and pathogen virulence factors.

Biotrophic pathogen infection activates salicylic acid (SA) responses, while necrotrophic pathogen infection activates jasmonic acid (JA) and ethylene (ET) responses.

Adapted from Tanaka et al., 2015.

Pathogen virulence factors take advantage of the antagonistic crosstalk between salicylic acid and jasmonic acid to inhibit plant defence against infection (Tanaka, Han and Kahmann, 2015), as also seen in figure 1.5. The phytotoxin coronatine produced by *P. syringae* is a mimic of jasmonic acid with more potent effects (Koda *et al.*, 1996), using the antagonism of jasmonic acid and salicylic acid to repress biotrophic pathogen defences by degrading JAZ proteins. In a similar manner HopX1 and HopZ effector proteins also from *P. syringae* target JAZ proteins for degradation in order to repress biotrophic pathogen defences (Jiang *et al.*, 2013; Gimenez-Ibanez *et al.*, 2014). In contrast, *B. cinerea* uses a chemical virulence factor exopolysaccharide to increase salicylic acid levels within tomato plants which indirectly represses necrotrophic pathogen defences (El Oirdi *et al.*, 2011).

1.3.7 Hormone crosstalk between stress responses and development

Jasmonic acid and JAZ proteins also influence the balance of plant development and stress responses though abscisic acid and gibberellic acid (Liu and Timko, 2021), as detailed in figure 1.6.

In abscisic acid signalling, the PYRABACTIN RESISTANCE/REGULATORY COMPONENT OF ABSCISIC ACID RECEPTOR (PYL/RCAR) receptors of ABA repress type 2C protein phosphatases (PP2Cs), which relieves repression of SNF1-related kinases 2 (SnRK2s) by PP2C (Ng *et al.*, 2014). This enables SnRK2 to activate the transcription factor ABSCISIC ACID-INSENSITIVE 5 (ABI5), which inhibits seed germination and post-germination growth in stress conditions (Collin, Daszkowska-Golec and Szarejko, 2021).

JAZ proteins are able to bind to ABI5 to repress it (Ju *et al.*, 2019). The RING E3 ligase KEEP ON GOING (KEG) ubiquitinates ABI5 to target it for degradation. However, KEG is autoubiquitinated in the presence of ABA. KEG is also able to bind to a single JAZ protein: JAZ12 (Pauwels *et al.*, 2015). However, instead of ubiquitinating JAZ12 KEG protects JAZ12 from degradation by COI1, stabilising JAZ12. This mechanism enables abscisic acid and jasmonic acid crosstalk to specify stress responses.

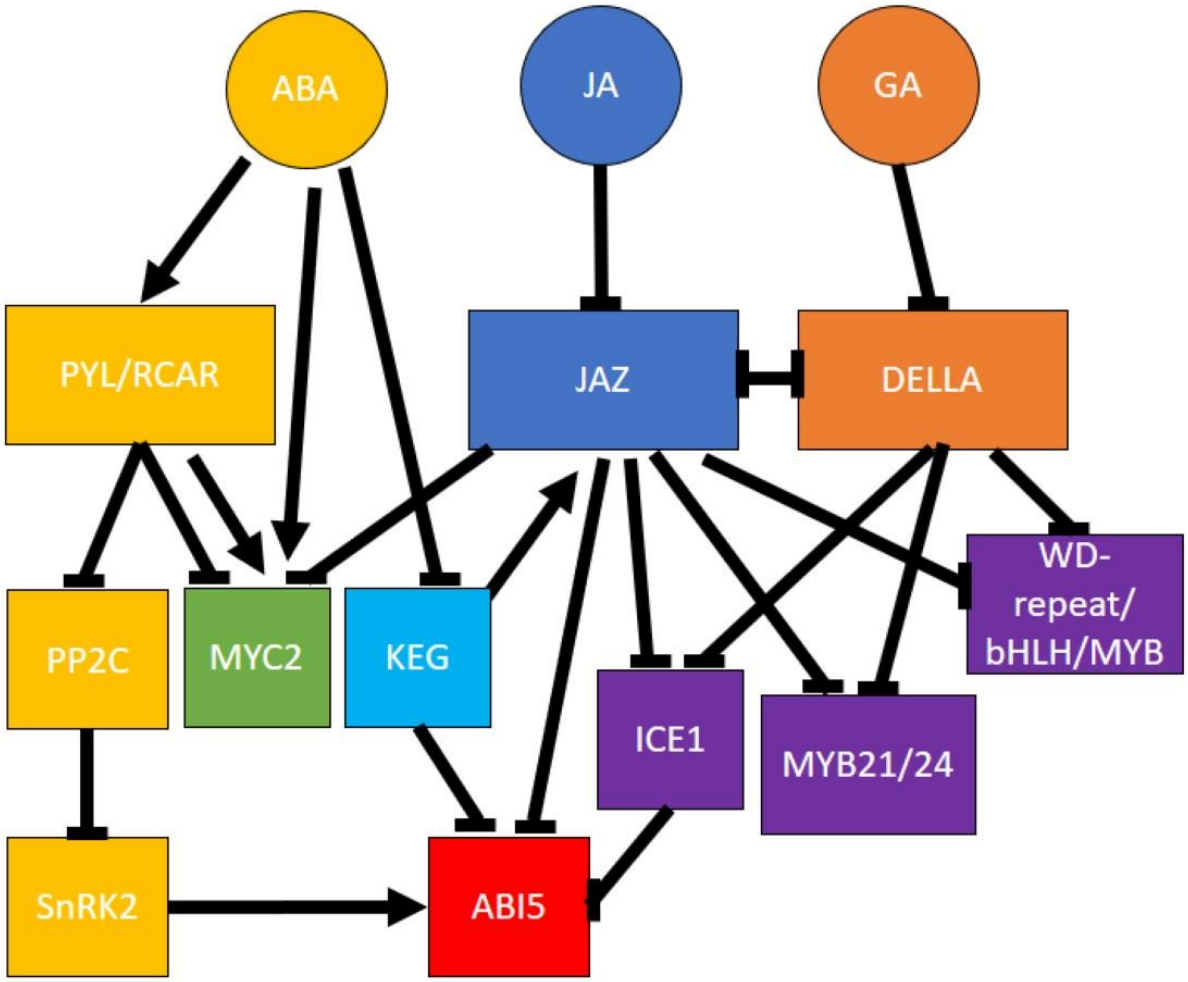


Figure 1.6: Simplified schematic of JA, GA, and ABA crosstalk involving JAZ proteins. JAZ and DELLA proteins compete to bind to their targets ICE1, MYB21/24 and the WD-repeat/bHLH/MYB proteins. ABA promotes MYC and indirectly changes its DNA binding affinity with PYL6. PYLs indirectly promote ABI5, which is repressed by KEG, ICE1, and JAZ proteins. Adapted from Liu and Timko, 2021.

Gibberellic acid also interacts with jasmonic acid signalling. Of the DELLA receptors of gibberellic acid, RGA interacts with JAZ1 (Hou *et al.*, 2010). In a comparable manner to MYC2/EIN3 antagonism, the binding of JAZ1 and RGA inhibits both of them from functioning. Curiously, both DELLAs and JAZ proteins inhibit common targets. The WD-repeat/bHLH/MYB transcription factors TT8/GL3/EGL3/MYB75/GL1 are some of these targets (Qi *et al.*, 2011; Tian *et al.*, 2016), controlling anthocyanin production and trichome development. MYB21/24 are other targets of both DELLAs and JAZ proteins which act to promote stamen development (Song *et al.*, 2011; Huang *et al.*, 2020). Finally, ICE1 is a target of both DELLAs and JAZ proteins which acts in the response to cold (Hu *et al.*, 2013, 2019). ICE1 represses ABI5, which as mentioned earlier is regulated by abscisic acid. This places ABI5 as the centre of a complex system of crosstalk to control growth in the face of environmental stress.

The classical growth hormone auxin antagonises jasmonic acid responses in leaf senescence and defence. Auxin represses the action of Aux/IAA proteins, which relieves repression of the WRKY transcription factor WRKY57 by IAA29 (Jiang *et al.*, 2014). IAA29 and JAZ4/8 competitively interact with WRKY57, while it is degraded by jasmonic acid. This complex series of interactions enables WRKY57 to repress jasmonic acid induced senescence by acting in crosstalk between jasmonic acid and salicylic acid. WRKY57 represses defence against *B. cinerea*, by directly inducing the expression of JAZ1 and JAZ5 (Jiang and Yu, 2016). WRKY57 also promotes drought tolerance by promoting abscisic acid biosynthesis by activating transcription of NCED3 (Jiang, Liang and Yu, 2012).

1.3.8 Links between defence and flowering

Flowering is the defining physiological process for Angiosperm plant reproduction, and Angiosperms are the most numerous and diverse monophyletic group of plant species consisting of over 95% of plant species alive today (Simpson, 2010). The genetic control of the transition to flowering is controlled by environmental signals including pathogens (Cho, Yoon and An, 2017). Notably, the key defence hormone salicylic acid was found to induce flowering in the 1970's (Cleland and Ajami, 1974), and so presents a clear link between the regulation of defence against infection and flowering.

The most comprehensive analysis of the effect of salicylic acid on flowering showed clear hastening of flowering time, as measured by leaf number, by both direct salicylic acid application and salicylic acid-inductive UV treatment (Martínez *et al.*, 2004). Using NahG-expressing plants as negative controls, salicylic acid can induce flowering irrespective of whether plants were grown in long or short-day growth conditions. These NahG-expressing plants constitutively express a bacterial salicylate hydroxylase enzyme which significantly lowers both endogenous and induced levels of salicylic acid (Friedrich *et al.*, 1995).

Further studies using molecular biology to investigate how salicylic acid induces flowering, concluded that the salicylic acid could affect the circadian clock (covered in Section 1.4). Mutagenesis screening identified *PATHOGEN AND CIRCADIAN CONTROLLED 1 (PCC1),* which responded to pathogen infection of Arabidopsis as well as UV exposure (Segarra *et al.*, 2010). From the physiology of mutant *pcc1* plants it was evidently a regulator of flowering time, though the regulatory mechanism is unclear. While its circadian regulation would suggest PCC1 is a member of the photoperiod dependent pathway of flowering induction, it does not directly interact with any obvious member of this pathway (Mir and León, 2014).

FLOWERING LOCUS D (FLD) from the autonomous pathway is a flowering gene which has been related to defence (Banday and Nandi, 2015). *FLD* was found to have a key role in systemic acquired resistance, with *fld* mutants unable to respond to the translocating signal (Singh *et al.*, 2013). FLD is also known to bind to HISTONE DEACETLYASE 6 (HDA6) to repress the expression of FLC through histone deacetylation (Yu *et al.*, 2011), and HDA6 is known to be recruited by JAZ proteins to repress *EIN3* transcription (Zhu *et al.*, 2011). The SUMO E3 ligase SIZ1 SUMOylates FLD to influence *FLOWERING LOCUS C (FLC)* chromatin structure (Jin *et al.*, 2008), and *siz1* mutants are early flowering with increased levels of salicylic acid.

A comprehensive analysis of the interaction of defence and flowering investigated Arabidopsis infection by the fungal pathogen *Fusarium oxysporum* (Lyons *et al.*, 2015). Mild infection accelerated the onset of flowering, and the susceptibility of a range of ecotypes negatively correlated with flowering time. Flowering mutants for a single ecotype exhibited

a stronger negative correlation. This was evident for several regulators of *FLC* in the autonomous pathway, particularly *FVE* and *FPA*. Given the involvement of FPA in regulating flagellin perception, this is perhaps not surprising (Lyons *et al.*, 2013). Genes which were responsive to infection by *F. oxysporum* include the circadian clock genes *EARLY FLOWERING 4 (ELF4)* and *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1),* as well as *FLC* and *FT.*

The SWR1 chromatin remodelling complex consists of PHOTOPERIOD INSENSITIVE EARLY FLOWERING 1 (PIE1), EARLY IN SHORT DAYS 1 (ESD1), and SWC6 (Noh and Amasino, 2003; Martin-Trillo *et al.*, 2006; Lázaro *et al.*, 2008). This complex acts to epigenetically control both flowering and defence against pathogens, with slightly different roles for each of the members (March-Díaz *et al.*, 2008; Berriri, Gangappa and Kumar, 2016). One of the ways in which it functions is the incorporation of the H2A.Z histone variant to replace standard H2A around transcription start sites. This is known to be a significant factor in flowering control, the presence of H2A.Z promoting *FLC* expression (Deal *et al.*, 2007).

PLANT U-BOX PROTEIN 13 (PUB13) post-translationally controls protein activity by ubiquitination (Li *et al.*, 2012). As part of the ARM U3 ubiquitin ligase complex with *PLANT U-BOX PROTEIN 12 (PUB12)* , it targets specific flowering related genes for degradation. However, they also have a very clear influence on plant defence, repressing salicylic acid biosynthesis and action. This is perhaps complemented by their role in the attenuation of flagellin sensing in PTI (Lu *et al.*, 2011). When the bacterial MAMP flagellin is sensed by the FLS2 receptor complex, it induces recruitment of PUB12 and PUB13 which then polyubiquitinate FLS2 for degradation. *PUB13* exhibits functional conservation with its rice orthologue which also controls defence and flowering, *SPOTTED LEAF 11*, which provided the first evidence that ubiquitination controls resistance and programmed cell death in plants (Zeng *et al.*, 2004).

HOPW1-1-INTERACTING3 (*WIN3*), a member of the firefly luciferase family, promotes defence against both *P. syringae* and *B. cinerea* (Lee *et al.*, 2007; G.-F. Wang *et al.*, 2011). It contributes to defence against *P. syringae* as a key element of RPS2 driven defence in ETI.

However, it also represses flowering synergistically with NPR1 by promoting *FLC* and repressing *FT* expression.

Among jasmonic acid responsive genes, the MYC transcription factors and ERF1 have established roles in regulating flowering. MYC2, 3, and 4 act cooperatively to induce flowering (H. Wang *et al.*, 2017). The induction of flowering by MYC transcription factors may be related to the promotion of flowering by its protein-protein binding partner MED25 (Iñigo *et al.*, 2012).

ERF1 can directly bind to the *FT* promoter and repress *FT* transcription to delay flowering (Chen *et al.*, 2021). It is as yet unclear if indirect repression of *ERF1* through inhibition of EIN3 is the mechanism by which MYC transcription factors induce flowering.

JAZ4 and JAZ8 proteins were found to bind TARGET OF EAT1 and TARGET OF EAT2 (TOE1 and TOE2) to repress flowering (Zhai *et al.*, 2015a). Additionally, they were also found to bind FAR1 RELATED SEQUENCE 7 (FRS7) and FRS12 to control flowering, growth, and glucosinolate biosynthesis (Ritter *et al.*, 2017). JAZ3, 4, 6, 8, 9, and 10 are able to bind to *LIKE HETEROCHROMATIN PROTEIN 1 (LHP1)* (Li *et al.*, 2021), an epigenetic regulator of the key flowering repressor *FLC* (Mylne *et al.*, 2006).

FERONIA is a master regulator of plant disease resistance, acting to influence both pathogen perception signalling and jasmonate responses (H. Guo *et al.*, 2018; Duan *et al.*, 2022). It was also recently discovered that *FERONIA* also has a role in promoting flowering time through *FLC*, using an alternative splicing mechanism (Wang *et al.*, 2020).

1.4 The circadian clock

1.4.1 The circadian clock regulates gene expression

The behaviour of almost all organisms oscillates diurnally to account for environmental changes (Bell-Pedersen *et al.*, 2005), notably temperature and light. Diurnal changes in plant physiology were first noted several thousand years ago, when the Greek philosopher Androsthenes described the diurnal movement of tamarind tree leaves (McClung, 2006). However, these observations did not distinguish between external light-dependent

mechanisms and internal endogenous mechanisms. In the 18th century, investigations showed that the control of leaf motion is circadian as the behaviour persisted in the absence of external signals (McClung, 2006). It was in the following century that the precise period length of these leaf movements was measured, and shown to be approximately (but significantly different to) 24 hours (McClung, 2006). This was another piece of evidence for an endogenous timekeeping system, as the period length matched no geophysical signal. Hence, the mechanism was named 'circadian', which is derived from Latin words for 'about' and 'day' to reflect the approximately 24-hour period for diurnal behaviour (McClung, 2006). Circadian clocks are of benefit to the plant, allowing it to coordinate physiological and biochemical changes with external conditions to optimise fitness (Dodd *et al.*, 2005). The clock also allows a plant to prepare for more long-term seasonal changes, particularly useful for limiting flowering to Spring to coordinate with pollinator activity (Song *et al.*, 2015).

The mechanism behind the circadian clock is a system of several transcription factors which inhibit each other in negative feedback loops so as to create oscillations in gene expression (Harmer, 2009a). These transcription factors respond to external signals of light and temperature to allow plants to anticipate regular environmental changes (Hsu and Harmer, 2014). The central mechanism of the clock consists of an oscillator with a few groups of genes co-expressed at different times of day (Hsu and Harmer, 2014). The central loop consists of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which repress expression of the pseudo-response regulator *TIMING OF CAB EXPRESSION 1 (TOC1)* (Harmer, 2009a). TOC1 indirectly promotes the expression of *CCA1* and *LHY,* causing daily oscillations in the expression (and therefore protein production) for all three genes.

The central clock genes have been extensively studied, which enables computer models to be constructed using ordinary differential equations (Locke, Millar and Turner, 2005; Pitchford and Avello, 2022). Other genes involved in the central circadian clock include the pseudo-response regulator paralogues of *TOC1*: *PSEUDO-RESPONSE REGULATOR 5, 7, 9 (PRR5, 7, 9)* (Eriksson *et al.*, 2003; Farré *et al.*, 2005), and the evening complex which

consists of *EARLY FLOWERING 3, 4 (ELF3, ELF4),* and *LUX ARRYTHMO (LUX)* (Doyle *et al.*, 2002; Yoshida *et al.*, 2009; Helfer *et al.*, 2011). These genes have been incorporated into more complex models of the circadian clock (De Caluwé *et al.*, 2016), as detailed in figure 1.7.

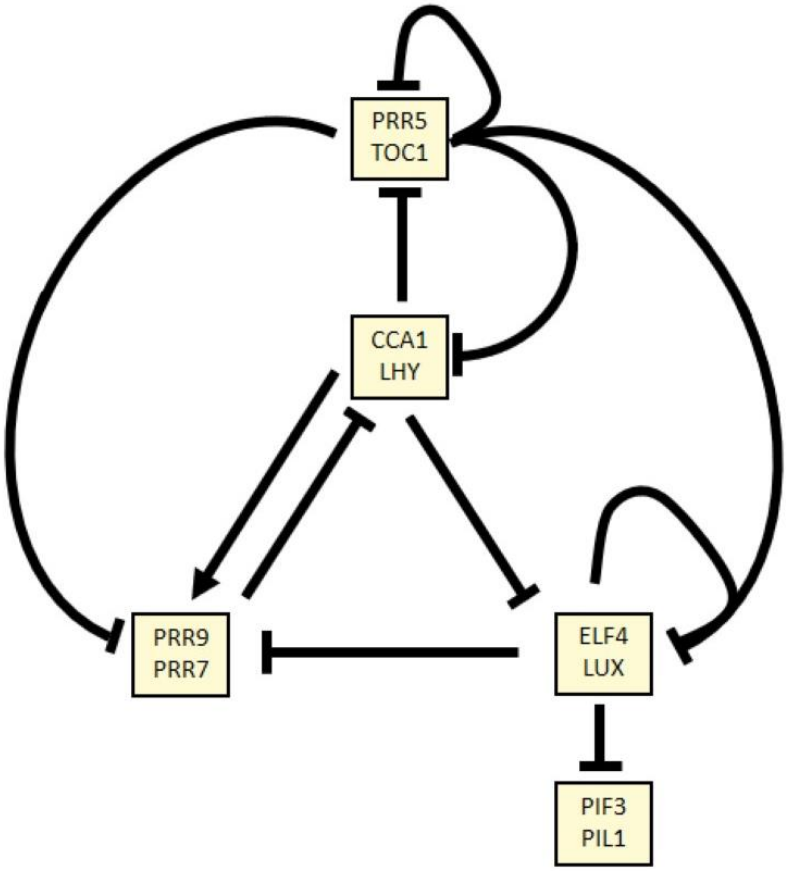


Figure 1.7: Schematic diagram illustrating the main features of the circadian clock model (De Caluwé et al., 2016).

The pseudo response regulators PRR5, 7, and 9 as well as TOC1 are consolidated into two distinct modules of transcriptional repression. CCA1 and LHY form a dawn-phased module. The evening complex is represented by a module consisting of ELF4 and LUX. PIF3 and PIL1 are included as transcriptional outputs of the circadian clock to influence growth via hypocotyl elongation.

Inputs to the circadian clock entrain the mechanism, ensuring it matches the external conditions outside. Light entrainment is managed by photoreceptors, proteins which interact with light of different wavelengths to change their conformational properties and behaviour (Casal and Smith, 1989). These include phytochromes and cryptochromes which interact with red or blue light respectively (Casal and Smith, 1989; Ahmad, 2016).

Temperature is another major input, which has recently been shown to be integrated with light by phytochromes (Jung *et al.*, 2016). Signal integration into the clock occurs via FHY3 and PHYTOCHROME INTERACTING FACTORS (PIFs) (Allen *et al.*, 2006; Pedmale *et al.*, 2016), though PIFs are also regulated by the circadian clock themselves. The circadian clock F-box protein ZEITLUPE (ZTL) is also a blue light photoreceptor responding to blue light (Kim *et al.*, 2007), which directly integrates light signals by post-translationally regulating TOC1 protein levels with another circadian clock protein GIGANTEA (GI) (Kim *et al.*, 2007). Circadian clock models have been developed to include entrainment by environmental factors (Avello *et al.*, 2019; Avello, Davis and Pitchford, 2021), and work continues in pursuit of more accurate and informative reflections of real biological systems.

It has been estimated that around 1/3 of Arabidopsis genes are under the transcriptional control of the circadian clock (Michael and McClung, 2003). This reflects the importance of the circadian clock in regulating a broad range of physiological properties of Arabidopsis, including photosynthesis, stomatal opening, and starch consumption. The transition to flowering and defence are processes under the control of the clock which are particularly relevant to this work, and so are expanded upon below.

1.4.2 The circadian clock in flowering

The key flowering regulator *FLOWERING LOCUS T (FT)* acts to regulate flowering in many plants including: Arabidopsis, rice, wheat, cucumber, and tomato (Kojima *et al.*, 2002; Lifschitz *et al.*, 2006; Yan *et al.*, 2006; Lin *et al.*, 2007). *CONSTANS (CO)* is a transcription factor which promotes *FT* expression in leaf phloem (An *et al.*, 2004), and so promotes flowering (Putterill *et al.*, 1995). CO and FT are part of the photoperiod dependent flowering pathway. The photoperiod dependent pathway induces flowering according to how long

plants are exposed to light, and depends on the circadian clock (Suárez-López *et al.*, 2001). Arabidopsis is a long day plant: long days enable CO to act, which in turn induces *FT* expression. CO protein accumulates across the day but is degraded in the dark (Valverde *et al.*, 2004). Therefore, it is the coincidence of high CO protein levels and light at the end of a long day which activates *FT* expression and so the transition to flowering, in what is called the coincidence model.

The mechanism behind photoperiod dependent flowering is driven by the negative feedback loop between the circadian clock genes *TOC1, ELF4, CCA1,* and *LATE ELONGATED HYPOCOTYL (LHY)* (Schaffer *et al.*, 1998; Wang and Tobin, 1998; Strayer *et al.*, 2000; Alabadí *et al.*, 2001; Doyle *et al.*, 2002). TOC1 and ELF4 induce the expression of *CCA1* and *LHY* in the evening, leading to a peak of *CCA1* and *LHY* expression at dawn. But as *TOC1* and *ELF4* are repressed by CCA1 and LHY, this results in a reversal in which TOC1 and ELF4 levels increase while CCA1 and LHY levels fall throughout the afternoon. The key flowering promotion gene *GIGANTEA (GI)* is similarly regulated by CCA1 and LHY, peaking in the evening (Mizoguchi *et al.*, 2005). GI can interact with the blue light photoreceptor FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1) to repress the *CO* repressor CYCLING DOF FACTOR 1 (CDF1), which in long days results in enough *CO* mRNA to induce flowering (Imaizumi *et al.*, 2005; Sawa *et al.*, 2007). FKF1 can also inhibit CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1)-dependent CO degradation, by inhibiting COP1 homo-dimerization (Lee *et al.*, 2017).

However, *CCA1* and *LHY* also accelerate flowering under continuous light independently of *CO* and *GI,* through *EARLY FLOWERING 3 (ELF3)* and the MADS box transcription factor *SHORT VEGETATIVE PHASE (SVP)* (Fujiwara *et al.*, 2008; Yoshida *et al.*, 2009). CCA1 and LHY inhibit *ELF3* expression, resulting in *SVP* expression. SVP then interacts with the key vernalisation pathway regulator *FLC (FLOWERING LOCUS C)* which represses *FT* to inhibit flowering (Michaels and Amasino, 1999; Helliwell *et al.*, 2006).

The vernalisation pathway (temperature-dependent inhibition of flowering before Spring) is central to flowering regulation, wherein Arabidopsis flowering is repressed until after a prolonged period of cold (Sheldon *et al.*, 2000). Vernalisation is centred on *FLC,* a repressor

of *FT* and *SOC1* (Michaels and Amasino, 1999; Helliwell *et al.*, 2006), which acts synergistically with *FRIGIDA (FRI)* (Lee and Amasino, 1995; Michaels and Amasino, 2001). *FLC* is, in part, regulated by RNA transcripts including its own antisense transcript (Liu *et al.*, 2010). *FLC* is repressed by the enrichment of chromatin with trimethylated histone H3 lysine 27 by polycomb repressive complex 2, which is targeted to *FLC* by another long RNA, the intronic noncoding RNA *COLDAIR* (Heo and Sung, 2011).The repression of FLC after vernalisation is due to vernalisation genes such as *VERNALIZATION 2 (VRN2),* which is a member of the polycomb complex that targets *FLC* (De Lucia *et al.*, 2008). Without *VRN2 FLC* transcription can still be repressed by a cold period, but increases afterwards (Gendall *et al.*, 2001).

Sometimes plants can flower without external cues. This is termed autonomous promotion. One such autonomous pathway also involves repressing *FLC* to allow flowering. This involves *FCA, FY, FVE, FPA, LD, FLD,* and *FLK* (Koornneef, Hanhart and Veen, 1991; Bäurle and Dean, 2006). Several of these encode RNA-interacting proteins, and it is known that FCA and FY physically interact with *FLC* chromatin and mediate processing of the *FLC* antisense transcript (Liu *et al.*, 2007). This requires *FLOWERING LOCUS D (FLD)*, which acts upstream of *FCA,* and is also known to effect flowering with *HDA6* as mentioned previously by mediating crosstalk between histone acetylation and deacetylation (Yu *et al.*, 2011).

1.4.3 The circadian clock in defence

As stated, the circadian clock controls the expression of approximately 1/3 of Arabidopsis genes (Michael and McClung, 2003). Therefore, it is not surprising that it affects genes involved in both abiotic and biotic stress responses.

The circadian clock appears to be tightly linked to levels of plant hormones, including the key defence hormones jasmonic acid, salicylic acid, and ethylene. Levels of jasmonic acid and salicylic acid were shown to oscillate diurnally (Goodspeed *et al.*, 2012), and antiphase of each other (peaking when the other is lowest). This may be explained by TOC1 repressing the transcription of jasmonic acid synthesis genes *LOX2, LOX3,* and *LOX4* (Huang *et al.*, 2012), while PRR5 represses the transcription of the salicylic acid synthesis genes *LFNR2* and *SIB1* (Nakamichi *et al.*, 2012). Ethylene levels also appear to oscillate diurnally in a variety of

plants including Arabidopsis (Kapuya and Hall, 1977; Thain *et al.*, 2004), which may similarly be due to activation of the ethylene synthesis genes *ACS2, ACS5,* and *ACS9* by PIF4 and PIF5 at night (Song *et al.*, 2018). The circadian regulation of plant hormone levels explains the circadian regulation of hormone-responsive genes (Covington *et al.*, 2008). Specifically there is circadian enrichment of genes regulated by ethylene, jasmonic acid, and salicylic acid for specific diurnal phases of expression (Covington *et al.*, 2008). This is exemplified by *ETHYLENE INSENSITIVE 3 (EIN3),* which is synergistically regulated by both ethylene and jasmonic acid, and peaks in expression around midday (Covington *et al.*, 2008).

Reciprocally, some of the hormones involved in defence have been identified as regulators of the circadian clock. Salicylic acid has been demonstrated to regulate the expression of central circadian clock genes (Zhou *et al.*, 2015). Here a combination of mutant analysis and computational modelling elucidated *CCA1/LHY, PRR7,* and *TOC1* were transcriptionally controlled by the salicylic acid receptor NPR1. Jasmonic acid also regulates the circadian clock, as shown by differential expression of clock genes in response to jasmonic acid treatment (Chong Zhang *et al.*, 2019). Here it was shown that both methyl-jasmonate and while jasmonic acid -isoleucine treatment dampened the amplitude of *CCA1* expression, but not in *coi1-17* mutant plants unable to respond to jasmonic acid treatment. The virulence factor coronatine, which functions as a mimic of jasmonic acid, also suppresses the expression of circadian clock genes (Gao, Zhang and Lu, 2020). In comparison to the detailed work with salicylic acid which identified specific targets of NPR1, the precise mechanism by which jasmonic acid alters clock gene expression is currently unknown.

Systemic acquired resistance (SAR) against plant pathogens is also affected by the circadian clock, through the interaction of the circadian clock with salicylic acid signalling as explained above (Jingjing Zhang *et al.*, 2019). Additionally, experimental data suggests that SAR is mediated by the cryptochrome and phytochrome light receptors which also happen to control the circadian clock (Griebel and Zeier, 2008; Wu and Yang, 2010). Specifically, *phyAphyB* mutants exhibit compromised induction of SAR and salicylic acid – dependent defences (Griebel and Zeier, 2008). Similarly, CRYPTOCHROME 1 positively regulates SAR

(Wu and Yang, 2010). As such, SAR and the circadian clock are both controlled by light signalling in a time of day dependent manner.

Some of the central oscillator genes of the circadian clock timekeeping mechanism have been directly linked to disease susceptibility. Mutating *CIRCADIAN CLOCK ASSOCIATED 1 (CCA1)* results in a loss of circadian susceptibility for infection by *H. arabidopsidis* (W. Wang *et al.*, 2011). In addition, *cca1 lhy* double mutants are more susceptible to *H. arabidopsidis* infection in general (Zhang *et al.*, 2013). For the bacterial pathogen *P. syringae*, there is greater susceptibility to infection after inoculation at night than during the day (Bhardwaj *et al.*, 2011). However, *elf3-1* clock gene mutants (Col0 background) and CCA1-overexpressing plants do not show the circadian susceptibility phenotype of WT Col0 plants. Again, *cca1 lhy* double mutants are more susceptible to infection in general (Zhang *et al.*, 2013). For *B. cinerea*, mutants of *cca1 lhy* are more susceptible to infection in general (Ingle *et al.*, 2015). They also lack a difference in the rate of infection between dawn and dusk. Circadian susceptibility to *B. cinerea* has also been linked to one of the transcriptional co-repressors involved in jasmonate signalling, known as *JAZ6 (AT1G72450)* (Ingle *et al.*, 2015).

1.5 Known properties of JAZ6

1.5.1 The TIFY family of proteins

JAZ proteins are a subset of the TIFY family, as detailed in figure 1.8. They are characterised by possessing both TIFY and JA-associated (Jas) domains (Vanholme *et al.*, 2007; Pérez *et al.*, 2014). The TIFY (or zinc-finger inflorescence meristem [ZIM]) domain is responsible for JAZ proteins homo- and hetero-dimerising with other JAZ proteins (Chini *et al.*, 2009), and the adapter protein Novel INteractor of JAZ (NINJA) which binds to the transcriptional corepressor TOPLESS (Pauwels *et al.*, 2010). The Jas domain, however, is central to JAZ binding to COI1 and transcription factors like MYC2 (Thines *et al.*, 2007; Zhang *et al.*, 2015)

A further subset of JAZ proteins possess ERF-associated amphiphilic repression (EAR) domains (Shyu *et al.*, 2012), capable of directly binding to transcription factors like TOPLESS in the absence of NINJA. EAR domains are not specific to the TIFY family, unlike TIFY and Jas, and are found in many other proteins (Kagale, Links and Rozwadowski, 2010), particularly regulators of defence and stress genes (Kazan, 2006). The amino acid sequences of these

domains are functional determinants of binding efficiency to other proteins (Zhang *et al.*, 2015), which shapes the activity and function of JAZ proteins. For example, JAZ8 does not contain a canonical degron in its Jas domain (Shyu *et al.*, 2012), which alters its propensity to be targeted for degradation by COI1. JAZ13 does not possess a canonical TIFY domain (Thireault *et al.*, 2015), and so does not interact with other JAZ proteins or NINJA.

TIFY8 belongs to the TIFY family but does not possess a Jas domain like the JAZ proteins (Pérez *et al.*, 2014). This prevents it from binding to transcription factors like MYC2 and prevents it from being degraded by COI1. However, it does possess a functional TIFY domain and can bind to all other JAZ proteins tested (JAZ13 was not tested), as well as the other TIFY family protein PEAPOD2 (PPD2) and the transcriptional co-repressor TPL through NINJA.

Figure 1.8: Schema for TIFY family proteins, illustrating major motifs.

A Diagram of the protein domain structure of TIFY family proteins. Jas (orange); Jas-like (red); TIFY/ZIM (blue); EAR (green); Cryptic MYC2-interacting (CMID, yellow); CONSTANS, CO-like, and TOC1 (CCT, purple); C2C2-GATA Zn-finger (GATA, lime green); and PEAPOD (brown) domains are labelled. Adapted from Pérez et al., 2014.

PEAPOD proteins are also members of the TIFY family, which possess a PEAPOD domain in addition to a TIFY and Jas-like domain (Pérez *et al.*, 2014). Their Jas domain differs from the JAZ proteins resulting in lower binding affinity for MYC transcription factors (Schneider *et al.*, 2021), and instead of COI1 they appear to be targeted for degradation by STERILE APETALA (SAP). The PEAPOD domain enables them to bind to KINASE-INDUCIBLE DOMAIN INTERACTING PROTEIN 8/9 (KIX8/9) in order to control leaf development as transcriptional co-repressors. Like JAZ proteins, PEAPODs can bind to LHP1 to repress gene expression by polycomb epigenetic silencing (Zhu *et al.*, 2020).

ZIM and its paralogues ZML1 and ZML2 possess TIFY domains, along with CCT and GATA box domains (Pérez *et al.*, 2014). They are thought to act as transcription factors involved in inflorescence and flower development (Shikata *et al.*, 2004).

1.5.2 The functional role of protein-protein binding for JAZ proteins

Jasmonic acid acts by targeting the JAZ proteins for degradation (Thines *et al.*, 2007), forming a physical link between a JAZ protein and the CORONATINE INSENSITIVE 1 (COI1) E3 ubiquitin ligase, as detailed in figure 1.3. The JAZ proteins act as transcriptional co-repressors (Zander, 2021), and so their degradation allows transcriptional reprogramming in response to jasmonic acid (W. Zhang *et al.*, 2017). JAZ proteins act as transcriptional repressors by binding to transcription factors like MYC2 (Zhang *et al.*, 2015), preventing them from activating transcription. MYC2 itself promotes transcription of JAZ genes in a negative feedback loop, resulting in upregulation of JAZ genes when JAZ proteins are degraded due to stresses like wounding which result in the production of jasmonic acid (Chung *et al.*, 2008). This is illustrated in chapter 3 of this thesis, where *JAZ6* is upregulated in response to wounding.

Table 1.1: Summary of known Arabidopsis JAZ protein-protein interactions. Data from Zander, 2021, and other references in the main text.

Proteins binding JAZ	JAZ proteins known to bind	Physiological role(s)
COI1	All, except JAZ13	Jasmonic acid perception, JAZ degradation
MYC2/3/4/5	All	Root growth, flowering, wounding, defence
TPL	JAZ1/5/6/7/8/10/13	Stress responses, development
NINJA	All, except JAZ7/8/13	Links TPL to JAZ proteins
MYB21/24	JAZ1/8/10/11	Stamen development
TT8/GL3/EGL3/ MYB75/GL1	All, except JAZ3/4/13	Anthocyanin production, trichome development
FIL/YAB1	JAZ1/3/4/9	Chlorophyll, defence
ECAP	JAZ6/8	Salt stress, anthocyanin production
ICE1/2	JAZ1/4/9	Freezing tolerance, JA/GA hormone crosstalk
WRKY57	JAZ4/8	Defence, senescence
EIN3/EIL1	JAZ1/3/9	Defence, root hair development
FHY3/FAR1	All, except JAZ4/5/7/12/13	Light perception, circadian clock gating
TOE1/2	JAZ1/3/4/9	Flowering
JAM1/2/3	All, except JAZ7/12/13	Defence, fertility, senescence
FRS7/12	JAZ4/8	Glucosinolate biosynthesis, flowering, growth
NPR3/4	All, except JAZ3/7/12/13	SA/JA hormone crosstalk
KEG	JAZ12	ABA/JA hormone crosstalk
ABI5	All, except JAZ1/4/13	Seed germination, ABA/JA hormone crosstalk
RGA	JAZ1	JA/GA hormone crosstalk
LHP1	JAZ3/4/6/8/9/10	Epigenetic chromatin modifications
HDA6	JAZ1/3/9	Epigenetic chromatin modifications

However, not all JAZ proteins bind to the same proteins. As the divergence in protein domains suggests, there is functional diversity among the JAZ proteins in terms of what they bind to. This is detailed in Table 1.1. While all JAZ proteins can bind to at least one of the MYC transcription factors, some protein-protein binding partners show more selective binding. In some publications on these proteins, only positive results were disclosed, perhaps most notably EIN3 (Zhu *et al.*, 2011). It may be the case that more JAZ proteins than JAZ1/3/9 can bind EIN3, or it may be the case that negative results for JAZ proteins binding EIN3 have not been reported. HDA6 forms part of the repressive protein complex with EIN3 and JAZ proteins (Zhu *et al.*, 2011), targeting genes for histone acetylation and so changes in gene expression.

Small differences in domain content and structure can influence the binding affinity of two proteins. While all JAZ proteins are able to bind MYC transcription factors, their affinity varies depending on the exact sequence of their Jas domains (Zhang *et al.*, 2015). As the Jas domain also mediates JAZ protein degradation by COI1 (Thines *et al.*, 2007), variation in the Jas domain can lead to drastically different protein activity. This is seen for JAZ13, JAZ8, and splice variants of JAZ10 and JAZ6, which all possess Jas domains compromised in binding to COI1 (Chung *et al.*, 2010; Shyu *et al.*, 2012). Recently the development of a jasmonic acid–mimicking chemical agonist specific to JAZ9 illustrates that the specific properties of the Jas domain can determine the target specificity of JAZ repression of transcription factors (Takaoka *et al.*, 2018). This may be what enables JAZ proteins to specifically effect either herbivorous insect defence or necrotrophic pathogen defence, as seen in their individual mutant phenotypes (Liu *et al.*, 2021).

Many JAZ proteins bind to transcription factors like GLABRA3 (GL3) and MYB75 which are involved in anthocyanin production and trichome development (Qi *et al.*, 2011). FIL/YAB were found to bind JAZ proteins to regulate defence and anthocyanin accumulation together with MYB75 (Boter *et al.*, 2015). Recently, ECAP was discovered as a novel interactor of JAZ proteins which links them to transcriptional co-repression via TPR2 (Li *et al.*, 2019), a paralogue of TPL, which also regulates anthocyanin accumulation.

Recently several JAZ proteins (including JAZ6) have been found to bind an element of the polycomb repressive complex 1, LHP1 (Li *et al.*, 2021). This is particularly interesting as LHP1 is known to regulate plant defence through repression of MYC2-dependent defences (Ramirez-Prado *et al.*, 2019), repressing defence against herbivorous insects but promoting salicylic acid and resistance to *P. syringae*.

1.5.3 Roles of potential interactors of JAZ6

Previously Dr Claire Stoker identified seven putative transcription factors as possible protein-protein binding partners of JAZ6 and not of JAZ5 (Stoker, 2016). These are ETHYLENE INSENSITIVE 3 (EIN3, AT3G20770), FAR RED HYPOCOTYL 3 (FHY3, AT3G22170), PHD DOMAIN CONTAINING PROTEIN (PFP, AT4G23860), WUSCHEL RELATED HOMEOBOX 12 (WOX12, AT5G17810), NAC DOMAIN CONTAINING PROTEIN 10 (NAC10, AT1G28470), a MYB like protein (AT5G56840) and ZINC FINGER PROTEIN 1 (ZF1, AT5G67450).

ZF1 has been noted to respond to abiotic stress, including water, heat, cold, and salt stress (Sakamoto *et al.*, 2000, 2004). In abiotic stress conditions, it appears to promote abscisic acid responses but repress auxin responses (Kodaira *et al.*, 2011). It also appears to be activated by PTI, as the expression of *ZF1* is induced by chitin (Libault *et al.*, 2007).

NAC10, also known as *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 3 (SND3),* promotes the formation of secondary cell walls (Zhong *et al.*, 2008). It is also a target for WRKY transcription factors during dark-induced senescence (Hsu *et al.*, 2022).

WOX12 acts in a combinatorial fashion with other WOX proteins during embryonic development to define cell type boundaries (Wu, Chory and Weigel, 2007). When activated by auxin it acts together with WOX11 to promote the growth of adventitious roots by activating *WOX5* and *WOX7* expression (Hu and Xu, 2016), inducing waves of cell fate changes in root founder cells.

EIN3 is a well-established part of ethylene signalling, which as noted above (Section 1.4.3) is transcriptionally regulated by the circadian clock (Covington *et al.*, 2008). EIN3 was previously shown to bind JAZ1/3/9 (Zhu *et al.*, 2011), though no negative results were

published for other JAZ proteins. EIN3 is known to promote resistance to necrotrophic pathogens (Alonso *et al.*, 2003), possibly through its promotion of plant defensins (He *et al.*, 2017). However, EIN3 also inhibits the expression of salicylic acid synthesis enzyme *ICS1* to inhibit PTI and salicylic acid based defences in an example of antagonistic crosstalk between plant hormones (Chen *et al.*, 2009). EIN3 also targets ORA59 for degradation in the presence of salicylic acid (He *et al.*, 2017), limiting the induction of plant defensins in another example of antagonistic crosstalk.

FHY3 gates red light inputs into the circadian clock, limiting maximum responsiveness to light to specific times of day (Allen *et al.*, 2006). As a positive regulator of the Phytochrome A signalling pathway FHY3 activates the transcription of the circadian clock gene ELF4 (Li *et al.*, 2011), connecting light signalling to the circadian clock. Together with its paralogue FAR-RED IMPAIRED RESPONSE 1 (FAR1), FHY3 represses salicylic acid production and so represses resistance to *P. syringae* (Wang *et al.*, 2016). FHY3 also interacts with MYC2 to regulate JA induced defence genes (Liu *et al.*, 2017), and recently FHY3 and FAR1 were shown to interact with JAZ1-3,6,8-11 (Y. Liu *et al.*, 2019). FHY3 may also act on defence through binding to EIN3, which regulates defence against necrotrophic pathogens as above (Alonso *et al.*, 2003). FHY3 and FAR1 have recently been shown to regulate senescence by acting in tri-protein complexes with EIN3 and PIF5 (Xie *et al.*, 2021), which may be indicative of possible roles in necrotophic pathogen defence as senescence is also controlled by jasmonic acid.

PFP is a member of the UBR (ubiquitin ligase N-recognin) protein family conserved across eukaryotes (Tasaki *et al.*, 2005), which are known for their role in N-end mediated protein degradation (Garzón *et al.*, 2007). The N-end rule determines proteins to be degraded on perception of external abiotic stresses (Vicente *et al.*, 2017), but has also been linked to pathogen responses (de Marchi *et al.*, 2016). Specifically, the UBR proteins recognise the oxidation state of cytosine residues at the ultimate N-terminus of proteins (Vicente *et al.*, 2017), and target these proteins for degradation through ubiquitination. The proteins targeted to degradation are largely ERFVII transcription factors which activate responses to hypoxia stress like flooding (Vicente *et al.*, 2017). As they are degraded in the presence of

oxygen, it is only under hypoxic conditions are ERFVII TFs can act. However, PFP does not bind to any possible N-terminal residue of proteins (Garzón *et al.*, 2007), and is the only UBR protein to contain a PHD finger (Adhikary *et al.*, 2019), suggesting that it has a distinct function to other UBR proteins.

The function of PFP was recently elucidated, as a repressor of flowering. It was shown that *pfp-1* mutants flowered early, while PFP-Ox over-expressors flowered late (Yokoyama, Kobayashi and Kidou, 2019). Investigation of gene expression in *pfp-1* mutants indicated that expression of the key flowering repressor *FLC* was promoted by PFP (Yokoyama, Kobayashi and Kidou, 2019). Further knowledge about the function of *PFP* comes from its homologues called *UBIQUITIN PROTEIN LIGASE E3 COMPONENT N-RECOGNIN 7 (UBR7)* in *N. benthamiana,* rice, and humans. Human HsUBR7 controls breast cancer development and is involved in transcriptional regulation as a chromatin editor by monoubiquitinating histone H2B (Adhikary *et al.*, 2019). In both Arabidopsis and humans, PFP and HsUBR7 can bind to histone H3 (Yokoyama, Kobayashi and Kidou, 2019; Hogan *et al.*, 2021), with a potential role in chaperoning histones. In *N. benthamiana* NbUBR7 negatively regulates virus disease resistance, by binding to and targeting the N protein for degradation (Yongliang Zhang *et al.*, 2019), which is an NB-LRR for resistance to tobacco mosaic virus (Marathe *et al.*, 2002). Recently, the rice orthologue of PFP (OsUBR7) was found to regulate plant growth by histone monoubiquitination (Zheng *et al.*, 2022), similar to human HsUBR7.

PFP is highly conserved across plant species (Yongliang Zhang *et al.*, 2019), and together with its orthologues it has been used to evaluate genome quality as a benchmarking universal single-copy orthologue (BUSCO) gene family (Simão *et al.*, 2015), as detailed at https://www.orthodb.org/?query=EOG09360BIG. Only 11.9% of plant BUSCO families are syntenic (Zhao and Schranz, 2019), which refers to evolutionary conserved relationships between genomic regions. *PFP* and its orthologues form one of these syntenic BUSCO families (Zhao and Schranz, 2019), highlighting the high degree of conservation of *PFP*.

In contrast to the putative transcription factors above, the MYB like protein (AT5G56840) has never been individually studied. It has been identified as a member of a novel group of

transcriptional repressors with R/KLFGV repression motifs (Ikeda and Ohme-Takagi, 2009). It is a target of MAPK kinase cascades involved in plant defence (Popescu *et al.*, 2009), and also a target of the flowering transcription factor SEPALLATA 3 (Kaufmann *et al.*, 2009).

1.5.4 JAZ proteins beyond Arabidopsis

JAZ proteins are not unique to Arabidopsis and can be found across the green plant lineage (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). From a single JAZ protein in the basal bryophyte liverwort *Marchantia polymorpha* (Monte *et al.*, 2019), the TIFY family has expanded several times resulting in 5 to 36 JAZ proteins in Angiosperm species (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). Comparative genomics approaches have identified JAZ proteins containing TIFY and Jas domains in many plant species, and just as in Arabidopsis these JAZ proteins exhibit conserved function in regulating disease resistance; abiotic stress resistance; and ethylene responses (Lv *et al.*, 2017; Zhu and Napier, 2017; Guofeng Zhang *et al.*, 2019; S. Liu *et al.*, 2019; Hu *et al.*, 2022). Specifically, wild grapevine VqJAZ4 promotes resistance to biotrophic powdery mildew and susceptibility to necrotrophic *B. cinerea* (Guofeng Zhang *et al.*, 2019) and apple JAZ controls fruit ripening by gating ethylene responses (Hu *et al.*, 2022). Moss PnJAZ1 promotes resistance to salinity stress (S. Liu *et al.*, 2019), while rice OsJAZ9 suppresses OsMYB30 to promote cold tolerance (Lv *et al.*, 2017).

1.6 Summary of JAZ6 and plant defence mechanisms

Plant diseases are a severe economic burden and threaten food security. By improving our understanding of plant defences systems against plant pathogens, more efficient means of plant pathogen control can be implemented. Consequently, this thesis focuses on the response of the JAZ6 protein to the presence of *B. cinerea, H. arabidopsidis,* and *S. sclerotiorum* in the model organism Arabidopsis.

The thesis will show how the JAZ proteins, especially JAZ6, regulate defence responses against *B. cinerea* and *S. sclerotiorum* in Arabidopsis as well as the activity of lettuce JAZ proteins. Nine lettuce JAZ proteins were identified, many of which respond to necrotrophic pathogen attack and abiotic cold stress.

1.7 Aims and objectives

Given the importance of understanding plant defences against pathogens, the main aim of this thesis is to understand the biological mechanisms underlying JAZ6 in host defence against plant pathogens. Initially presented is work detailing gene and protein regulation involving JAZ6, specifically what genes may regulate JAZ6 itself in a circadian manner, and how JAZ6 may act to regulate gene expression through binding to novel proteins. The roles of JAZ6 and such novel protein-protein binding partners in affecting plant physiological phenotypes and gene expression were then considered, particularly with regards to pathogen disease phenotypes of necrotrophic and biotrophic pathogen infection. With a view to translate the knowledge of JAZ6 into crop species, comparative analysis of the Arabidopsis and lettuce orthologous JAZ genes and proteins was then performed.

Specific objectives were to:

- Predict circadian regulation of JAZ6 (Chapter 2).
- Validate novel protein-protein binding partners of JAZ6, and the specificity of this binding (Chapter 2).
- Examine the role of these novel protein-protein binding partners of JAZ6 involved in plant defence responses (Chapter 3).
- Elucidate the mechanisms of JAZ6 controlling plant defence (Chapter 3).
- Identify JAZ orthologues in lettuce and investigate their potential roles in plant pathogen defence (Chapter 4).

When considered together, these chapters constitute a significant advancement in our understanding of how JAZ6 functions inside the plant, the roles JAZ6 and its protein-protein binding partners can take in the plant, and what orthologues exist for Arabidopsis JAZ genes in the important crop plant lettuce. These advancements may be leveraged to improve crop plant production, particularly lettuce.

2: Investigating the mechanism of JAZ6 time-of-day impact on disease resistance

2.1 Introduction

This chapter details the investigation into aspects underlying JAZ6 diurnal activity in regulating disease susceptibility. The first aim is to discover the potential circadian regulation mechanisms of *JAZ6*, while the second is to validate protein-protein binding targets which JAZ6 potentially regulates. This research extends a previously published study which was focussed on the role of JAZ genes in regulating diurnal oscillations in susceptibility to necrotrophic pathogen infection (Ingle *et al.*, 2015).

Given the time-of-day impact of JAZ6 on defence, it was hypothesised that circadian expression of JAZ6 could underlie this, and modelling was used to predict components of the circadian clock that could regulate JAZ6. Modelling gene activity and behaviour is a tool to reach otherwise inaccessible research questions by simplifying the system and using a clear set of assumptions (Le Novère, 2015). Computational mathematical modelling of gene regulation is a recent area of research interest, particularly with regards to building gene regulatory networks encompassing and predicting transcription factor behaviour across the transcriptome (Karlebach and Shamir, 2008). A series of differential equations can be used to build a predictive circadian model of gene expression for diurnally oscillating changes in expression and activity of a small number of genes (Gonze, 2011). Circadian models of gene expression have been used to explain and assess the diurnal expression and activity of key circadian clock genes (Locke, Millar and Turner, 2005). This includes the identification of previously unknown functions for genes acting to control clock gene transcription, such as GIGANTEA (GI) as an activator of TIMING OF CAB EXPRESSION 1 (TOC1) (Pokhilko *et al.*, 2010).

There are several models of the circadian clock in Arabidopsis, ranging from the simple Locke model with 8 ordinary differential equations (Locke, Millar and Turner, 2005), to the complex Pokhilko model with 32 (Pokhilko, Mas and Millar, 2013). These models can also be expanded to account for inputs and outputs of the clock in other processes, such as

predicting which clock genes are regulated by NPR1 which has a major role in defence (Zhou *et al.*, 2015). The De Caluwé model is a simplified model of the circadian clock genes and their transcriptional control of downstream genes (De Caluwé *et al.*, 2016), which can be readily expanded to account for the role of the circadian clock in other processes like heat compensation (Avello *et al.*, 2019). The use of modelling allows for refinement of hypotheses for experimental testing, the results of which could feed back into a more precise model which better reflects the biological system. While models have their limitations, and may be incorrect, nevertheless they can be useful in giving further insight into processes needing additional laboratory analysis. In fact, none of the major circadian clock models to date (Pokhilko *et al.*, 2010; Pokhilko, Mas and Millar, 2013; De Caluwé *et al.*, 2016) have incorporated JAZ proteins.

JAZ6 acts as a co-repressor by binding to transcription factors and preventing them from promoting gene transcription. Therefore, protein-protein binding partners of JAZ6 are of considerable interest for understanding how JAZ6 controls gene expression. Overexpression of target genes can be induced transiently using infiltration of *A. tumefaciens* into *N. benthamiana* (Bally *et al.*, 2018), which is of particular use for investigating protein-protein interactions and their subcellular localisation *in planta*. One approach consists of tagging proteins with fragments of fluorescent proteins in a technique called bimolecular fluorescence complementation (BiFC), in which binding of the tagged proteins brings the fluorescent protein fragments close enough together to fluoresce (Azimzadeh *et al.*, 2008; Miller *et al.*, 2015). While this technique gives useful information on subcellular localisation of protein-protein binding, it is subject to both false positives and false negatives (Horstman *et al.*, 2014). False positives can arise from a 'sticky' target protein at high abundance, relatively saturating a subcellular compartment and so *YELLOW FLUORESCENT PROTEIN (YFP)* fluorescing due to high concentrations of each half of YFP instead of protein binding. On the other hand, false negatives can arise from steric hindrance of target proteins by the fluorescent protein fragments, which may be larger than the target proteins themselves, preventing target proteins from assuming the steric positioning for protein-protein binding. Despite these caveats BiFC remains a valid source of information on subcellular localisation of protein-protein binding (Miller *et al.*, 2015), particularly when supported by other

protein-protein binding data from yeast-2-hybrid and co-immunoprecipitation (Xing *et al.*, 2016).

Using yeast-2-hybrid, BiFC, and co-immunoprecipitation JAZ proteins have previously been shown to bind to transcription factors like MYC2 (Withers *et al.*, 2012), the E3 ubiquitin ligase COI1 (Sheard *et al.*, 2010), as well as transcriptional co-repressors like NINJA and TOPLESS (Pauwels *et al.*, 2010). Yeast-2-hybrid screening has previously suggested that seven putative transcription factors may uniquely bind to JAZ6 and no other JAZ proteins (Stoker, 2016), which are of interest for testing *in planta*. They are ETHYLENE INSENSITIVE 3 (EIN3, AT3G20770), FAR RED HYPOCOTYL 3 (FHY3, AT3G22170), PHD DOMAIN CONTAINING PROTEIN (PFP, AT4G23860), WUSCHEL RELATED HOMEOBOX 12 (WOX12, AT5G17810), NAC DOMAIN CONTAINING PROTEIN 10 (NAC10, AT1G28470), ZINC FINGER PROTEIN 1 (ZF1, AT5G67450) and a MYB like protein (AT5G56840). These are candidates for controlling the diurnal variation in susceptibility to *B. cinerea*, as JAZ6 is a co-repressor which acts by preventing transcriptional activation by bound transcription factors.

2.2 Aims and objectives

The objectives of this chapter are to identify circadian clock genes regulating JAZ6, and binding partners of JAZ6 which may be responsible for regulating diurnal variation (oscillations which repeat on a 24-hour time period) in susceptibility to *B. cinerea*. The first objective will be met through *in silico* analysis and modelling, while the second will involve *in planta* protein-protein binding testing with a focus on bimolecular fluorescence complementation (BiFC).

Assessing the diurnal patterns of JAZ6 expression and regulatory elements present in the JAZ6 promoter will provide candidates for direct regulation of JAZ6 transcription by the circadian clock. Modelling will assist in identifying clock genes from these candidates which may regulate JAZ6, and how JAZ6 protein abundance may be regulated by diurnal variation in jasmonic acid levels.

The seven protein-protein binding candidates for JAZ6 are of particular interest for *in planta* testing, which will be initially conducted with BiFC. Positive interactions will be verified using JAZ6 domain deletions and mutants, as well as co-immunoprecipitation. This approach should give great confidence in the protein-protein binding partners of JAZ6.

The analysis should therefore predict core circadian clock gene(s) regulating JAZ6, and protein-protein binding partners of JAZ6 which are candidates for the regulation of diurnal variation in susceptibility to *B. cinerea.*

2.3 Methods

2.3.1 Plant material

Nicotiana benthamiana (*N. benthamiana*) RA-4 accession (Goodin *et al.*, 2008) was used as a platform for transient transformation with *Agrobacterium tumefaciens* as detailed further below.

2.3.2 Plant growth

Plants were grown in F2 compost (Levington), stratified for 2 days at 4°C covered with tinfoil, and then placed in the glasshouse, under ambient photoperiod, humidity, CO_2 concentration, and light intensity. Temperature varied from 20°C (day), to 17°C (night). *N. benthamiana* was grown in $7cm^2$ (7K) square pots (Desch plant-pak). The University of York horticulture team assisted in pot filling and plant cultivation.

2.3.3 Microbial growth and subculturing

Escherichia coli DH5α (ThermoFisher) was cultured in LB (lysogeny broth) liquid broth (Bertani, 1951) or on LB agar, as LB liquid broth with 1% bacto-agar (Sigma). Antibiotics were added as appropriate to select for bacteria transformed with specific vectors.

Agrobacterium tumefaciens GV3101 (Holsters *et al.*, 1980) was cultured in YEB (yeast extract beef) liquid broth (Vervliet *et al.*, 1975) or on YEB agar, as YEB liquid broth with 1% Bacto-agar (Sigma). Rifampicin and gentamycin were added to growth media to select for *A.*

tumefaciens growth. Other antibiotics were added as appropriate to select for bacteria transformed with specific vectors.

Saccharomyces cerevisiae yeast AH109 and Y187 (Clontech) was cultured in SC (synthetic complete) liquid broth (Sigma) or on SC agar with 1% agar, with dropout amino acids specific to the vectors carried by the yeast. Yeast was also cultured on YPDA (yeast extract peptone dextrose agar) mating plates (Sigma).

2.3.4 Molecular cloning

The Gateway Invitrogen cloning system was used to clone putative transcription factor protein coding sequences out of a yeast-2-hybrid library (Pruneda-Paz *et al.*, 2014) and into destination vectors with pDONRzeo (ThermoFisher) as the backbone for entry clones. This was performed as detailed in the Gateway Technology users guide (Invitrogen) with Gateway BP Clonase II enzyme mix. DH5α *Escherichia coli* transformation was then performed according to the manufacturer's instructions.

After verification of the entry clones, coding sequences were then transferred into pBIFP, pEG201, and pK7FWG2 (Karimi, Inzé and Depicker, 2002; Earley *et al.*, 2006; Azimzadeh *et al.*, 2008), with nYFP, cYFP, HA, and GFP tags respectively. This was performed as detailed in the Gateway Technology users guide (Invitrogen) with Gateway LR Clonase II enzyme mix. After confirmation by PCR and sequencing, these binary vectors were transformed into *A. tumefaciens*, GV3101, for plant transformation (Tinland, Hohn and Puchta, 1994).

2.3.5 *Agrobacterium* infiltration

Bimolecular fluorescence complementation (BiFC) assays were performed in *N. benthamiana* as previously described (Azimzadeh *et al.*, 2008). Briefly, *A. tumefaciens* harboring a pair of pBIFP vectors were grown overnight at 28°C in YEB media, along with *A. tumefaciens* harboring the P19 silencing suppressor (Lombardi *et al.*, 2009). The cultures were then harvested by 30 minutes centrifugation at 3000xg and resuspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$ pH 5.7) with 100 µM acetosyringone and incubated at room temperature in the dark for 4 hours. They were then mixed in a 3:3:1 ratio for a final

optical density of $O.D._{600}$ 0.6, Flat-top syringes were used to infiltrate the underside of *N. benthamiana* leaves. *N. benthamiana* plants were three weeks old when infiltrated.

Stability assays with pEG201 containing PFP and pK7FWG2 containing JAZ6 were performed as described for stability assays with JAZ12 and KEG (Pauwels *et al.*, 2015). However, instead of methyl-jasmonic acid pBin19PLUS containing HA tagged COI1 was co-infiltrated as the JAZ6-degrading treatment (Devoto *et al.*, 2002), provided by Professor Alessandra Devoto. *A. tumefaciens* culturing and growth was performed as above for BiFC assays.

For co-immunoprecipitation assays *A. tumefaciens* was infiltrated into *N. benthamiana* as described previously (Steinbrenner *et al.*, 2014). pEG201 containing PFP and pK7FWG2 containing JAZ6 were the same as used for stability assays, while pBin-35S-mGFP5 provided by Dr Fabian Vaistij was used as a control (Haseloff *et al.*, 1997).

2.3.6 Confocal microscopy

Three days post infiltration, $1cm^2$ sections of infiltrated leaf tissue were cut from *N. benthamiana* leaves and placed on glass slides for imaging. Imaging was performed using a Zeiss 710 or 780 LSM confocal microscope in the York Technology Facility. Images were taken using an EC Plan-Neofluar 20x/0.50 M27 lens. Argon laser light at a wavelength of 514 nm excited the sample, with the eYFP detector wavelength range from 520 to 561 nm and the chlorophyll detector wavelength range from 640 to 692 nm, and transmitted light was also captured.

Stability assay imaging utilised the same Zeiss 710 LSM confocal microscope in the York Technology Facility as in BiFC assays, and the detector wavelength range was set for eGFP instead of eYFP at 520 to 535 nm.

Confocal microscopy images were analysed and processed in FiJi ImageJ (Schindelin *et al.*, 2012).

2.3.7 Protein extraction

Protein extraction from *N. benthamiana* tissue three days after infiltration was as previously described (Liu *et al.*, 2008). Briefly, infiltrated leaves were homogenised and mixed with protein extraction buffer including 2% PVPP protease inhibitor on ice. Protein concentration was then quantified using a Bradford assay with Bradford reagent (BioRad) as per the manufacturer's instructions.

2.3.8 Co-immunoprecipitation

For co-immunoprecipitation, Pierce anti-HA (Thermo) and anti-GFP (ChromoTek) agarose beads were used following the manufacturer's instructions. Beads washed three times with wash buffer and then incubated with protein extracts for 2 hours at 4°C, as previously described (Liu *et al.*, 2008). Then, beads were washed again, and samples processed for immunoblotting.

2.3.9 Immunoblotting

Samples were denatured in Laemmli buffer (Laemmli, 1970), separated by 10% SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were reversably stained with Ponceau Red to check equal loading. Membranes were blocked with 4% BSA TBST (tris-buffered saline, 0.1% Tween) overnight at 4°C. Horseradish peroxidase (HRP)-conjugated anti-GFP antibody (MACS, 130-091-833) and anti-HA antibody (Roche, 12013819001) were used to detect GFP and HA tagged proteins respectively, at concentrations of 1/5000 in 4% BSA TBST. After incubation with antibody for 2 hours, membranes were washed 5 times with TBST.

Protein bands were visualised by chemiluminescence using ECL reagents (GE Healthcare), as per the manufacturer's instructions. Blue Precision Plus protein standards (BioRad) were used as size markers.

2.3.10 Yeast-2-hybrid

Yeast-2-hybrid was performed with DB:JAZ6 in pDEST32 in AH109 yeast cloned by Dr Sarah Harvey and AD:PFP in pDEST22 in Y187 yeast from a transcription factor library (Pruneda-Paz *et al.*, 2014). Yeast containing pDEST32 was grown on leucine dropout SC-Leu media,

while yeast containing pDEST22 was grown on tryptophan dropout SC-Trp media. Yeast-2-hybrid was performed as described previously (Niu, Figueroa and Browse, 2011). Briefly, 10 µL of each of pairs of pDEST32 and pDEST22 yeast were spotted onto YPDA mating media. YPDA mating plates were replica plated with sterile velvet onto SC-Leu-Trp plates to check mating, and SC-Leu-Trp-His (histidine) to select for protein-protein interactions. Positive interactions were assessed by growth on the selective media lacking histidine. As negative controls, yeast harbouring empty vectors with AD or DB were also mated with DB:JAZ6 and AD:PFP yeast.

2.3.11 Computational modelling

2.3.11.1. Gene regulatory network prediction

To predict gene regulatory networks for JAZ6, the Causal Structure Inference (CSI) and Transcriptional Regulation Switch (TRS) programs developed at Warwick were used (Penfold *et al.*, 2015; Minas *et al.*, 2017). CSI and TRS were downloaded from the Warwick Systems Biology Centre website and run using Matlab R2018a.

TRS and CSI were used to infer which of several central clock regulation factors was transcriptionally regulating JAZ6. To this end, the input data consisted of gene expression data for JAZ6 and the central circadian clock transcription factors which were experimentally found to be capable of binding upstream of JAZ6. This transcriptional data came from the Windram et al. 2012 dataset, mock treatment, because the Botrytis treatment data would be overwhelmed by JAZ6 transcriptional promotion through MYC2.

TRS and CSI are similar in that they both use gene expression data to infer putative transcriptional regulatory relationships by Bayesian inference. However, they differ in that TRS considers regulators to be binary ON/OFF switches for target gene expression, while CSI considers the pattern of gene expression on a continuous scale in a regulator over time should be reflected in the target gene with a delay. In simple terms, TRS is focusing on locating a “switch” in target gene expression, and looking for prior or simultaneous changes in putative transcriptional regulators which best reflect the timing of the transcriptional “switch”. On the other hand, CSI places a greater importance on matching the general

pattern of how gene expression changes in the target gene to the shapes of changes in gene expression in putative regulators. Because it was unknown which of these two discrete or continuous modes of gene regulation best fitted putative JAZ6 gene regulation by the circadian clock, both were used.

Simulations of gene and protein interactions were performed based on an ordinary differential equation (ODE) model of the circadian clock (De Caluwé *et al.*, 2016), run in Matlab R2018a using ODE solver `ode45` (but also checking for convergence and accuracy using the stiff solver `ode23`). The main components of the clock system correspond to *CCA1/LHY [CL], PRR9/PRR7 [P97], PRR5/TOC1 [P95], ELF3/ELF4/LUX [EL],* and *PIFs [PIF],* with an additional protein P [P] to simulate light dependent regulation of CCA1. The equations and parameter values are listed below, where L is set to 1 in light a 0 in dark, while D is inversely 0 in light and 1 in dark.

In this model, there is an ODE corresponding to the rate of change of the transcription or translation of each component, such as *CL*. Genes are grouped together into these components to simplify the model. The rates of change of each component are dependent on how much of their regulatory components were present. For example, the higher the amount of the transcriptional repressor of *CL*, the lower the rate of *CL* transcription. To 'run' the ODE model, the equations are given initial starting values for each of the components and the results can be plotted over time.

As previously described with respect to temperature compensation, the De Caluwé model can be extended to reflect additional to transcriptional regulation by the central circadian clock (Avello *et al.*, 2019). Like this prior art, the ODE equations described by De Caluwe et al. and listed below were programmed into Matlab code (De Caluwé *et al.*, 2016).

Specifically, for JAZ6 regulation by circadian clock genes experimentally determined to bind upstream of JAZ6 and who were deemed by CSI and TRS to be possible regulators of JAZ6 given their expression patterns. In this regard, new equations were added to represent JAZ6 transcriptional regulation by a variety of central clock genes, as listed below in section 2.30.

As controls, regulation by light and no circadian regulation were also tested. The rates and magnitudes of clock gene transcriptional regulation of JAZ6 were assumed to be equivalent to the rates at which clock genes had been experimentally validated to regulate each other, as described in the original ODE model (De Caluwé *et al.*, 2016) .

2.3.11.2 Equations from the De Caluwé clock model

$$\frac{d[CL]m}{dt} = (v_1 + v_{1L} * L * [P]) * \left(\frac{1}{1 + \left(\left(\frac{[P97]p}{K_1}\right)^2\right) + \left(\left(\frac{[P51]p}{K_2}\right)^2\right)} \right) - (k_{1L} * L + k_{1D} * D) * [CL]m$$

$$\frac{d[CL]p}{dt} = (p_1 + p_{1L} * L) * [CL]m - d_1 * [CL]p;$$

$$\frac{d[P97]m}{dt} = \left(v_{2L} * L * [P]p + v_{2A} + v_{2B} * \left(\frac{[CL]p^2}{(K_3)^2 + ([CL]p)^2} \right) \right) * \left(\frac{1}{1 + \left(\left(\frac{[P51]p}{K_4}\right)^2\right) + \left(\left(\frac{[EL]p}{K_5}\right)^2\right)} \right) - k_2 * [P97]m;$$

$$\frac{d[P97]p}{dt} = p_2 * [P97]m - (d_{2D} * D + d_{2L} * L) * [P97]p;$$

$$\frac{d[P51]m}{dt} = v_3 * \left(\frac{1}{1 + \left(\left(\frac{[CL]p}{K_6}\right)^2\right) + \left(\left(\frac{[P51]p}{K_7}\right)^2\right)} \right) - k_3 * [P51]m;$$

$$\frac{d[P51]p}{dt} = p_3 * [P51]m - (d_{3D} * D + d_{3L} * L) * [P51]p;$$

$$\frac{d[EL]m}{dt} = L * v_4 * \left(\frac{1}{1 + \left(\left(\frac{[CL]m}{K_8}\right)^2\right) + \left(\left(\frac{[P51]p}{K_9}\right)^2\right) + \left(\left(\frac{[EL]p}{K_{10}}\right)^2\right)} \right) - k_4 * [EL]m;$$

$$\frac{d[EL]p}{dt} = p_4 * [EL]m - (d_{4D} * D + d_{4L} * L) * [EL]p;$$

$$\frac{d[P]}{dt} = 0.3 * (1 - [P]) * D - [P] * L;$$

$$\frac{d[PIF]m}{dt} = v_5 * \left(\frac{1}{1 + \left(\left(\frac{[EL]p}{K_{11}}\right)^2\right)} \right) - k_5 * [PIF]m;$$

$$\frac{d[PIF]p}{dt} = p_5 * [PIF]m - (d_{5D} * D + d_{5L} * L) * [PIF]p;$$

Table 2.1: Parameter values from the De Caluwé clock model

Parameter description	Name	Value	Units
CL synthesis	v1	4.6	$nM.h^{-1}$
CL light-induced synthesis	v1L	3.0	$nM.h^{-1}$
P97 synthesis	v2A	1.3	$nM.h^{-1}$
P97 CL-induced synthesis	v2B	1.5	$nM.h^{-1}$
P97 light-induced synthesis	v2L	5.0	$nM.h^{-1}$
P51 synthesis	v3	1.0	$nM.h^{-1}$
EL synthesis	v4	1.5	$nM.h^{-1}$
CL mRNA degradation (light)	k1L	0.53	h^{-1}
CL mRNA degradation (dark)	k1D	0.21	h^{-1}
P97 mRNA degradation	k2	0.35	h^{-1}
P51 mRNA degradation	k3	0.56	h^{-1}
EL mRNA degradation	k4	0.57	h^{-1}
CL translation	p1	0.76	h^{-1}
CL light-induced translation	p1L	0.42	h^{-1}
P97 translation	p2	1.0	h^{-1}
P51 translation	p3	0.64	h^{-1}
EL translation	p4	1.0	h^{-1}
CL degradation	d1	0.68	h^{-1}
P97 degradation (dark)	d2D	0.50	h^{-1}

P97 degradation (light)	d2L	0.30	h^{-1}
P51 degradation (dark)	d3D	0.48	h^{-1}
P51 degradation (light)	d3L	0.78	h^{-1}
EL degradation (dark)	d4D	1.2	h^{-1}
EL degradation (light)	d4L	0.38	h^{-1}
Inhibition: CL by P97	K1	0.16	nM
Inhibition: CL by P51	K2	1.2	nM
Activation: P97 by CL	K3	0.24	nM
Inhibition: P97 by P51	K4	0.23	nM
Inhibition: P97 by EL	K5	0.30	nM
Inhibition: P51 by CL	K6	0.46	nM
Inhibition: P51 by itself	K7	2.0	nM
Inhibition: EL by CL	K8	0.36	nM
Inhibition: EL by P51	K9	1.9	nM
Inhibition: EL by EL	K10	1.9	nM
PIF synthesis	v5	0.10	nM.h h^{-1}
PIF mRNA degradation	k5	0.14	h^{-1}
PIF translation	p5	0.62	h^{-1}
PIF protein degradation (light)	d5L	4.0	h^{-1}
PIF protein degradation (dark)	d5D	0.52	h^{-1}
Inhibition: PIF by EL	K11	0.21	nM
Activation: growth by PIF	K12	0.56	nM

2.4 Results

2.4.1 *JAZ6* expression is rhythmic and regulated by the circadian clock

To first address the involvement of *JAZ6* with circadian variation in disease susceptibility, it was considered whether *JAZ6* is transcriptionally regulated by the circadian clock. While previous analysis has considered that *JAZ6* expression peaks at CT3 (Stoker, 2016). CT refers to the time in hours after the last subjective day according to the entrained circadian clock, while ZT refers to the time in hours since the last external signal of light or temperature. The circadian or light regulation aspects of *JAZ6* expression regulation are further explored here by interrogating publicly available datasets for *JAZ6* expression data from temperature entrainment and circadian clock gene and light sensing mutants *lux-2* and *phyb-9* (Bläsing *et al.*, 2005; Mockler *et al.*, 2007; Covington *et al.*, 2008).

As seen previously (Stoker, 2016), there is a significant peak ($p<0.05$) of CT3 for *JAZ6* expression in free-running conditions in these publicly available datasets of gene expression (Figure 2.4.1). This data also shows that the rhythmic pattern of *JAZ6* expression can be entrained by either photoperiodic light cycling (Figure 2.4.1A), or temperature cycling in constant light (Figure 2.4.1B), which implies that *JAZ6* transcription is controlled by the circadian clock as opposed to other light or temperature sensing mechanisms. The significant peak ($p<0.05$) at ZT3 for rhythmic expression is also seen outside of free-running conditions in seedlings in 12-hour photoperiods (Figure 2.4.2A). *lux-2* clock gene mutants in the same conditions display different patterns of gene expression, with an oscillating expression profile significantly peaking ($p<0.05$) at ZT12 (Figure 2.4.2B). On one hand this further supports *JAZ6* transcriptional regulation by the circadian clock, as the mutation of the core circadian clock gene *LUX* altered the pattern of *JAZ6* expression. However, expression is still rhythmic despite the lack of rhythm in circadian outputs in the *lux-2* mutant (Hazen *et al.*, 2005), so it also highlights that JAZ6 expression is under the influence of environmental factors such as light and temperature. This result also does not distinguish direct from indirect transcriptional regulation, as many other transcription factors are altered in circadian clock gene mutants which could be regulating JAZ6 expression in knock-on effects (Harmer *et al.*, 2000).

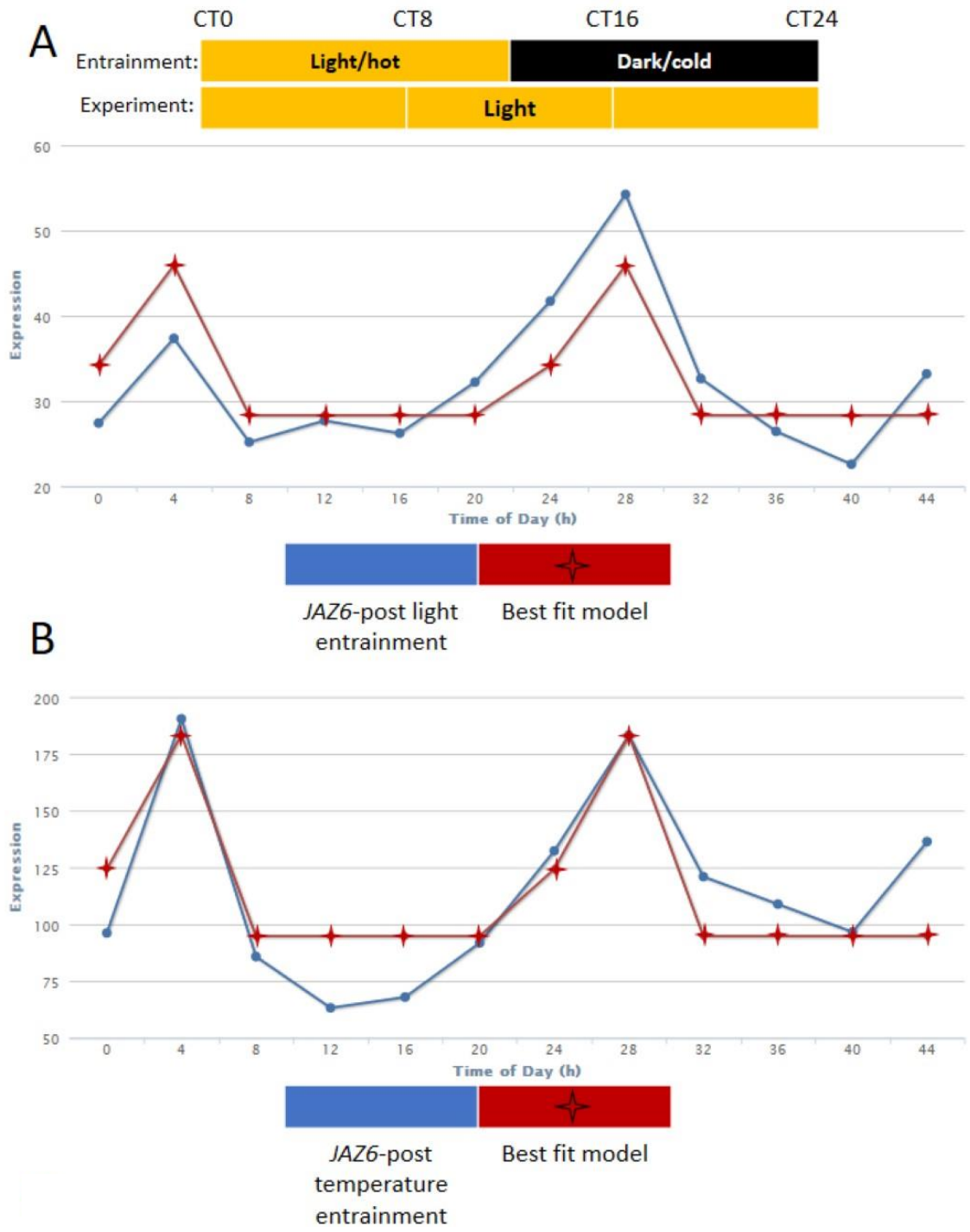


Figure 2.4.1: JAZ6 expression is rhythmic in free-running expression experiments.

JAZ6 expression is rhythmic with a peak at ZT3 in free-running conditions, light entrained (A, 12:12h light:dark), and temperature entrained (B, 22:12°C hot:cold). For this public dataset, gene expression data is from seedlings grown in Petri dishes for 9 days under the free-running and entrainment conditions described. Circadian rhythmicity and pattern matching determined using DIRURNAL (mocklerlab.org/tools).

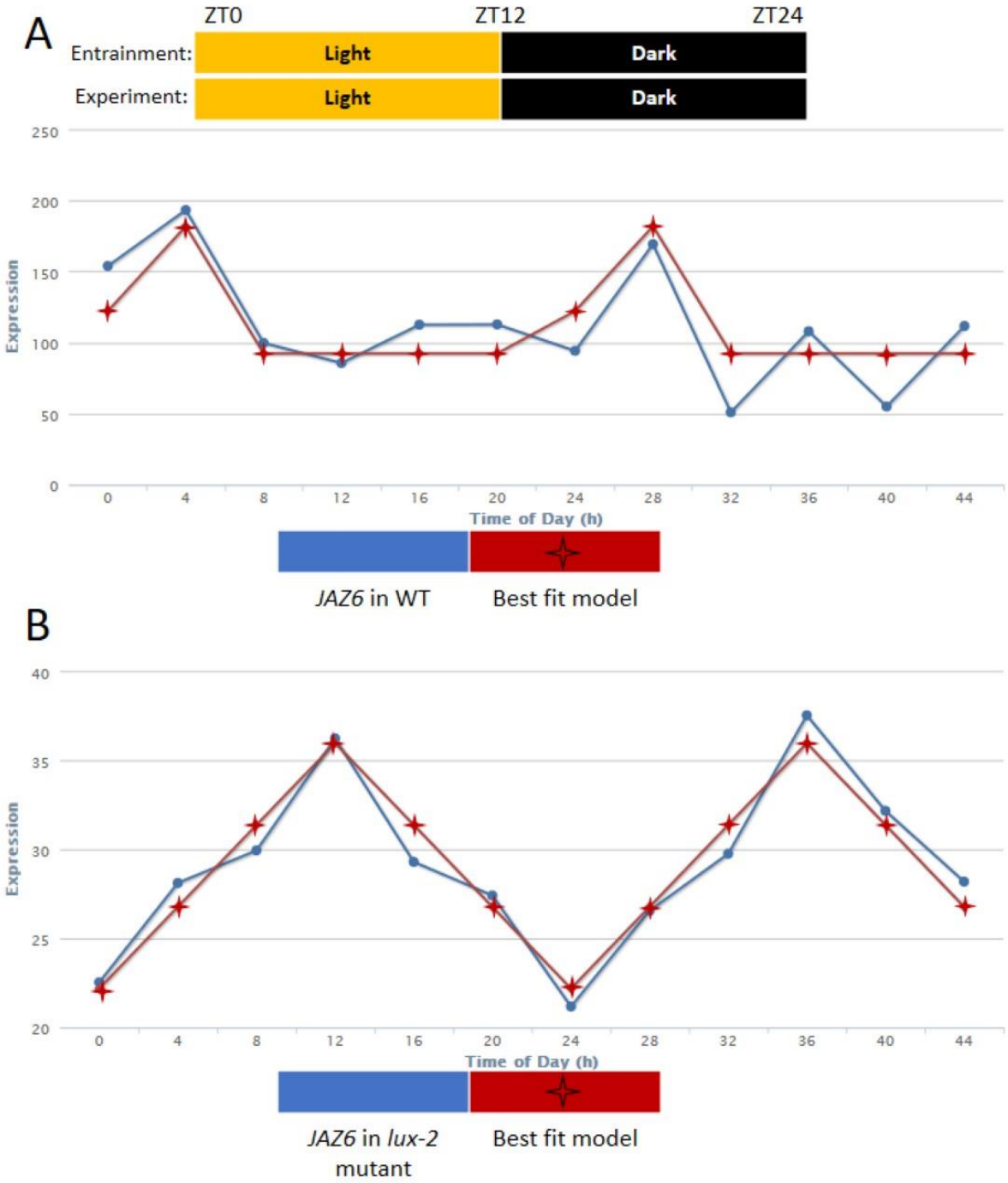


Figure 2.4.2: JAZ6 expression rhythmic patterns are affected by clock gene mutants.
JAZ6 expression is rhythmic with a peak at ZT3 in constant temperature (A), and CT12 in *lux-2* mutants (B). For this public dataset, gene expression data is from 35 day old plants grown on soil under the light conditions described. Circadian rhythmicity and pattern matching determined using DIRURNAL (mocklerlab.org/tools).

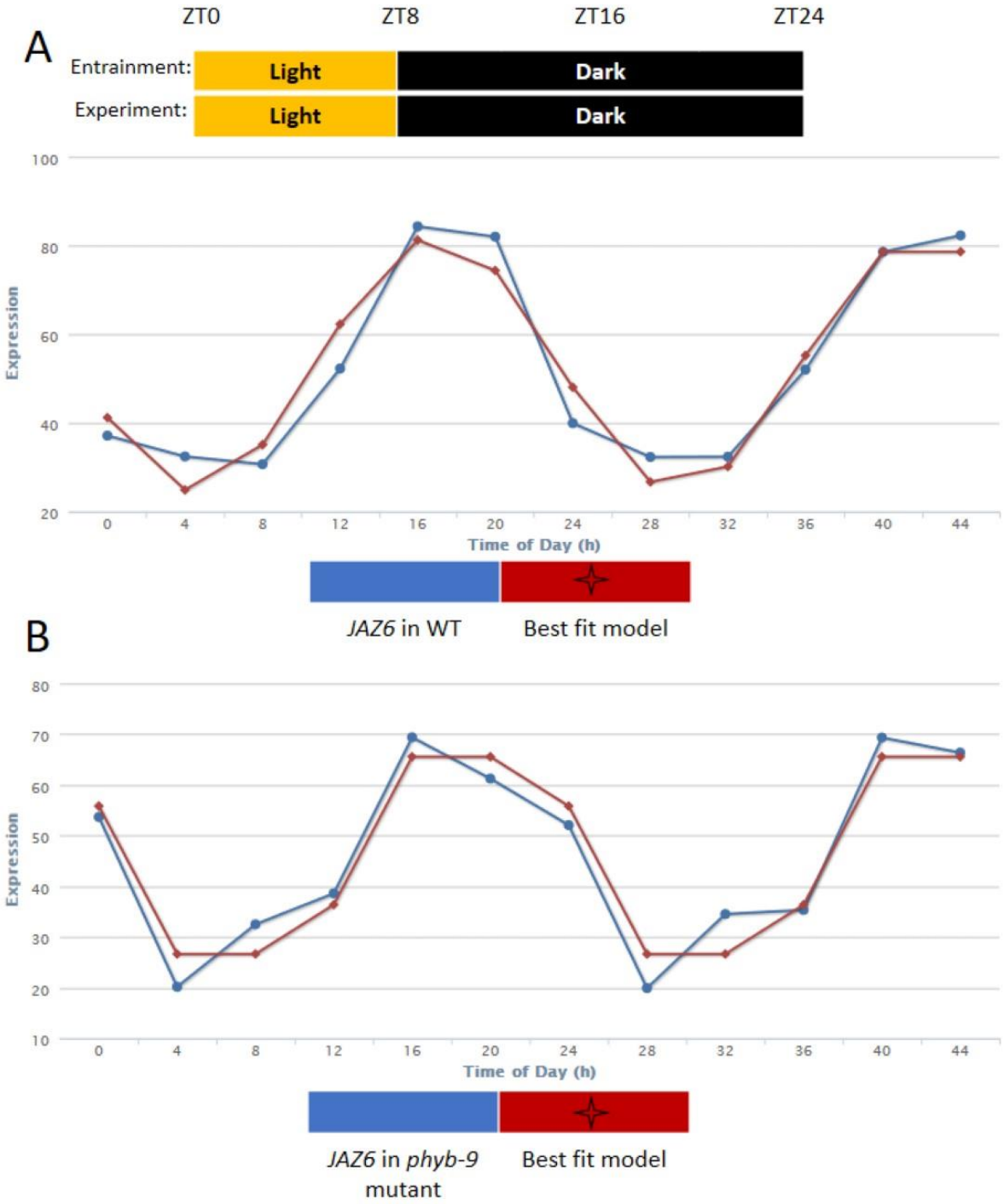
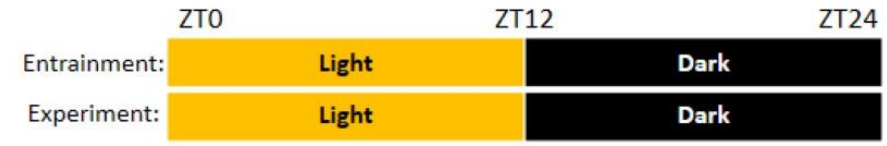


Figure 2.4.3: JAZ6 expression is rhythmic in short day conditions, and only mildly affected by light sensing through PHYB.

JAZ6 expression is rhythmic with a peak at ZT17 in short day conditions (A), and ZT19 in *phyb-9* mutants (B). For this public dataset, gene expression data is from seedlings grown in Petri dishes for 7 days under short day conditions. Circadian rhythmicity and pattern matching determined using DIRURNAL (mocklerlab.org/tools).

Plants in short day conditions display a significantly different rhythmic *JAZ6* expression pattern (Figure 2.4.3), which peaks at ZT17 (Figure 2.4.3A). In *phyb-9* mutants, the rhythmic JAZ6 expression pattern is very similar but peaks slightly later at ZT19 (Figure 2.4.3B). This implies a slight effect on JAZ6 transcription through light sensing, which could occur indirectly through the light-dependent activity of core circadian clock transcription factors (Tsumoto *et al.*, 2011).

The expression of *JAZ6* can also be seen to cycle at the translatome level using CAST-R (Bonnot, Gillard and Nagel, 2022), showing RNA available to be translated which is associated with ribosomes. Here we see that JAZ6 significantly ($p<0.05$) peaks with a phase of ZT1.5 (Figure 2.4.4), like previous free-running data (Figure 2.4.1), but also with a second peak at the beginning of subjective night. This indicates that *JAZ6* expression remains rhythmic as it is translated. The two peak nature of JAZ6 diurnal expression has previously been reported under long day growth conditions (Michael *et al.*, 2008), suggesting this is a facet of JA6 transcriptional regulation dependent on the photoperiod or other external environmental conditions.



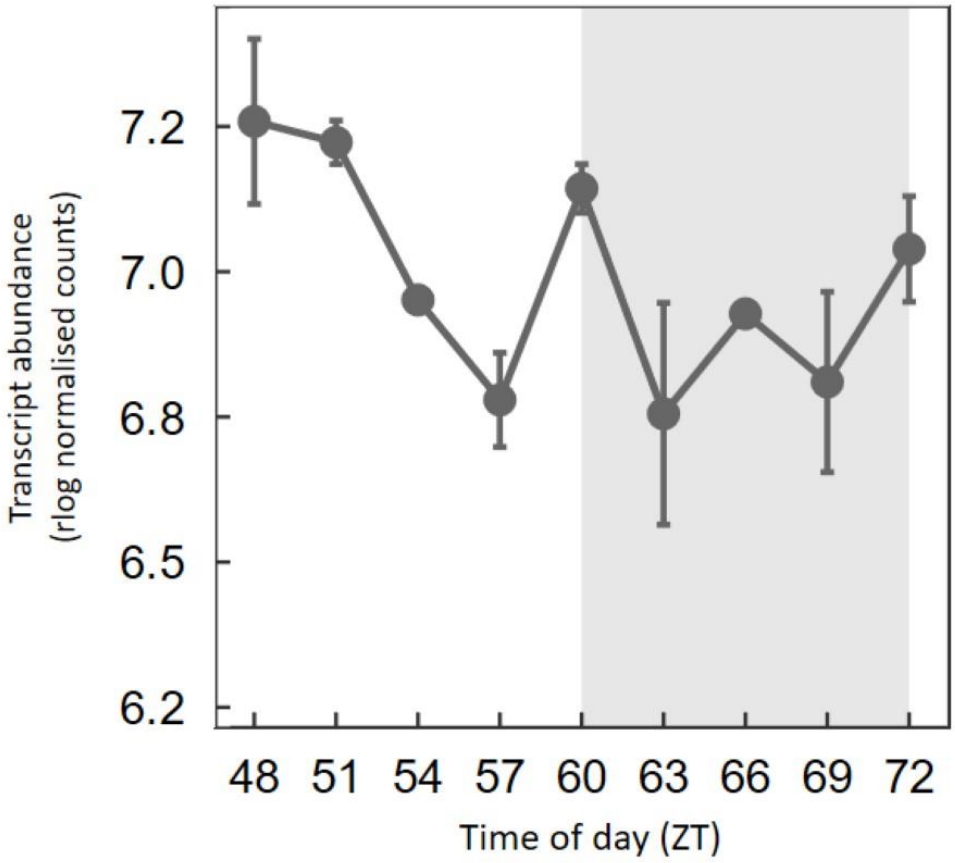


Figure 2.4.4: JAZ6 expression is rhythmic at the translatome level.

JAZ6 transcript abundance over time associated with ribosomes, data from Bonnot and Nagel (2021). Data are means +/- standard deviation for n = 3 biological replicates.

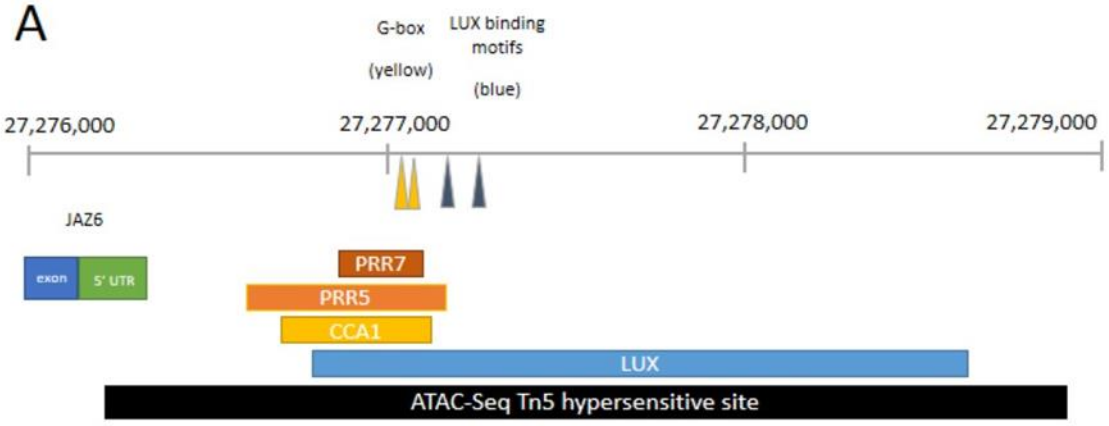
2.4.2 *JAZ6* is transcriptionally repressed by the circadian clock gene *LUX*

The circadian clock is a system of interlocking loops of repression by transcription factors (Harmer, 2009b). The daily oscillations in expression which are exhibited by these transcription factors control physiological processes through regulating gene expression. As the mutants for key genes in the circadian clock timekeeping mechanism exhibit the same circadian susceptibility phenotype as the *jaz6-3* mutant (Ingle *et al.*, 2015), modelling a network which incorporates both the clock mechanism and *JAZ6* could aid in understanding how *JAZ6* is regulated.

The first step Is to identify possible mechanisms by which *JAZ6* might be transcriptionally regulated by the circadian clock. A literature search was conducted to identify clock transcription factors binding DNA upstream of the transcriptional start site for *JAZ6.* The clock transcription factors PRR5, PRR9, PIF4, PIF5, LUX, CCA1, and LHY can all bind within 1kb upstream of *JAZ6* (Pfeiffer *et al.*, 2014; Kamioka *et al.*, 2016; T. L. Liu *et al.*, 2016; Ezer *et al.*, 2017; Adams *et al.*, 2018) (Figure 2.4.5). These ChIP-seq binding peaks appear within an area of open chromatin upstream of *JAZ6* determined by ATAC-Seq (Dr Richard Hickman, personal communication), which is accessible for transcription factor binding.

Where ChIP-seq peak width is known, peaks include the double palindromic G-box (CACGTG) regulatory element region -720 bp upstream of the JAZ6 transcriptional start site (Figure 2.4.5A). The G-box motif is enriched at LHY, CCA1, LUX, PRR, and PIF binding sites (Pfeiffer *et al.*, 2014; Kamioka *et al.*, 2016; T. L. Liu *et al.*, 2016; Ezer *et al.*, 2017; Adams *et al.*, 2018). Therefore, the G-box represents a candidate binding site of LHY, CCA1, LUX, PRRs, and PIFs in the *JAZ6* promoter. The G-box motifs are also the site of MYC2 and the MED25 subunit of the Mediator transcriptional co-activator complex binding to the *JAZ6* promoter (Wang *et al.*, 2019), which is known to transcriptionally promote *JAZ6*. As such, there may be competition for binding at the G-box motifs between circadian transcription factors and MYC2. The LUX ChIP-seq peak also covers the LUX binding DNA sequences ATATCG and the near-palindromic sequence AAGAGTCTT (Figure 2.4.5A), experimentally determined LUX DNA binding motifs (Silva *et al.*, 2020).

A



B

Clock gene short name	Clock gene ID	Binding peak start in JAZ6 promoter	Binding peak end in JAZ6 promoter	Binding peak summit to TSS	Data source
PRR5	*AT5G24470*	27276600	27277154	621	Liu et al. 2016
CCA1	*AT2G46830*	27276700	27277121	594	Kamioka et al. 2016
LHY	*AT1G01060*	N/A	N/A	624	Adams et al. 2018
PRR7	*AT5G02810*	27276864	27277088	731	Liu et al. 2016
LUX	*AT3G46640*	27276792	27278617	N/A	Ezer et al. 2017
PIF1	*AT2G20180*	27276986	27276987	669	Pfeiffer et al. 2014
PIF4	*AT2G43010*	27276955	27276956	638	Pfeiffer et al. 2014
PIF5	*AT3G59060*	27277044	27277045	727	Pfeiffer et al. 2014

Figure 2.4.5: *JAZ6* promoter region contains G-box elements and LUX binding motifs, bound by key circadian clock genes.

(A) Circadian clock regulatory elements are present in the *JAZ6* promoter. Yellow arrows indicate the location of the two palindromic G-box (GTGCAC) motifs, and blue arrows indicate the location of two LUX binding motifs (ATATCG and AAGAGTCTT). Ruler represents location on Arabidopsis chromosome 1. Brown PRR7 ChIP peak, orange PRR5 ChIP peak, yellow CCA1 ChIP peak, blue LUX ChIP peak.

(B) List of Circadian clock transcription factors which bind the JAZ6 promoter, and locations of binding peaks from the transcriptional start site.

However, while these circadian transcription factors can bind upstream of *JAZ6*, they may not regulate *JAZ6* expression directly in the native context. Therefore, in order to predict which of these transcription factors could regulate *JAZ6* in a circadian manner in uninfected plants, the network inference algorithms Causal Structure Inference (CSI) (Penfold *et al.*, 2015) and TRS (Minas *et al.*, 2017) were run on time-series gene expression data for JAZ6 as a target of the clock genes binding upstream of JAZ6 as detailed above (Figure 2.4.5). For CSI, time-series gene expression data was an RNA-seq time series dataset from mock inoculated detached Arabidopsis leaves, with samples taken every 2 hours for 48 hours (Windram *et al.*, 2012). Using this dataset CSI predicted the most likely regulator(s) using patterns in gene expression (Figure 2.4.6), proposing LUX as the most likely single regulator of JAZ6 with a combined edge weight over 0.4 and a probability of 0.41. For multiple regulators, PIF5 is the most likely to co-regulate *JAZ6* with LUX, with a probability of 0.36 and combined edge weight over 0.3. PIF1, CCA1, and LHY are then the next most likely co-regulators of JAZ6 with combined edge weights over 0.2 and probabilities of 0.28, 0.26 and 0.27, respectively.

The output of CSI broadly concurs with the output of TRS where JAZ6 was set as a target of all other genes, in contrast to CSI where all genes are potential regulators and targets. Also in contrast, TRS used input from time series sampling of plants in free-running or short day conditions for 48 hours (Mockler *et al.*, 2007). TRS predicted *JAZ6* was the target of regulation by LUX and PIF1 with probabilities over 0.8 (Figure 2.4.7A), and that there were most likely two regulators (Figure 2.4.7B).

In summary, the clock gene proteins LUX, PIFs, and CCA1/LHY were proposed as likely *JAZ6* transcriptional regulators by CSI and TRS independently using different sets of gene expression data. This investigation covers the circadian regulation of JAZ6 in uninfected leaves, under different conditions such as biotic stress JAZ6 would be expected to be under the control of other stress-responsive genes, particularly MYC2.

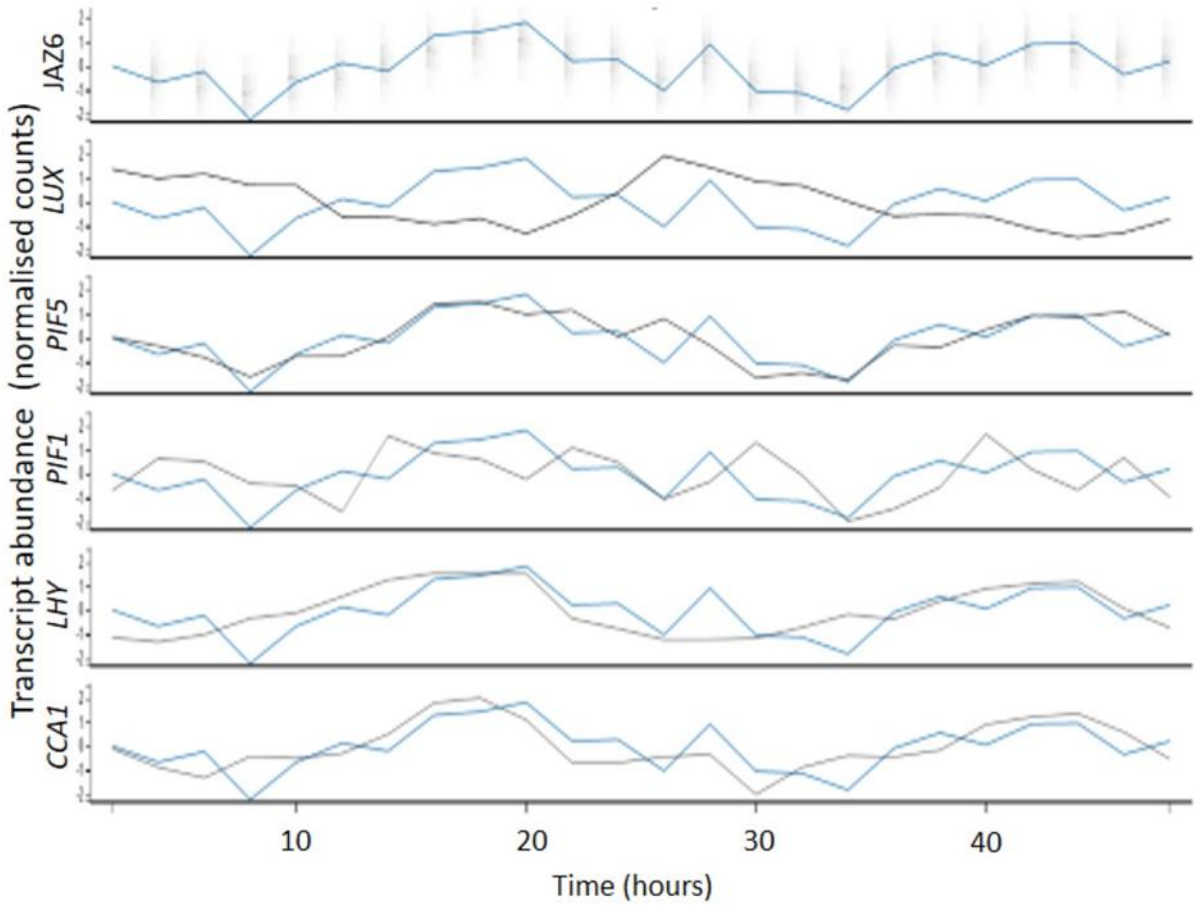


Figure 2.4.6: *JAZ6* is predicted to be regulated by LUX, PIFs, and LHY/CCA1 by the Causal Structure Inference (CSI) algorithm.

Time series expression of *JAZ6* (blue) overlaid with *LUX, PIF5, PIF1, LHY*, and *CCA1* (black), ordered by the probability the transcription factor regulates *JAZ6* expression, by CSI. Expression data from mock treated plants parallel to a *B. cinerea* assay used (Windram et al, 2012).

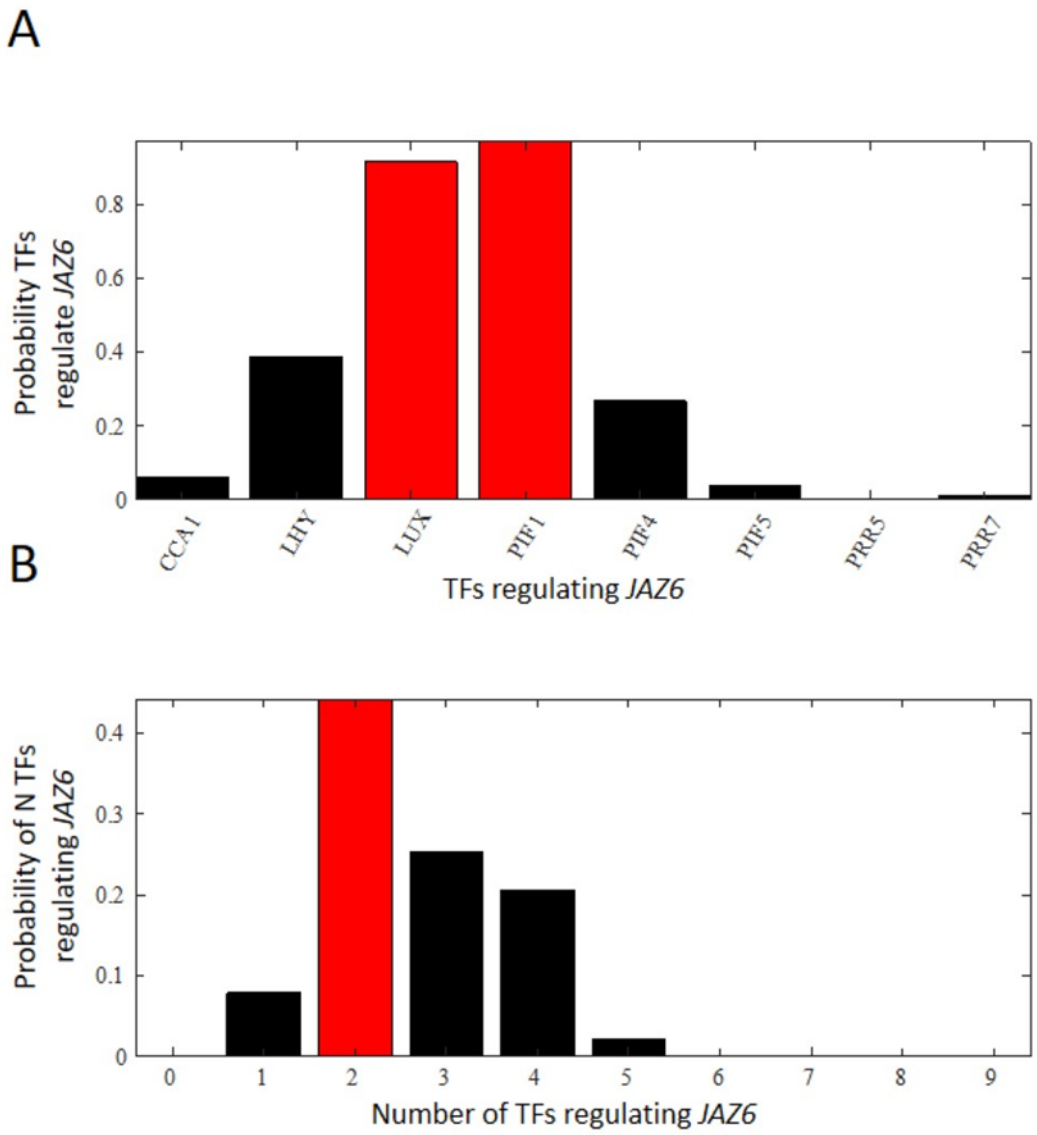


Figure 2.4.7: *JAZ6* is predicted to be regulated by LUX and PIF1 by the Transcriptional Regulation Switch algorithm.

(A) The probability circadian clock genes which can bind the JAZ6 promoter are regulating JAZ6 expression, by TRS. Expression data from free-running and short-day conditions used (Mockler et al 2007).

(B) The number of circadian clock genes likely to regulate *JAZ6* expression, by TRS.

Balancing the need to simulate realistic clock outputs with keeping the mathematical system as simple as possible, the De Caluwé 2016 model was used (De Caluwé *et al.*, 2016). In contrast to the predictive network inference algorithms which predict regulators to fit known expression data, the clock model can predict gene expression as the result of multiple different models of regulation. The De Caluwé model was used as it is able to predict and recapitulate many characteristics of the real Arabidopsis circadian clock, including temperature compensation (Avello *et al.*, 2019). This model includes components corresponding to combinations of clock oscillator genes: *CCA1/LHY, TOC1/PRR5, PRR9/PRR7, ELF3/ELF4/LUX,* and *PIFs*.

JAZ6 expression under the regulation of combinations of LUX, PIFs, and CCA1/LHY were simulated as an extension of the De Caluwé clock model in Matlab (De Caluwé *et al.*, 2016), by adding JAZ6 as a 'node' in the model (Figure 2.4.8). This practically means adding additional equations to the model, which use the existing parameter values for the clock genes and how they regulate each other, to extend such predictions to their regulation of *JAZ6*. Several different models were constructed and run to predict JAZ6 regulation by the circadian clock, each encoding *JAZ6* regulation by different combination of the circadian clock transcription factors *LUX, PIFs,* and *CCA1/LHY* (Figure 2.4.8). These specific clock genes were chosen as potential regulators of JAZ6 because of their demonstrated ability to bind upsteam of *JAZ6*, combined with the predictions from CSI and TRS that such clock genes exhibit gene expression patterns consistent with regulating *JAZ6*. For the regulation of *JAZ6*, parameters were reused from existing regulatory relationships elsewhere in the model, in the absence of kinematic data. *JAZ6* expression (regulated negatively by LUX alone) results in predicted peaks of expression at ZT3 (Figure 2.4.9), which is closest to the real patterns of *JAZ6* expression in both 12-hour photoperiod and free-running conditions (Figures 2.4.1 & 2).

If direct transcriptional regulation of JAZ6 by LUX was correct, differential expression of *JAZ6* would be expected in *LUX* mutants. This has been reported, with upregulation of *JAZ6* expression in *lux-6* mutants compared to WT (Y. Zhang *et al.*, 2018). This may have changed the circadian pattern of expression of JAZ6 as seen for the *lux-2* mutant (Figure 2.4.2), but this was not analysed. Additionally, no differential expression of *JAZ6* was seen in *PIF* or *LHY*

mutant plants compared to WT (Adams *et al.*, 2018; Martín *et al.*, 2018). Therefore, gene expression data from circadian clock mutants further supports the model of *JAZ6* transcriptional repression by *LUX* alone. While evidence of *JAZ5* direct regulation by LUX has been demonstrated by ChIP-qPCR *JAZ6* was not tested (Chong Zhang *et al.*, 2019), and attempts at yeast-1-hybrid to test LUX binding to the JAZ6 promoter by Dr Fabian Vaistij were unsuccessful. The pattern of JAZ6 expression was also noted to change in the *lux-2* mutant as shown previously (Figure 2.4.1), though it was still oscillating diurnally. These oscillations could reflect additional transcriptional regulation of JAZ6 transcription by light or temperature, because *lux-2* mutants exhibit severely disrupted clock outputs (Hazen *et al.*, 2005).

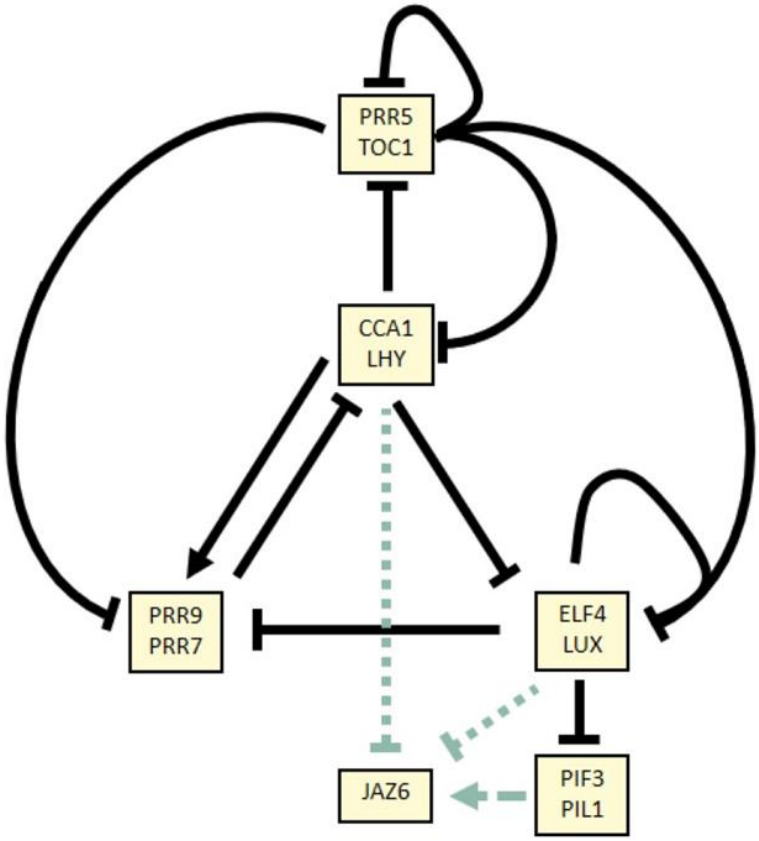


Figure 2.4.8: Schematic diagram illustrating the main features of the circadian clock model (De Caluwé et al., 2016), with added modes of JAZ6 transcriptional repression.

In the model (De Caluwé et al., 2016), similar genes are merged into the single variables, representing the pairs of genes *PRR5/TOC1*, *CCA1/LHY, PRR9/PRR7, ELF4/LUX*, and *PIF3/PIL1* respectively. Edges in black represent transcriptional repression or activation of the target by the source, as in the model (De Caluwé et al., 2016). Hashed green edges were added to represent the multiple possible forms of transcriptional repression and activation of *JAZ6* by LHY, CCA1, LUX, and PIF transcription factors in the model (De Caluwé et al., 2016).

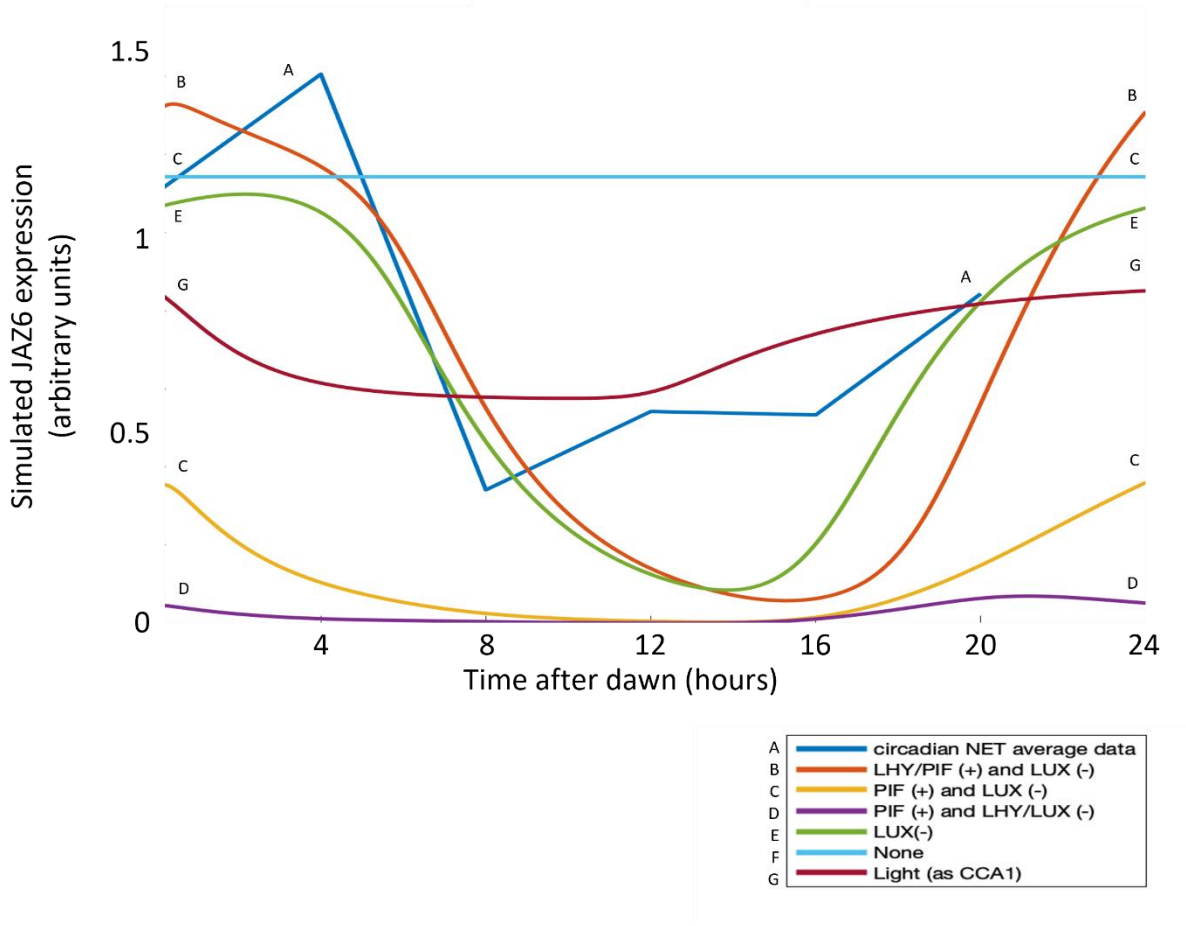


Figure 2.4.9: *JAZ6* expression most closely matches negative transcriptional regulation by LUX.

Average of JAZ6 expression across multiple circadian timeseries (dark blue), with multiple simulations of JAZ6 expression under circadian regulation made in Matlab with a circadian clock model (De Caluwé et al., 2016). Simulations include: Activation by LHY and PIFs (+) and repression by LUX (-) (orange); activation by PIFs (+) and repression by LUX (-) (yellow); activation by PIFs (+) and repression by LHY and LUX (-) (purple), repression by LUX (-) alone (green), no circadian regulation (turquoise), and regulation by light like CCA1 (red). Repression by LUX alone best matches the peak of JAZ6 expression.

2.4.3 JAZ6 is predicted to be post-translationally regulated by diurnal fluctuations in jasmonic acid

Post-translational regulation of JAZ proteins through targeted degradation is key to relieve repression of transcription by JAZ proteins (Chini *et al.*, 2007; Sheard *et al.*, 2010). The targeted degradation of JAZ6 proteins is dependent on the concentration of the bioactive form of the phytohormone jasmonic acid (JA), jasmonic acid isoleucine. The level of jasmonates varies across the day diurnally (Goodspeed *et al.*, 2012). Stress in the form of wounding, infection, or altered photoperiods stimulate jasmonic acid accumulation in the plant and consequently degrade JAZ proteins (Chung *et al.*, 2008; Campos, Kang and Howe, 2014; Nitschke *et al.*, 2016), though the level of JA induction by these stresses has not been directly compared to diurnal variation in JA levels. While recent work has assessed circadian control of protein rhythms (Krahmer *et al.*, 2021), none of the JAZ proteins were quantified in this analysis. Hence, modelling JA-dependent degradation of JAZ6 allows the evaluation of how diurnal patterns of gene expression might translate into diurnal protein rhythms.

Assuming that the diurnal fluctuations of JA in Arabidopsis are sufficient to degrade JAZ6, this effect was modelled. In order to model JAZ6 degradation by JA, JA accumulation through the day was fitted to a mathematical equation using experimental data from (Goodspeed *et al.*, 2012). Initially diurnal oscillations in JA were fitted to simple trigonometric equations with periods of 24 hours (Figure 2.4.10A), which take the form:

$$y = a\sin(\pi t/12 + b) + c$$

Where *t* is time in hours, *y* is chemical concentration, and *a, b*, and *c* are parameters specific to each equation. However, despite a high adjusted R-square value for the fit (0.8725), jasmonic acid values increase faster than they decrease in the experimental data, indicating a more complex relationship between time of day and JA. Therefore, a more complex Fourier series was fit to the data (Figure 2.4.10B), resulting in a higher R-square value (0.9402) and a better fit by eye. The equation for the two term Fourier series is:

$$y = y0 + a \times \cos(t \times w) + b \times \sin(t \times w) + c \times \cos(t \times w) + d \times \sin(t \times w)$$

Where *t* is time in hours, *y* is chemical concentration or susceptibility, and y0, *a, b, c, d* and *w* are parameters specific to each equation.

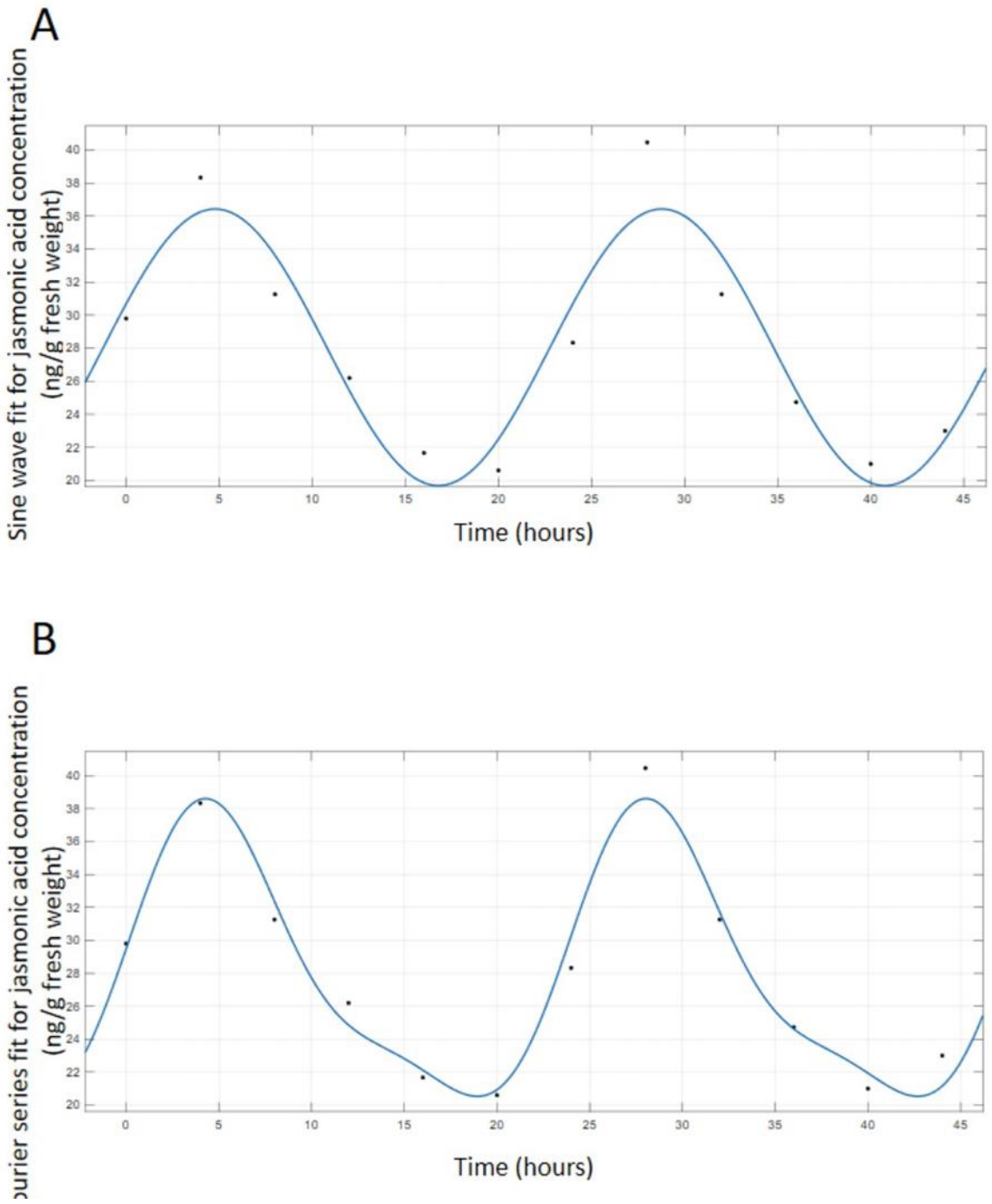


Figure 2.4.10: Jasmonic acid oscillates diurnally over the day.

Defence hormone jasmonic acid concentration fitted to sine wave oscillations with 24 hour periodicity. Data from (Goodspeed et al., 2012). (A) JA fits a sine curve (JA=8.372*sin((pi/12)*Time+b)+c) with an adjusted R-square of 0.8725. (B) jasmonic acid fits a two-term Fourier fit (JA= 28.1+ 2.238*cos(Time*0.2646)+8.119*sin(Time*0.2646)-0.8815*cos(Time*0.2646)+2.14*sin(Time*0.2646) with an adjusted R-square of 0.9402.

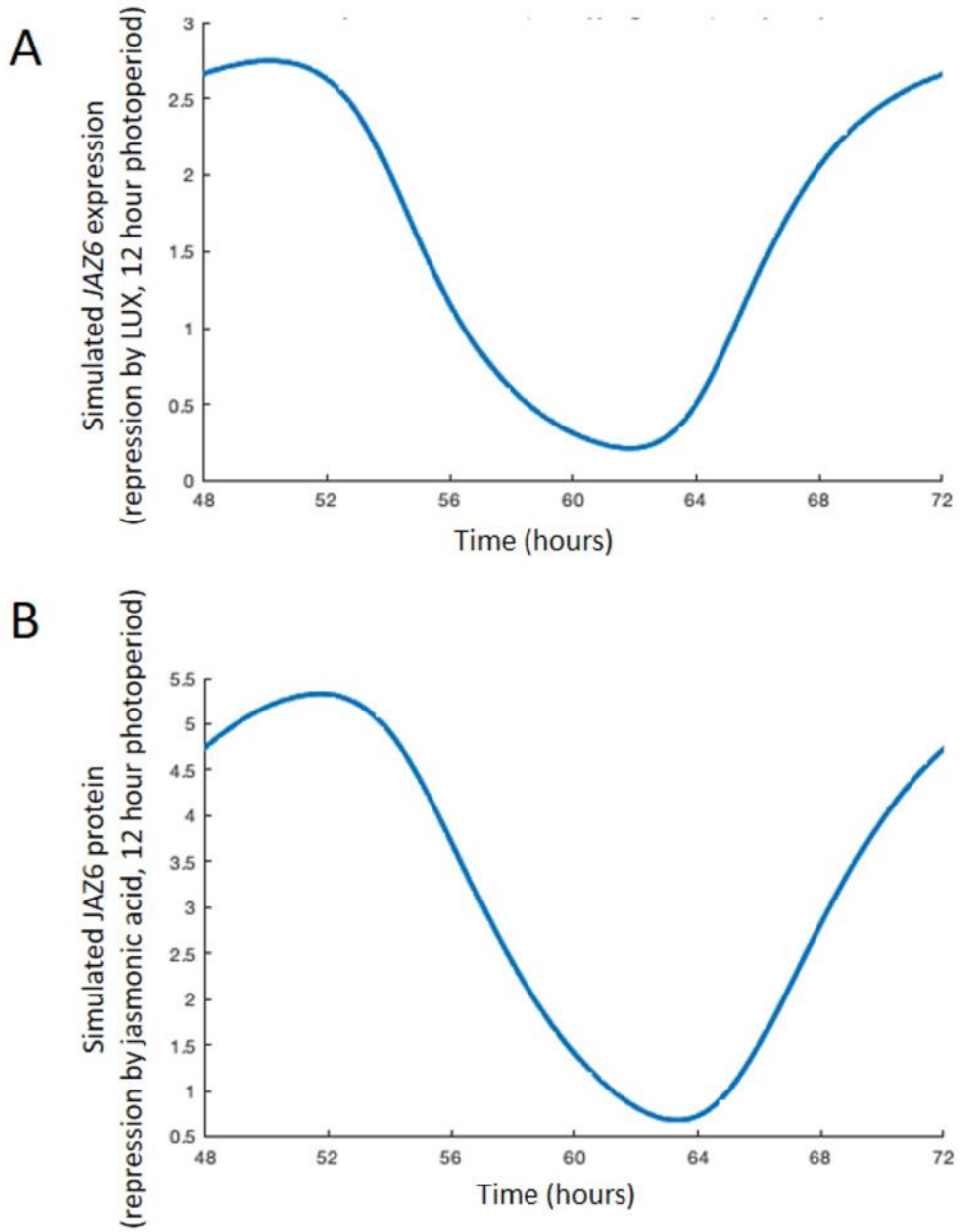


Figure 2.4.11: Diurnal variation in jasmonates might not influence JAZ6 protein levels

(A) *JAZ6* gene expression when negatively regulated transcriptionally by LUX.

(B) JAZ6 protein levels over time when negatively regulated by jasmonates. Data covers 24 hours with a 12/12 hour light/dark photoperiod.

Both transcriptional inhibition by LUX and the promotion of degradation by jasmonic acid were incorporated into a model for predicting JAZ6 protein levels over time (Figure 2.4.11). With estimated values of JAZ6 production and degradation rates, the pattern of JAZ6 protein abundance appears like *JAZ6* mRNA levels, suggesting diurnal variation in jasmonic acid does not drive diurnal activity of JAZ6. However, JAZ6 degradation by native plant levels of jasmonic acid is yet to be determined experimentally. More refined parameter values, from *in planta* JAZ6 tagged protein accumulation experiments in *N. benthamiana*, and JAZ6 protein level analysis with a specific antibody could improve this prediction.

2.4.4 JAZ6 binds the epigenetic regulator PFP in the nucleus *in planta*

JAZ6 is a transcriptional co-repressor which regulates gene expression by binding to other proteins to inhibit them from activating gene expression (Zander, 2021). As such, control of diurnal variation in susceptibility to infection is likely dependent on the transcription factors which bind to JAZ6. Previous work on JAZ6 by Dr Claire Stoker established 7 putative transcription factors which bound to JAZ6 (Stoker, 2016): ETHYLENE INSENSITIVE 3 (EIN3, AT3G20770), FAR RED HYPOCOTYL 3 (FHY3, AT3G22170), PHD DOMAIN CONTAINING PROTEIN (PFP, AT4G23860), WUSCHEL RELATED HOMEOBOX 12 (WOX12, AT5G17810), NAC DOMAIN CONTAINING PROTEIN 10 (NAC10, AT1G28470), ZINC FINGER PROTEIN 1 (ZF1, AT5G67450) and a MYB like protein (AT5G56840). These were found by yeast-2-hybrid screening, with a library of putative plant transcription factors (Pruneda-Paz *et al.*, 2014) screened against JAZ6, JAZ5, and a pool of other JAZs. These seven putative transcription factors were shown to bind to JAZ6 but not to the most similar protein to JAZ6, JAZ5 (Stoker, 2016). Determining if these interactions occur *in planta* is a vital initial step in assessing what transcription factors JAZ6 binds to and represses.

While attempts were made to use publicly available alpha fold models for protein structure to predict how the above putative transcription factors may bind to JAZ6 (Jumper *et al.*, 2021), the protein structure for JAZ6 is of very low confidence outside of short regions largely corresponding to the Jas and ZIM domains, making it unsuitable for use in binding prediction modelling.

Interactions were tested *in planta* using a split YFP BiFC assay. This results in eYFP fluorescence in *N. benthamiana* leaves if the two proteins cloned into vectors with the N- and C-terminal halves of YFP interact with each other (Figure 2.4.12A). There are 4 pBIFP vectors (Azimzadeh *et al.*, 2008), with different halves of YFP at the beginning or end of the protein of interest, for a total of 8 possible combinations for each JAZ6-interactor binding pair (Figure 2.4.12B). This is useful as steric hindrance from fluorescent tags will prevent protein-protein binding in certain orientations (Horstman *et al.*, 2014), so protein-protein binding will typically only be seen for certain configurations or pairs of pBIFP vectors.

Each of the pBIFP vectors for JAZ6 and the seven putative interactors were generated using Gateway cloning. All four pBIFP constructs were made for this initial screening, for JAZ6 and each of the seven interactors. This was as all four were required to test all 8 possible combinations for each JAZ6-interactor binding pair (Figure 2.4.12B), as steric hindrance could result in false negative results for some orientations of YFP bound to proteins (Horstman *et al.*, 2014). All pBIFP constructs were verified by PCR amplifying with one vector-specific and one gene-specific primer, to check content and direction of insertion, and then sequenced.

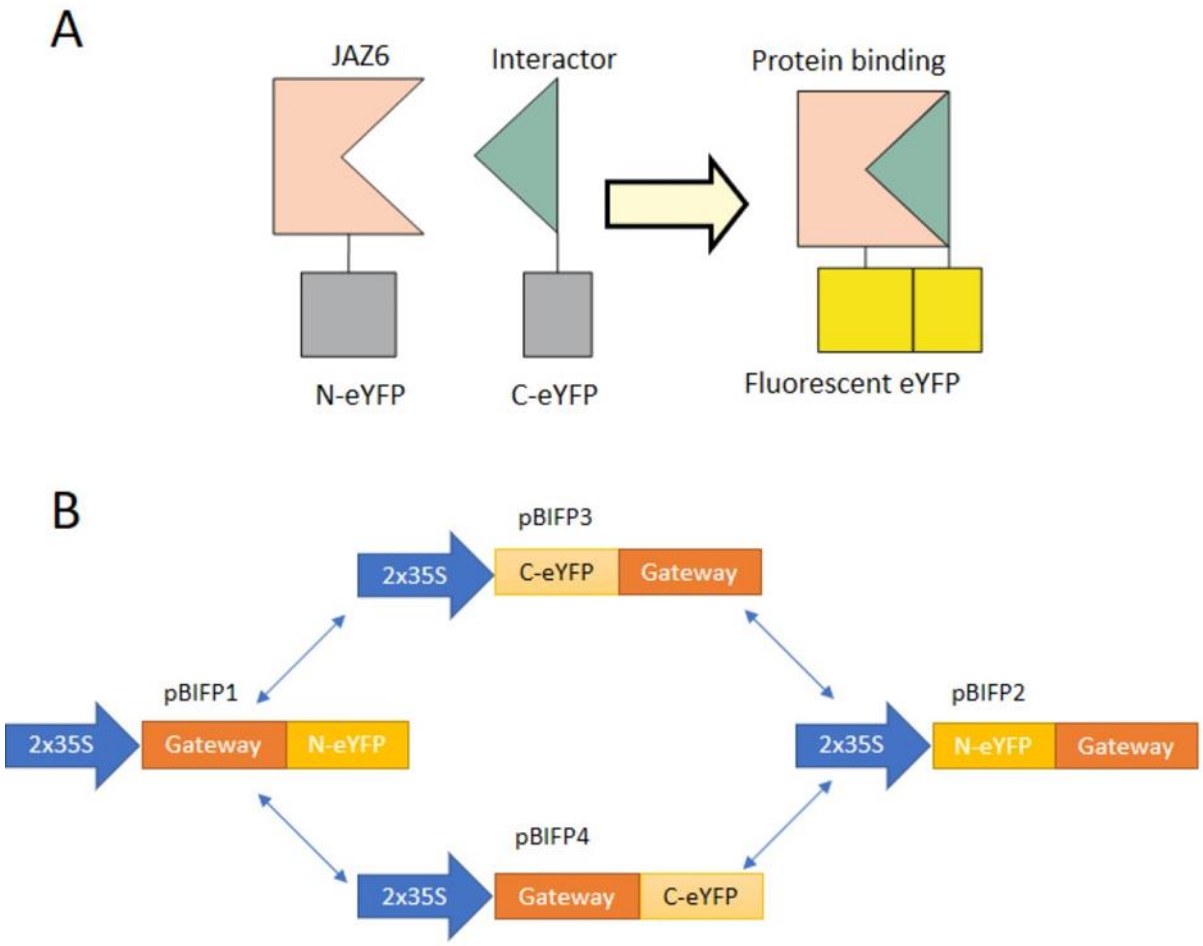


Figure 2.4.12: Schema for BiFC protein-protein binding testing *in planta*, using the pBIFP vectors expressing N and C fragments of YFP.

(A) BiFC schema with N-eYFP and C-eYFP resulting in fluorescent eYFP, if the proteins being tested bind.

(B) pBIFP Gateway-cloning compatible bimolecular fluorescence complementation assay vectors. All 7 potential interactors and JAZ6 were cloned into the Gateway part of all 4 vectors. Arrows indicate possible pairwise combinations for fluorescence to occur when the two proteins interact.

Each pair of pBIFP vector combinations were *Agrobacterium*-infiltrated with the silencing suppressor P19 at equal concentrations (Lombardi *et al.*, 2009), into three week old *N. benthamiana* leaves. All possible pBIFP vector combinations were tested for interaction of JAZ6 with the seven candidate transcription factors (Table 2.4.1), with at least 3 repeats in independent experiments. As initial negative controls, JAZ6 was infiltrated alone with N or C-terminal halves of YFP, showing they do not fluoresce alone (Figure 2.4.13A & B). Then, as a positive control, JAZ6-JAZ6 homodimerization was also assessed as yeast-2-hybrid experiments suggest it can homodimerize mediated by its TIFY / JAZ domain (Vanholme *et al.*, 2007; Pérez *et al.*, 2014). As expected, yellow fluorescence in the nuclei was observed (Figure 2.4.13C). This matches the known localisation of JAZ proteins to the nucleus due to MYC2 binding (Withers *et al.*, 2012).

Regarding the seven putative transcription factors previously found to interact in the Y2H system; only the JAZ6 PFP interaction (in three combinations) was confirmed in the pBIFP system (Table 2.4.1), with yellow fluorescence observed in the nuclei (Figure 2.4.13E & F). PFP also appeared to homodimerize, which has not been previously reported and the functional implications are yet unclear.

Since it has been shown previously with BiFC (Liu *et al.*, 2017), the FHY3-EIN3 interaction was also used as a positive pBIFP system control and, as expected, yellow fluorescence in the nuclei was observed (Table 2.4.2, Figure 2.4.14). Though tests with JAZ6 and FHY3 did not show an interaction (Table 2.4.1, Figure 2.4.14), literature produced since the time of testing has shown JAZ6 and FHY3 binding with BiFC testing (Y. Liu *et al.*, 2019).

As JAZ6 and FHY3 had been shown to bind (Y. Liu *et al.*, 2019), FHY3 and PFP binding testing was performed to investigate if they could be present in the same protein complexes. Unexpectedly, FHY3 and PFP did appear to interact (Table 2.4.2, Figure 2.4.14), with a fluorescent signal detected in multiple orientations.

Table 2.4.1: Pairwise bimolecular fluorescence binding pairs tested with JAZ6 in BiFC. JAZ6 homodimerizing (pBIFP2 JAZ6 + pBIFP3 JAZ6) was observed in N. *benthamiana*. Of the heterodimers tested, only JAZ6 and PFP were observed to interact in *N. benthamiana*, in three orientations (pBIFP2 JAZ6 + pBIFP3 PFP, pBIFP2 PFP + pBIFP3 JAZ6, pBIFP2 PFP + pBIFP4 JAZ6). Evidence of interaction is indicated by F, while a lack of detectable interaction is indicated by X. White space in the table corresponds to combinations which were not tested as the constructs contain the same halves of YFP.

pBIFP insert:		JAZ6		EIN3				FHY3				WOX12			
pBIFP vector:		3	4	1	2	3	4	1	2	3	4	1	2	3	4
JAZ6	1	X	X			X	X			X	X			X	X
	2	F	X			X	X			X	X			X	X
	3			X	X			X	X			X	X		
	4			X	X			X	X			X	X		

pBIFP insert:		NAC10				ZF1				Mybl				PFP			
pBIFP vector:		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
JAZ6	1			X	X			X	X			X	X			X	X
	2			X	X			X	X			X	X			F	X
	3	X	X			X	X			X	X			X	F		
	4	X	X			X	X			X	X			X	F		

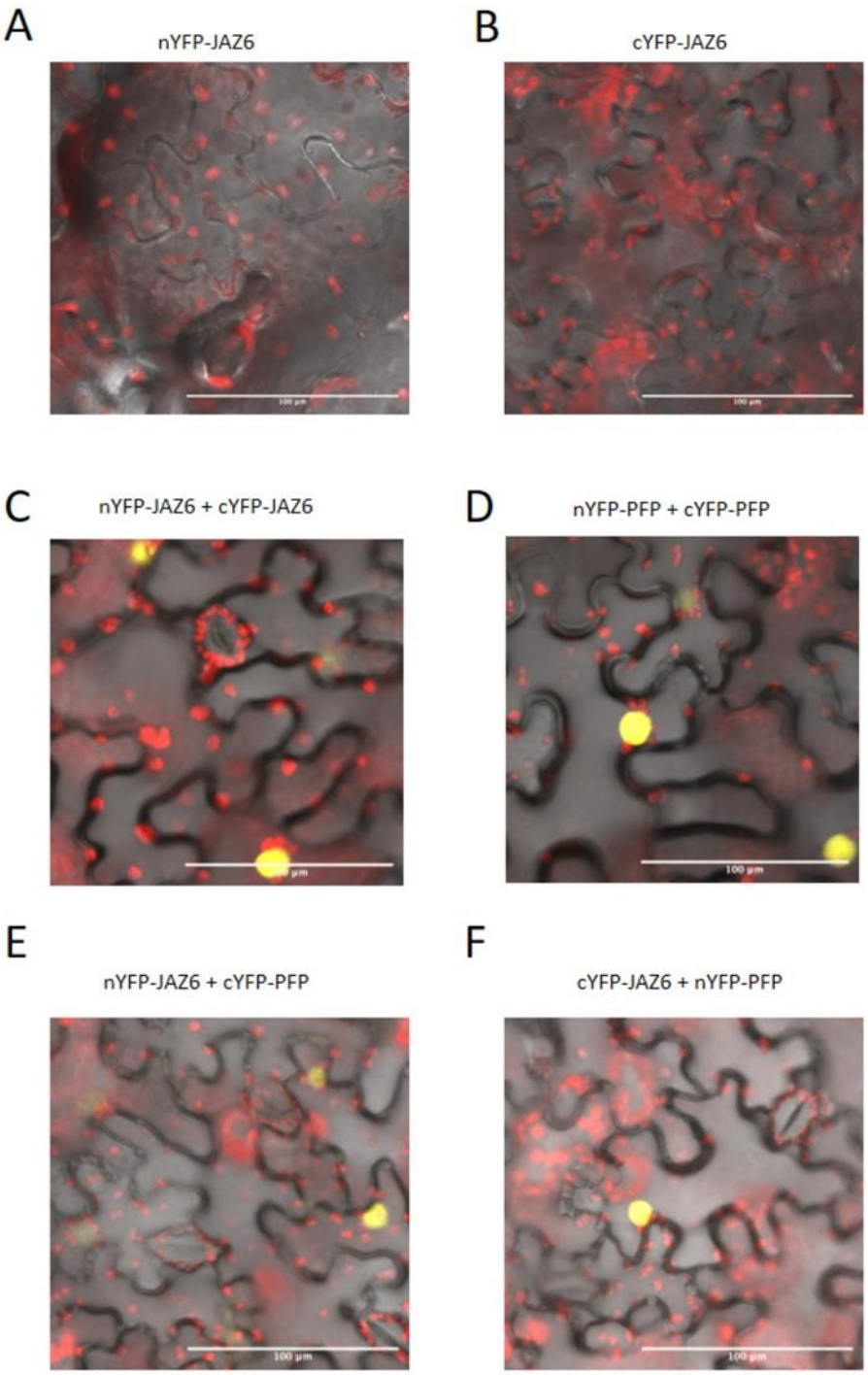


Figure 2.4.13: PFP homodimerizes and interacts directly with JAZ6 in BiFC assays in *N. benthamiana* leaves.

Overlay of transmitted light and fluorescent images of *N. benthamiana* leaf epidermal cells 3 days post infiltration with *A. tumefaciens* containing the indicated pBIFP vectors: nYFP-JAZ6 (A); cYFP-PFP (B); nYFP-JAZ6 and cYFP-JAZ6 (C); nYFP-PFP and cYFP-PFP (D); nYFP-JAZ6 and cYFP-PFP (E); nYFP-PFP and cYFP-JAZ6 (F). Yellow (indicating YFP) and red (indicating chloroplasts) are overlaid on a DIC image. Experiments were performed three times on a Zeiss 710 confocal microscope. Scale bars represent 100 µm.

Table 2.4.2: Pairwise bimolecular fluorescence binding pairs tested with PFP and FHY3 in BiFC. PFP homodimerizing (pBIFP2 PFP + pBIFP3 PFP) was observed in N. *benthamiana*. Of the heterodimers tested, JAZ6 and FHY3 were observed to interact with PFP in N. *benthamiana*, in three orientations each. FHY3 was also observed to bind EIN3 in N. *benthamiana*, in two orientations. Evidence of interaction is indicated by F, while a lack of detectable interaction is indicated by X. White space in the table corresponds to combinations which were not tested as the constructs contain the same halves of YFP.

	pBIFP insert:	PFP		JAZ6				FHY3				EIN3			
	pBIFP vector:	3	4	1	2	3	4	1	2	3	4	1	2	3	4
PFP	1	X	X			X	X			X	X			X	X
PFP	2	F	X			F	F			F	F			X	X
PFP	3			X	F			X	F			X	X		
PFP	4			X	X			X	X			X	X		

	pBIFP insert:	JAZ6				PFP				EIN3			
	pBIFP vector:	1	2	3	4	1	2	3	4	1	2	3	4
FHY3	1			X	X			X	X			X	X
FHY3	2			X	X			F	X			X	X
FHY3	3	X	X			X	F			X	X		
FHY3	4	X	X			X	F			F	F		

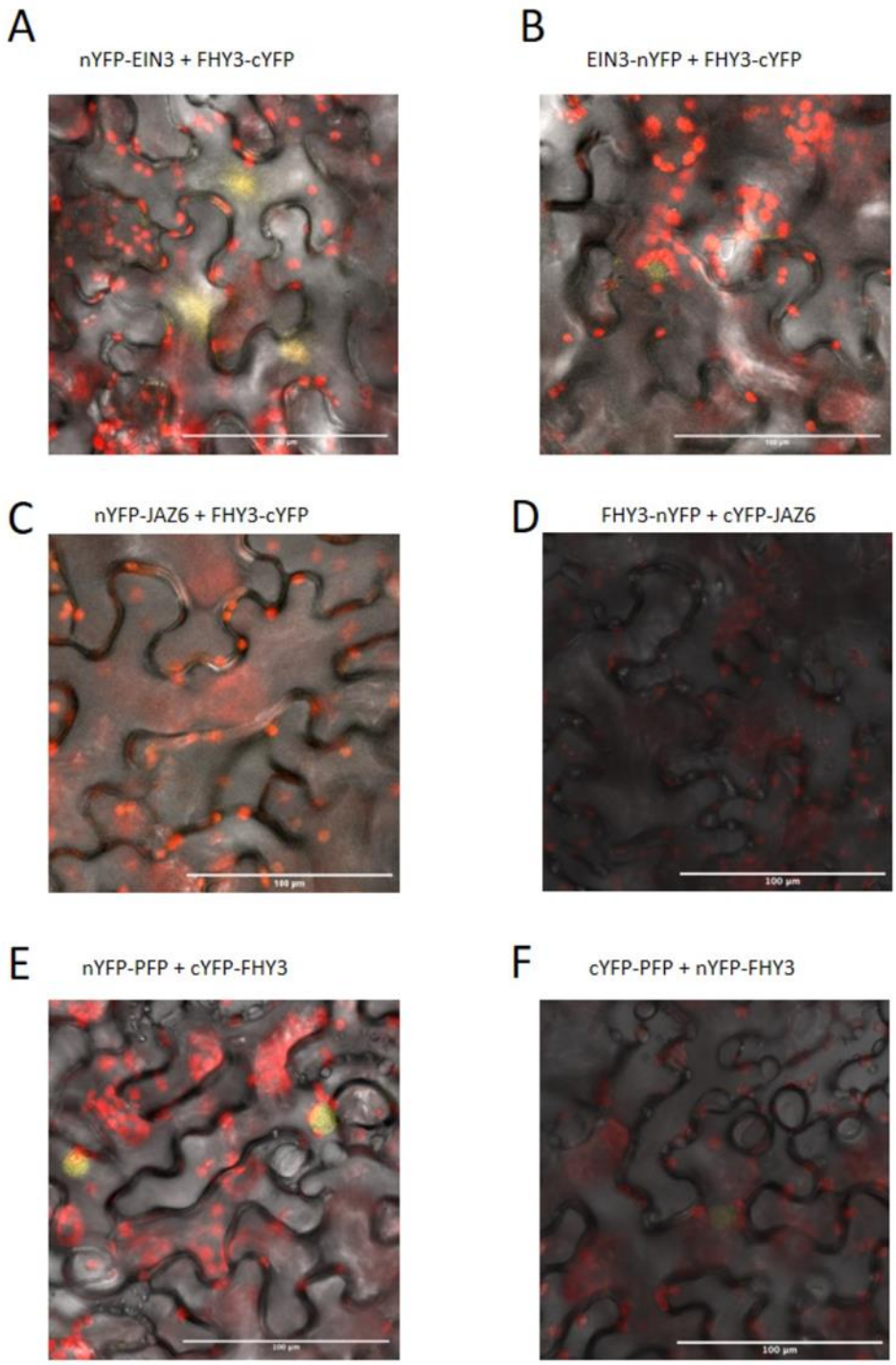


Figure 2.4.14: FHY3 interacts with EIN3 and PFP, JAZ6 is not observed to bind to FHY3. Overlay of transmitted light and fluorescent images of *N. benthamiana* leaf epidermal cells 3 days post infiltration with *A. tumefaciens* containing the indicated pBIFP vectors: nYFP-EIN3 and FHY3-cYFP (A); EIN3-nYFP and FHY3-cYFP (B); nYFP-JAZ6 and FHY3-cYFP (C); FHY3-nYFP + cYFP-JAZ6 (D); nYFP-PFP + cYFP-FHY3 (E); cYFP-PFP + nYFP-FHY3 (F). Yellow (indicating YFP) and red (indicating chloroplasts) are overlaid on a DIC image. Experiments were performed three times on a Zeiss 710 confocal microscope. Scale bars represent 100 µm.

2.4.5 The EAR domains of JAZ6 are essential for binding PFP

JAZ6 is known to possess zinc-finger inflorescence meristem (ZIM), JA-associated (Jas) domain, and ERF-associated amphiphilic repression (EAR) protein-protein binding domains which are crucial for JAZ proteins to repress gene transcription through binding to other proteins (Chini *et al.*, 2009; Pauwels *et al.*, 2010; Sheard *et al.*, 2010; Fernández-Calvo *et al.*, 2011). Recently the cryptic MYC interacting domain (CMID) was been characterised for JAZ1, 2, 5, 6, and 10 (F. Zhang *et al.*, 2017; Takaoka *et al.*, 2021). As such, it is likely one of these domains is necessary for JAZ6-PFP protein binding. Therefore, the domain specificity of JAZ6-PFP protein binding was also tested using in-planta BiFC assays in *N. benthamiana*. This was to investigate the roles of the Jas, ZIM, and EAR domains in protein-protein binding to PFP (Figure 2.4.15 & 16), the CMID was not originally planned to be tested. It also serves as a 'gold standard' for negative controls of BiFC, if a mutated form of the protein is unable to bind (Horstman *et al.*, 2014).

For these experiments two domain deletion constructs were made for JAZ6: JAZ6ΔZIM and JAZ6ΔJas (Figure 2.4.15A). These constructs lack the ZIM and Jas domains respectively. 72 bp corresponding to the Jas domain were deleted from JAZ6ΔJas, while 108 bp corresponding to the ZIM domain were deleted from JAZ6ΔZIM. A construct with mutated EAR domains, but no disrupted Jas domain between them, was also made: JAZ6mEAR (Figure 2.4.15A). The EAR domains in JAZ6mEAR were mutated from DLNEPT to AMSEAT and LELKL to FEFKF, as these specific mutations eliminate their protein-protein binding activity as EAR domains while maintaining amino acid polarity, as reported previously for EAR domain mutations in other proteins (Harvey *et al.*, 2020; Darino *et al.*, 2021). These constructs were cloned into pBIFP2 and 3 (Figure 2.4.12), as JAZ6 only binds PFP in these orientations (Figure 2.4.13 & 14).

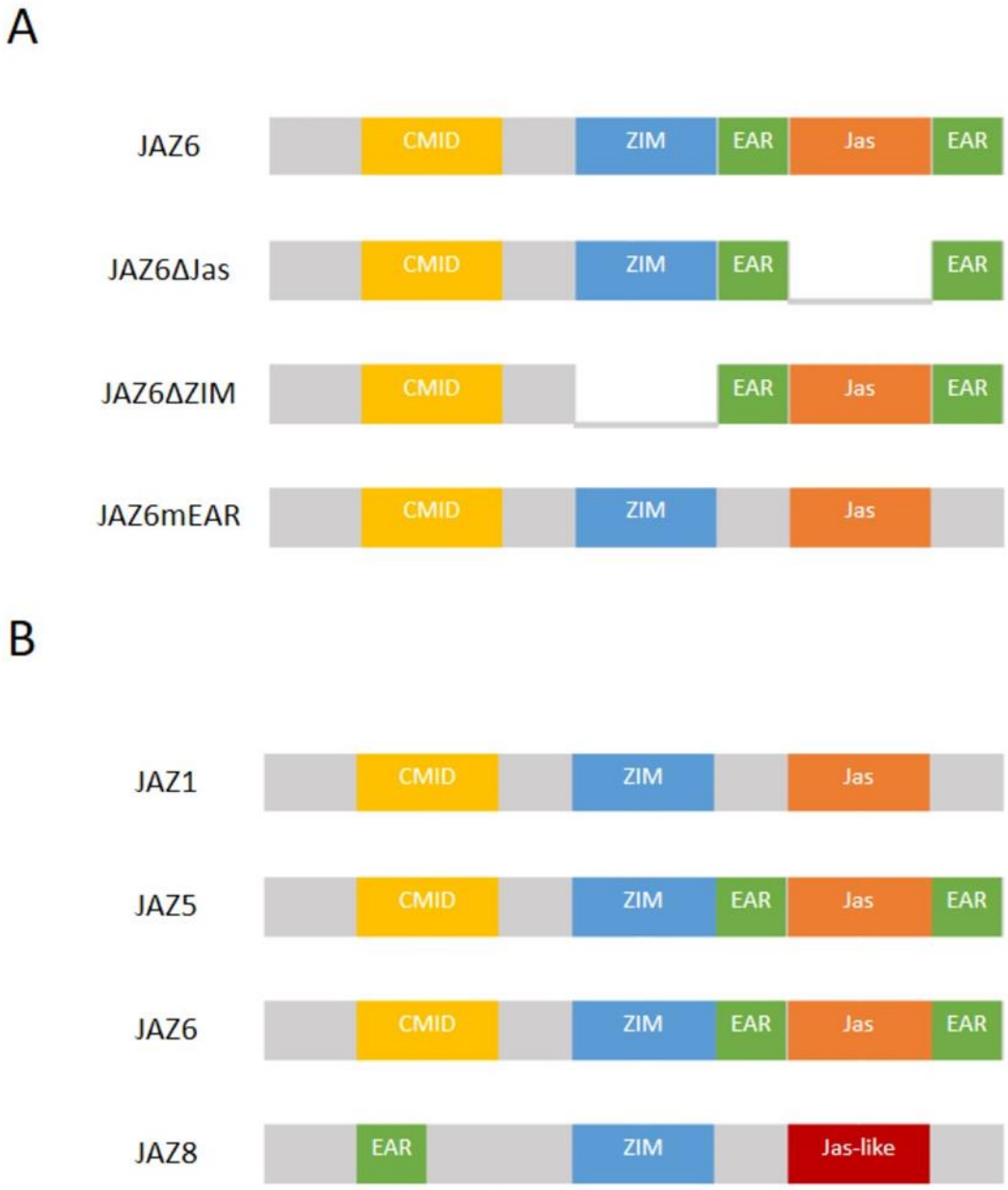


Figure 2.4.15: Schema for JAZ protein domain structure, and JAZ6 domain deletions, illustrating major motifs. A diagram of the protein domain coding structure of JAZ6 and the JAZ6 domain deletions JAZ6ΔJas and JAZ6ΔZIM, as well as the JAZ6mEAR mutant, used to create BiFC constructs. Jas (orange); ZIM (blue); and EAR (green) domains are labelled. 72bp corresponding to the Jas domain were deleted from JAZ6ΔJas, while 108bp corresponding to the ZIM domain were deleted from JAZ6ΔZIM. EAR domains in JAZ6mEAR were mutated from DLNEPT and LELKL to AMSEAT and FEFKF.

B JAZ1, 5, 6, and 8 protein domain structures. Jas (orange); Jas-like (red); ZIM (blue); EAR (green); and cryptic MYC2-interacting (CMID, yellow) domains are labelled. Adapted from Garrido-Bigotes et al. 2019.

Firstly, experiments were conducted to validate these domain deletion constructs (Figure 2.4.16). The JAZ6mEAR was shown to homodimerize in the nucleus (Figure 2.4.16A & B), just like JAZ6 (Figure 2.4.12C). For JAZ6ΔJas, the JAZ6 homodimer was originally expected to lose nuclear-specific localisation, as nuclear localisation is directed by protein-protein binding partners like MYC2 which interacts with the Jas domain of JAZ proteins (Withers *et al.*, 2012). However, JAZ6 possesses a CMID domain (F. Zhang *et al.*, 2017; Takaoka *et al.*, 2021) and so the deletion of the Jas domain did not stop it from binding to MYC2 and localising to the nucleus (Figure 2.4.15C). It will also still homodimerize as that is mediated by the TIFY domain (Chini *et al.*, 2009). A loss of nuclear-specific localisation due to the deletion of the Jas domain was previously reported for YFP:JAZ9ΔJas (Withers *et al.*, 2012), which does not possess a CMID domain (F. Zhang *et al.*, 2017; Takaoka *et al.*, 2021). The JAZ6ΔZIM construct is expected to lose the ability to homodimerize, as it lacks the ZIM domain which is necessary for JAZ proteins to homodimerize (Chini *et al.*, 2009). This is seen for JAZ6ΔZIM-JAZ6ΔZIM (Figure 2.4.15B), but also JAZ6-JAZ6ΔZIM binding (Figure 2.4.15B). The JAZ6mEAR construct is expected to lose the ability to bind to the transcriptional repressor TOPLESS (TPL), as JAZ6 binds to TPL via EAR domains (Pauwels *et al.*, 2010). While JAZ6 is seen to bind TPL (Figure 2.4.16A & B), JAZ6mEAR does not bind TPL (Figure 2.4.16C & D).

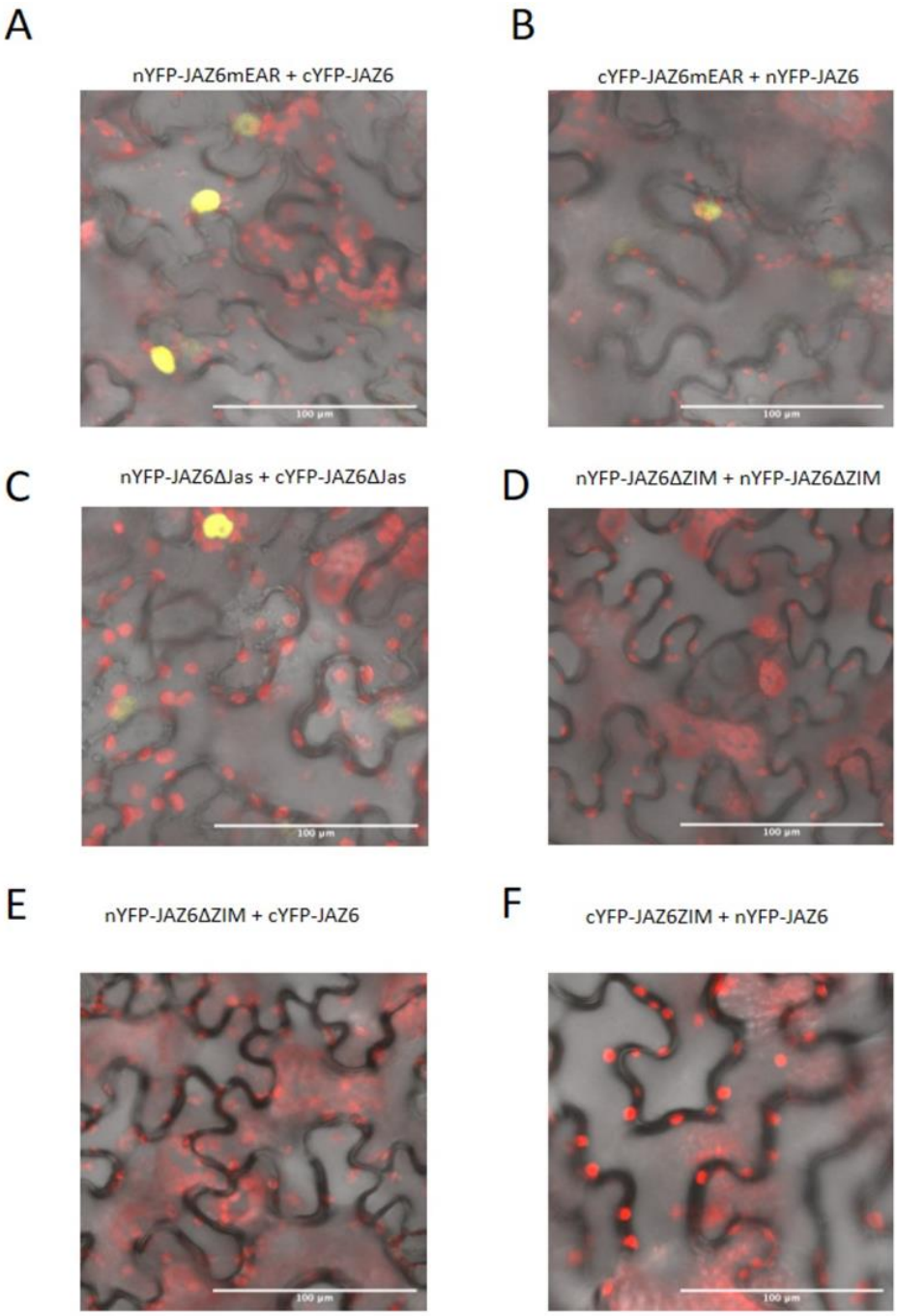


Figure 2.4.16: Validation of JAZ6 domain deletions and mutants.

Overlay of transmitted light and fluorescent images of *N. benthamiana* leaf epidermal cells 3 days post infiltration with *A. tumefaciens* containing the indicated pBIFP vectors: nYFP-JAZ6mEAR + cYFP-JAZ6 (A); cYFP-JAZ6mEAR + nYFP-JAZ6 (B); nYFP-JAZ6ΔJas + cYFP-JAZ6ΔJas (C); nYFP-JAZ6ΔZIM + nYFP-JAZ6ΔZIM

(D); nYFP-JAZ6ΔZIM + cYFP-JAZ6 (E); cYFP-JAZ6ZIM + nYFP-JAZ6 (F). Yellow (indicating YFP) and red (indicating chloroplasts) are overlaid on a DIC image. Experiments were performed three times on a Zeiss 780 confocal microscope. Scale bars represent 100 µm.

Testing nYFP-JAZ6ΔZIM and cYFP-PFP binding shows YFP signal (Figure 2.4.17A & B), indicating JAZ6 does not require a ZIM domain to bind to PFP. Similarly nYFP-JAZ6ΔJas and cYFP-PFP also results in YFP fluorescence (Figure 2.4.17C & D), showing that JAZ6 does not require a Jas domain to bind to PFP, which is where MYC2 binds JAZ6 (Fernández-Calvo *et al.*, 2011). However, when testing the JAZ6 domain deletions and mutants binding to PFP, it appeared that EAR domains were necessary for JAZ6-PFP protein-protein binding (Figure 2.4.17). This indicates that JAZ6 requires EAR domains to bind PFP.

2.4.6 PFP binds the transcriptional repressor TOPLESS

JAZ6 is able to bind to the transcriptional co-repressor TOPLESS (TPL) via its EAR domains (Pauwels *et al.*, 2010). As the EAR domains appeared to be important for JAZ6 binding PFP, and PFP possesses its own EAR domain (Kagale, Links and Rozwadowski, 2010), the binding of PFP to the TOPLESS (TPL) repressor was tested (Figure 2.4.18). To validate BiFC with TPL JAZ6 was shown to bind to TPL (Figure 2.4.18A & B), as previously reported (Pauwels *et al.*, 2010). JAZ6mEAR did not bind to TPL (Figure 2.4.17E & F), as it lacks functional EAR domains to bind TPL (Pauwels *et al.*, 2010). PFP was able to bind to TPL, possibly through the EAR domain it possesses (Kagale, Links and Rozwadowski, 2010).

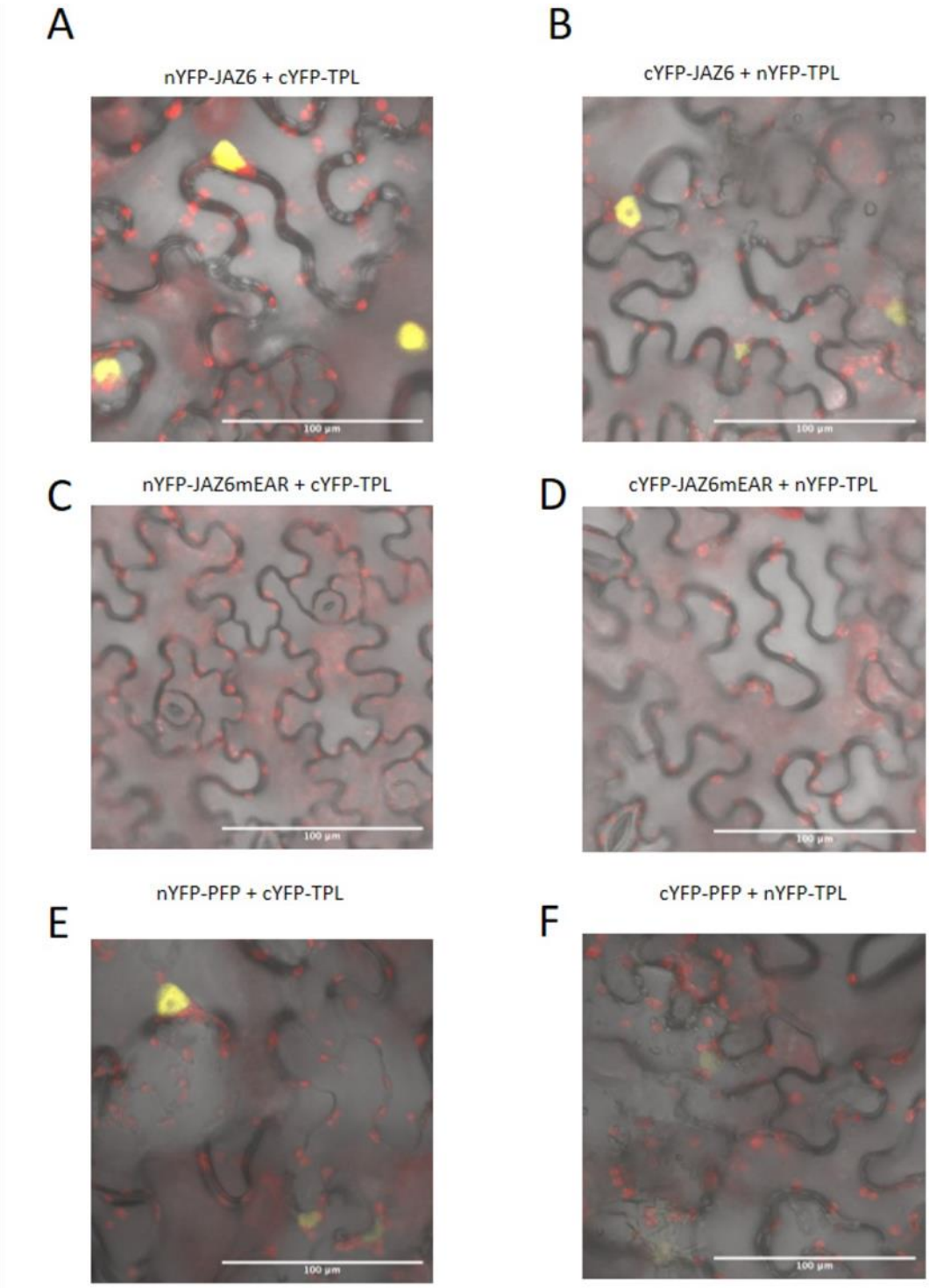


Figure 2.4.17: TPL binds JAZ6 and PFP.

Overlay of transmitted light and fluorescent images of *N. benthamiana* leaf epidermal cells 3 days post infiltration with *A. tumefaciens* containing the indicated pBIFP vectors: nYFP-JAZ6 + cYFP-TPL (A); cYFP-JAZ6 + nYFP-TPL (B); nYFP-JAZ6mEAR + cYFP-TPL (C); cYFP-JAZ6mEAR + nYFP-TPL (D); nYFP-PFP + cYFP-TPL (E); cYFP-PFP + nYFP-TPL (F). Yellow (indicating YFP) and red (indicating chloroplasts) are overlaid on a DIC image. Experiments were performed three times on a Zeiss 780 confocal microscope. Scale bars represent 100 µm.

2.4.7 Not all JAZ proteins bind PFP

As JAZ proteins are defined as containing similar protein-protein binding Jas and ZIM domains (Vanholme *et al.*, 2007), the potential for JAZ1, JAZ5, or JAZ8 to bind PFP was investigated (Figure 2.4.19). JAZ5 is very similar to JAZ6, with almost perfect matches for the sequences of their Jas, ZIM, and two EAR domains (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). JAZ1 contains Jas and ZIM domains, but not the EAR domains of JAZ6 (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019), while JAZ8 contains Jas and ZIM domains, but only an LxLxL EAR domain in a different structural configuration: closer to the N-terminal end of the protein, rather than the C-terminal end as in JAZ5 and JAZ6 (Figure 2.4.15). And so, this range of JAZ proteins tests a range of protein domain structures for PFP binding. Testing similar proteins using BiFC also serves as a good series of controls, particularly as a negative control if they do not bind (Horstman *et al.*, 2014). As with the JAZ6 domain deletion constructs, these JAZ constructs were cloned into pBIFP2 and 3 (Figure 2.4.12), as JAZ6 only binds PFP in these orientations (Figure 2.4.13 & 14).

As expected, there was no evidence for JAZ1 binding PFP (Figure 2.4.18A & B), which reflects the lack of the EAR domain in JAZ1. However, JAZ8 also did not bind PFP (Figure 2.4.17E & F), despite possessing one EAR domain. However, there is a nuclear YFP signal for JAZ5 binding PFP in both orientations (Figure 2.4.17C & D). This indicates that JAZ5 binds PFP *in planta*, which was not seen in yeast-2-hybrid (Stoker, 2016). To validate the JAZ1 and JAZ8 constructs, they were tested to see if they homodimerized (Figure 2.4.20). Both JAZ1 and JAZ8 showed a nuclear YFP signal as they homodimerized, validating the constructs.

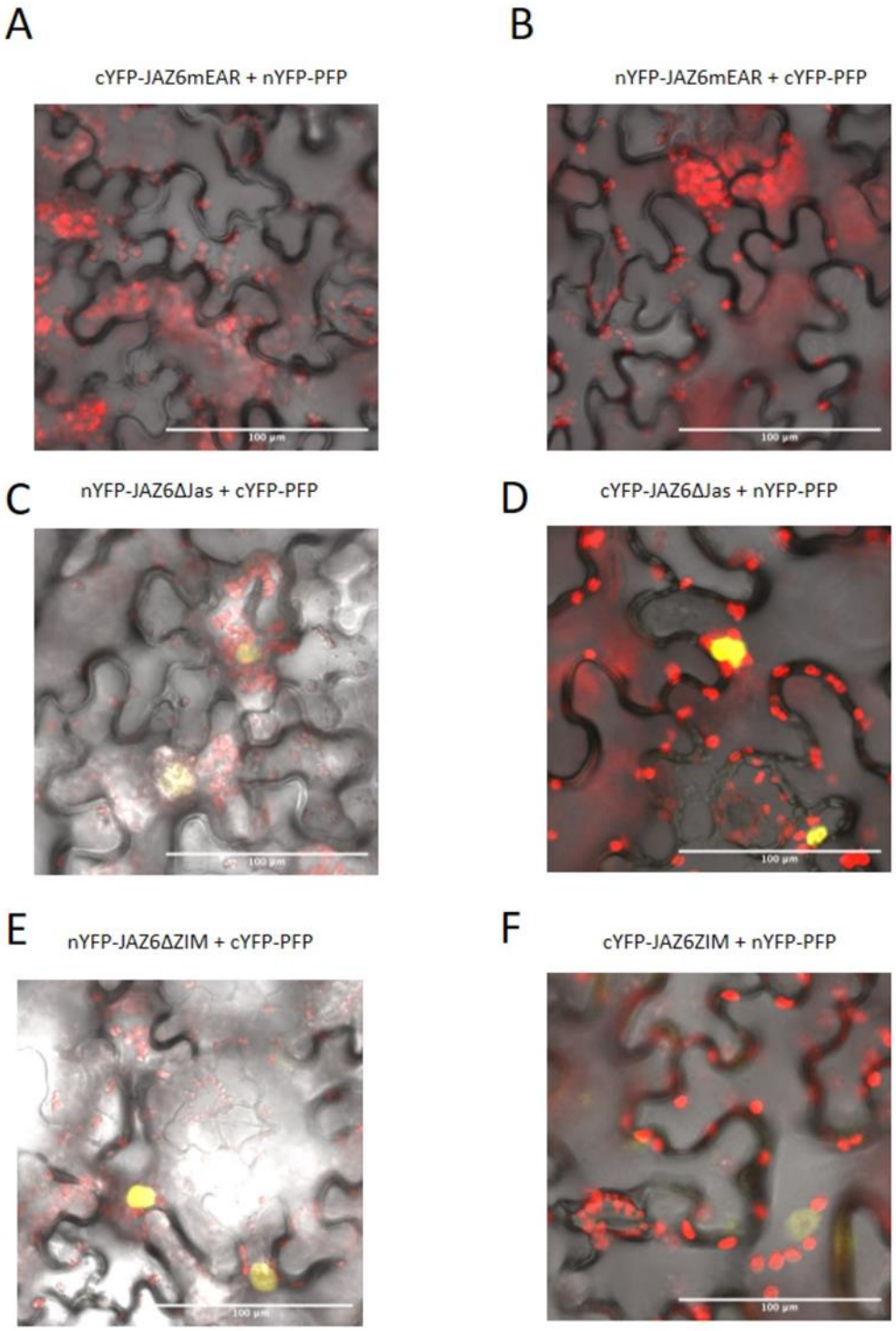


Figure 2.4.18: JAZ6 binds PFP via EAR domains. Overlay of transmitted light and fluorescent images of *N. benthamiana* leaf epidermal cells 3 days post infiltration with *A. tumefaciens* containing the indicated pBIFP vectors: cYFP-JAZ6mEAR + nYFP-PFP (A); nYFP-JAZ6mEAR + cYFP-PFP (B); nYFP-JAZ6ΔJas + cYFP-PFP (C); cYFP-JAZ6ΔJas + nYFP-PFP (D); nYFP-JAZ6ΔZIM + cYFP-PFP (E); cYFP-JAZ6ZIM + nYFP-PFP (F). Yellow (indicating YFP) and red (indicating chloroplasts) are overlaid on a DIC image. Experiments were performed three times on a Zeiss 780 confocal microscope. Scale bars represent 100 µm.

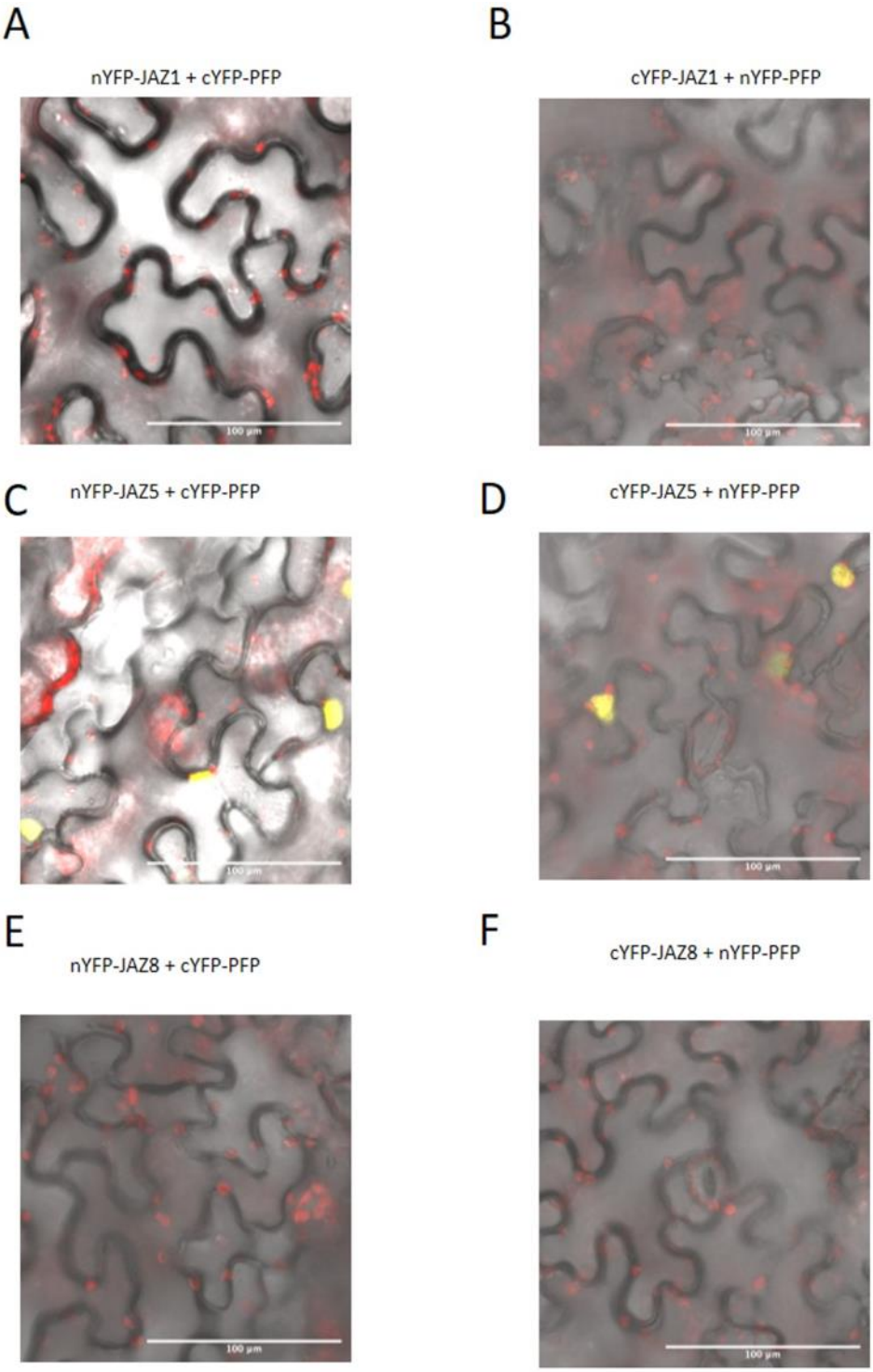


Figure 2.4.19: PFP binds JAZ5, but not JAZ1 or JAZ8.

Overlay of transmitted light and fluorescent images of *N. benthamiana* leaf epidermal cells 3 days post infiltration with *A. tumefaciens* containing the indicated pBIFP vectors: nYFP-JAZ1 + cYFP-PFP (A); cYFP-JAZ1 + nYFP-PFP (B); nYFP-JAZ5 + cYFP-PFP (C); cYFP-JAZ5 + nYFP-PFP (D); nYFP-JAZ8 + cYFP-PFP (E); cYFP-JAZ8 + nYFP-PFP (F). Yellow (indicating YFP) and red (indicating chloroplasts) are overlaid on a DIC image. Experiments were performed three times on a Zeiss 780 confocal microscope. Scale bars represent 100 µm.

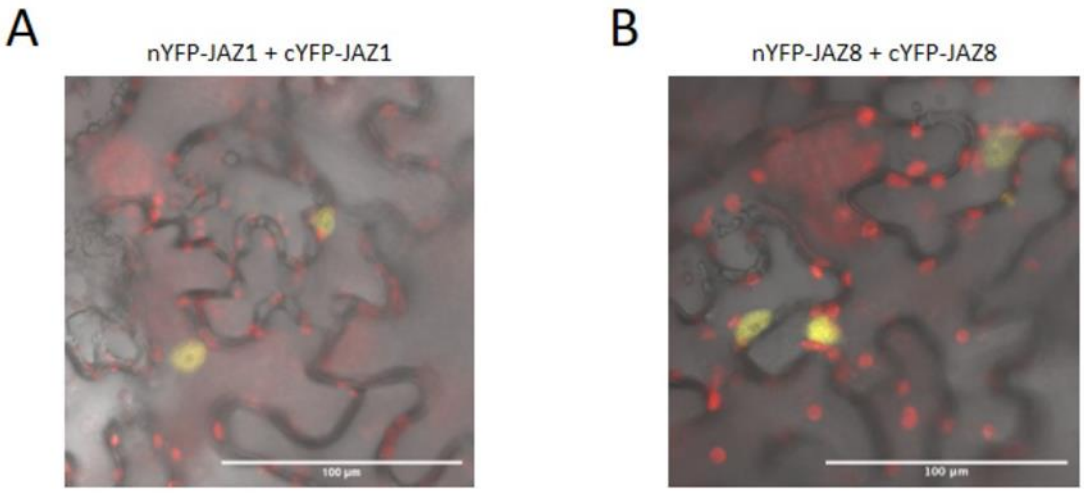


Figure 2.4.20: JAZ1 and JAZ8 homodimerize.
Overlay of transmitted light and fluorescent images of *N. benthamiana* leaf epidermal cells 3 days post infiltration with *A. tumefaciens* containing the indicated pBIFP vectors: nYFP-JAZ1 + cYFP-JAZ1 (A); cYFP-JAZ8+ nYFP-JAZ8 (B). Yellow (indicating YFP) and red (indicating chloroplasts) are overlaid on a DIC image. Experiments were performed three times on a Zeiss 780 confocal microscope. Scale bars represent 100 µm.

2.4.8 Confirmation of JAZ6 binding to PFP *in planta*

As BiFC can produce false positive and negative results (Horstman *et al.*, 2014), yeast-2-hybrid and co-immunoprecipitation techniques were used to thoroughly validate JAZ6 binding to PFP. While the yeast-2-hybrid assay is a repeat of the previous yeast-2-hybrid screening of JAZ6 binding partners (Stoker, 2016), co-immunoprecipitation is a more stringent technique to test if proteins interact *in planta* (Xing *et al.*, 2016).

Protein-protein binding of JAZ6 with PFP was verified using yeast-2-hybrid (Figure 2.4.21). JAZ6 was previously cloned into pDEST32 DNA binding domain (DB) vector and transformed into yeast by Dr Sarah Harvey, while PFP was present in the Y2H transcription factor library previously cloned into pDEST22 activation domain (AD) vectors in yeast (Pruneda-Paz *et al.*, 2014). Yeast was mated in AD/DB pairs on YPDA plates, alongside negative controls where

they were paired with empty vectors or no yeast at all. They were then replica plated onto SC-LT plates to check mating, and only pairs of AD/DB yeast grew because they had mated successfully, which included empty vectors. In parallel, the YPDA plate was replica plated onto protein-protein binding selection SC-LTH plates, where growth was only seen for the PFP-JAZ6 binding pair as the empty vectors were unable to activate the expression of the HIS3 reporter. This provides further evidence that JAZ6 binds PFP.

Co-immunoprecipitation experiments in *N. benthamiana* show further evidence for JAZ6-PFP protein binding (Figure 2.4.22 & 23), using transient expression of GFP fused to the C-terminus of JAZ6, and HA fused to the N-terminus of PFP, as well as a GFP only control. The JAZ6-GFP construct was cloned by Dr Sarah Harvey, while the GFP was provided by Dr Fabian Vaistij. Gateway cloning was used to make the HA-PFP construct by an LR reaction from the entry vector used for cloning PFP into pBIFP BiFC vectors. This vector was sequenced and HA-PFP accumulation in transiently transformed *N. benthamiana* was verified by immunoblotting.

Three days after *N. benthamiana* was infiltrated with *A. tumefaciens* carrying various combinations of these vectors, this tissue was processed to extract proteins. An aliquot of this protein extract was set aside to later run as the 'input' on an SDS-PAGE gel. Immunoprecipitation was performed with anti-HA and anti-GFP agarose beads on the remaining protein. The protein extract 'input' and immunoprecipitated protein were run on an SDS-PAGE protein gel and immunoblotted.

Here, there is enrichment of HA-PFP after anti-GFP pulldown, but only when JAZ6-GFP and HA-PFP are co-infiltrated (Figure 2.4.24). As while GFP is pulled down, no HA-PFP is co-immunoprecipitated. This indicates that PFP strongly and specifically binds JAZ6. We also show enrichment of JAZ6-GFP after anti-HA pulldown, only when JAZ6-GFP and HA-PFP are co-infiltrated (Figure 2.4.24). Similarly, this again indicates that PFP binds JAZ6.

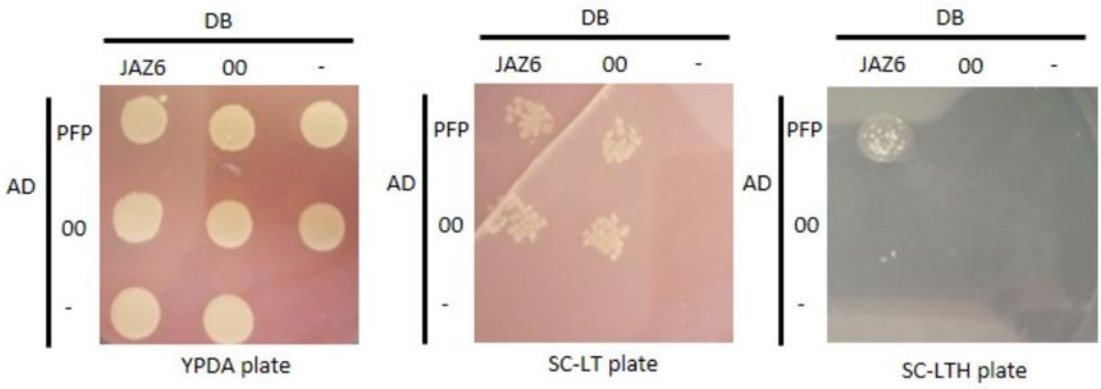


Figure 2.4.21: PFP interacts directly with JAZ6 in yeast-2-hybrid.

pDEST22 and pDEST32 containing JAZ6, PFP, or empty (00) were mated on YPDA (left), and then replica plated onto selection SC-LT (centre) and SC-LTH (right) plates. "-" indicates no yeast of that type was included. All colonies grew on the YPDA media (left) and were then replica plated with sterile velvet onto plates to check mating (SC-LT) and protein-protein binding (SC-LTH) plates. All colonies with a pair of AD and DB vectors grew on SC-LT mating selection media (centre), only the AD PFP and DB JAZ6 binding pair grew on SC-LTH binding selection media (right). Protein interactions are indicated by colony growth.

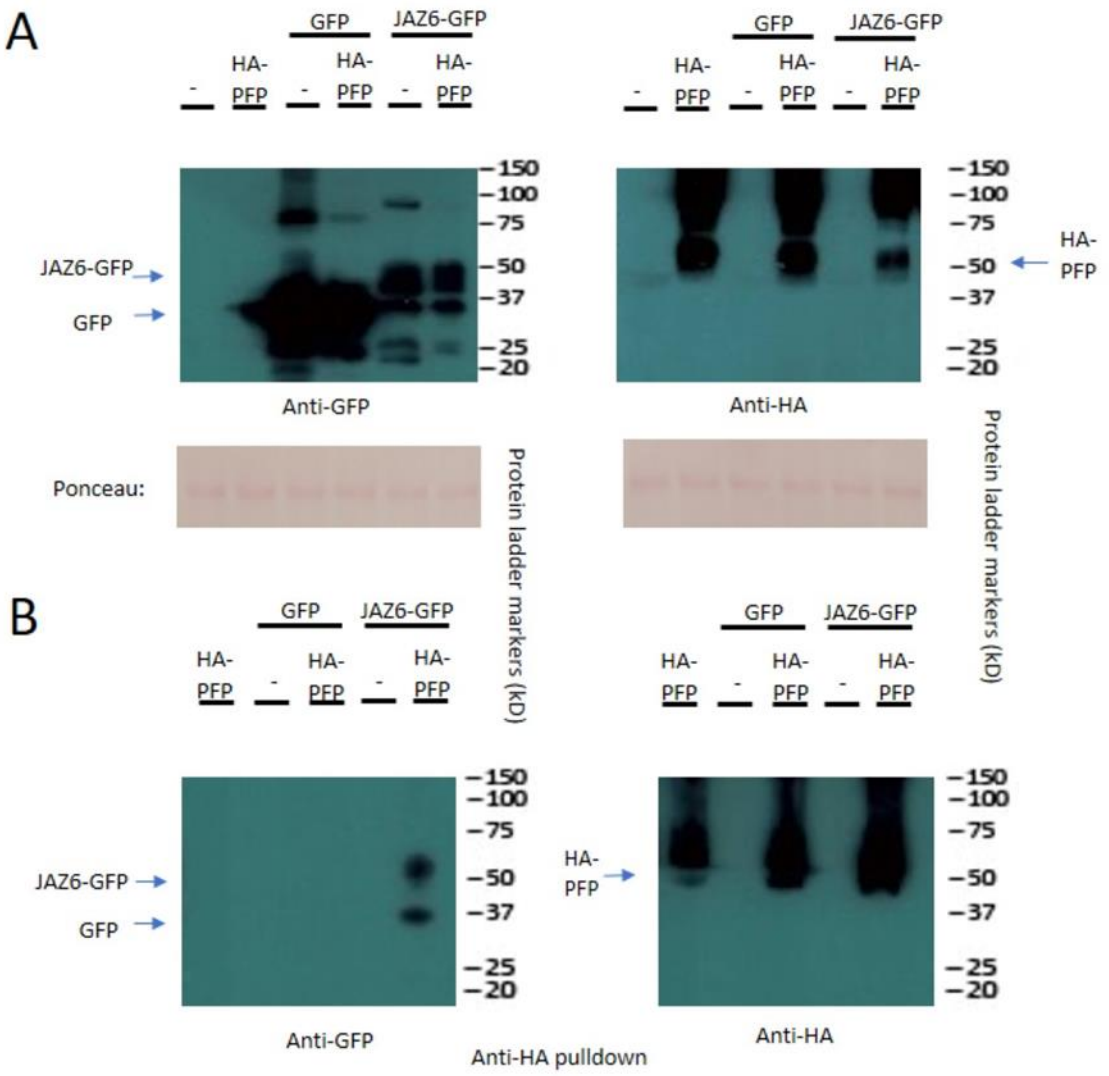


Figure 2.4.22: Anti-HA co-immunoprecipitation shows PFP interacts directly with JAZ6 *in planta*.

Input (A), and anti-HA immunoprecipitation (B) of protein extracts from *N. benthamiana.* Samples were taken three days after infiltration with GFP, JAZ6-GFP and/or HA-PFP. Both anti-GFP (left) and anti-HA (right) antibodies were used for immunoblotting. GFP was used as a negative control. Below the input (A) is Ponceau staining of the blotted membrane as an indication of total protein loading. BioRad PrecisionPlus blue ladders were used to indicate size.

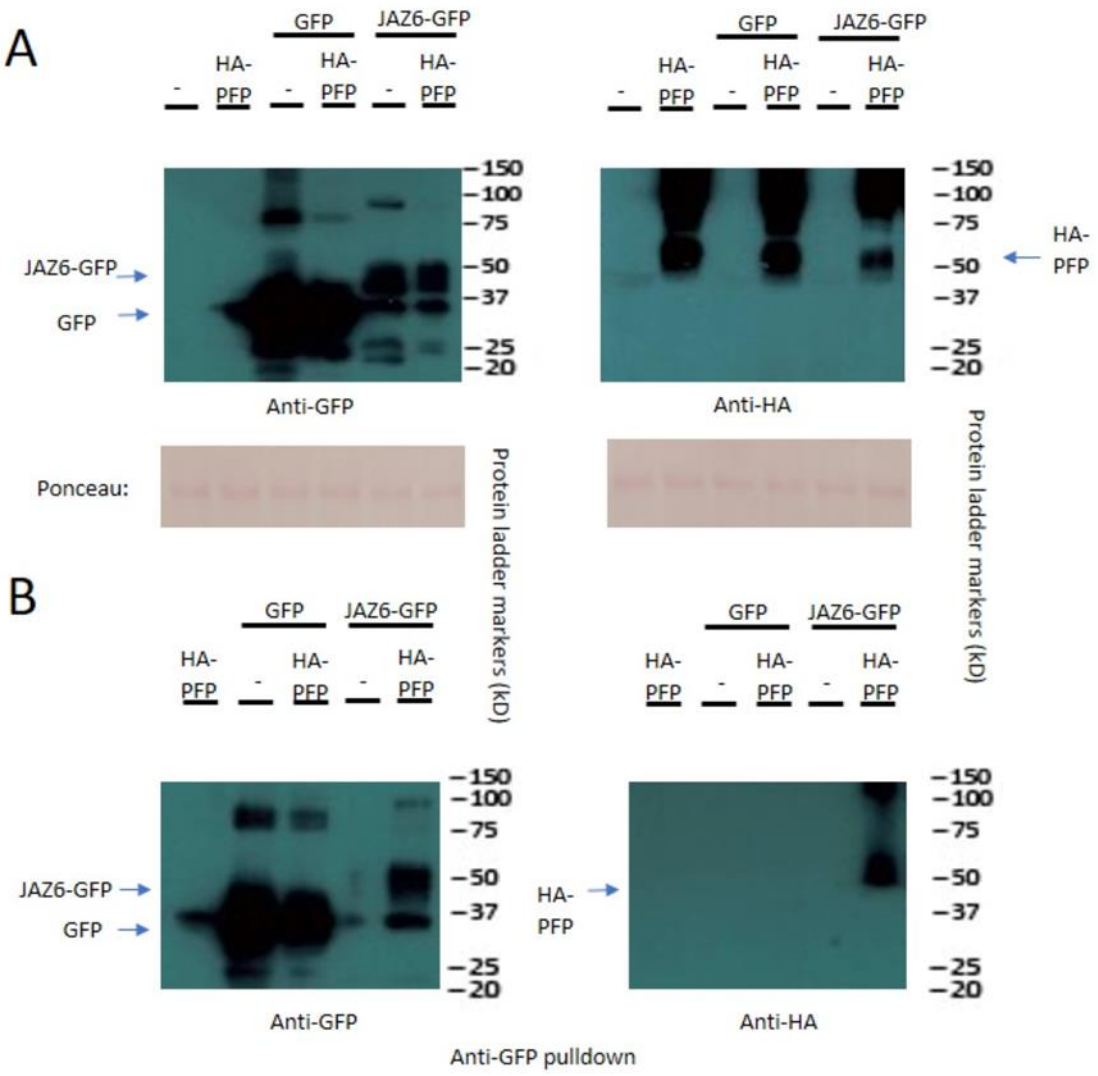


Figure 2.4.23: Anti-GFP co-immunoprecipitation shows PFP interacts directly with JAZ6 *in planta*.

Input (A), and anti-GFP immunoprecipitation (B) of protein extracts from *N. benthamiana.* Samples were taken three days after infiltration with GFP, JAZ6-GFP and/or HA-PFP. Both anti-GFP (left) and anti-HA (right) antibodies were used for immunoblotting. GFP was used as a negative control. Below the input (A) is Ponceau staining of the blotted membrane as an indication of total protein loading. BioRad PrecisionPlus blue ladders were used to indicate size. The expected sizes for proteins of interest are: JAZ6-GFP 55kDa, HA-PFP 55kDa, GFP 27KDa.

2.4.9 PFP does not stabilize JAZ6 against COI1-dependent degradation

As PFP interacts with JAZ6, I investigated if this could affect the stability of JAZ6 by targeting JAZ6 for degradation or protecting JAZ6 from degradation. It was initially supposed that PFP could destabilise JAZ6 by ubiquitination, as it is a member of the UBR box family of E3 ubiquitin ligases (Garzón *et al.*, 2007). JAZ6 protein is known to be targeted for degradation by the E3 ubiquitin ligase COI1 (Sheard *et al.*, 2010). Instead of targeting JAZ6 for degradation, PFP could make JAZ6 more stable by preventing COI1 targeting JAZ6 for degradation. An example of an E3 ubiquitin ligase stabilising a JAZ is JAZ12 which is protected from degradation as it binds to the E3 ubiquitin ligase KEG (KEEP ON GOING) (Pauwels *et al.*, 2015).

To test if PFP could stabilise JAZ6, a stability assay was conducted in *N. benthamiana* by transient expression of JAZ6-GFP, HA-PFP, and COI1 (Figure 2.4.24). JAZ6-GFP fluorescence and protein accumulation was measured with co-infiltration of HA-PFP and/or COI1. As expected, JAZ6-GFP is not present in the negative control with only HA-PFP infiltrated (Figure 2.4.22A). Without COI1 co-infiltration, JAZ6-GFP accumulates at similar levels in leaves infiltrated with JAZ6-GFP or JAZ6-GFP and HA-PFP together (Figure 2.4.24A). With COI1 co-infiltration, there was no difference in JAZ6-GFP accumulation in leaves infiltrated with JAZ6-GFP, compared to infiltration with both JAZ6-GFP and HA-PFP (Figure 2.4.24A). This suggests COI1 co-infiltration caused degradation of JAZ6; and this was not inhibited by the presence of HA-PFP. A band the expected size of cleaved GFP at 27kDa was also detected in all samples infiltrated with JAZ6-GFP.

Transiently transformed leaves were also observed under a confocal fluorescence microscope to observe JAZ6-GFP (Figure 2.4.24B). Concordant with the immunoblot results, JAZ6-GFP was visible when infiltrated alone or with HA-PFP. With COI1 co-infiltration, JAZ6-GFP was less visible with or without HA-PFP co-infiltration. In summary, PFP does not appear to stabilise or destabilise JAZ6.

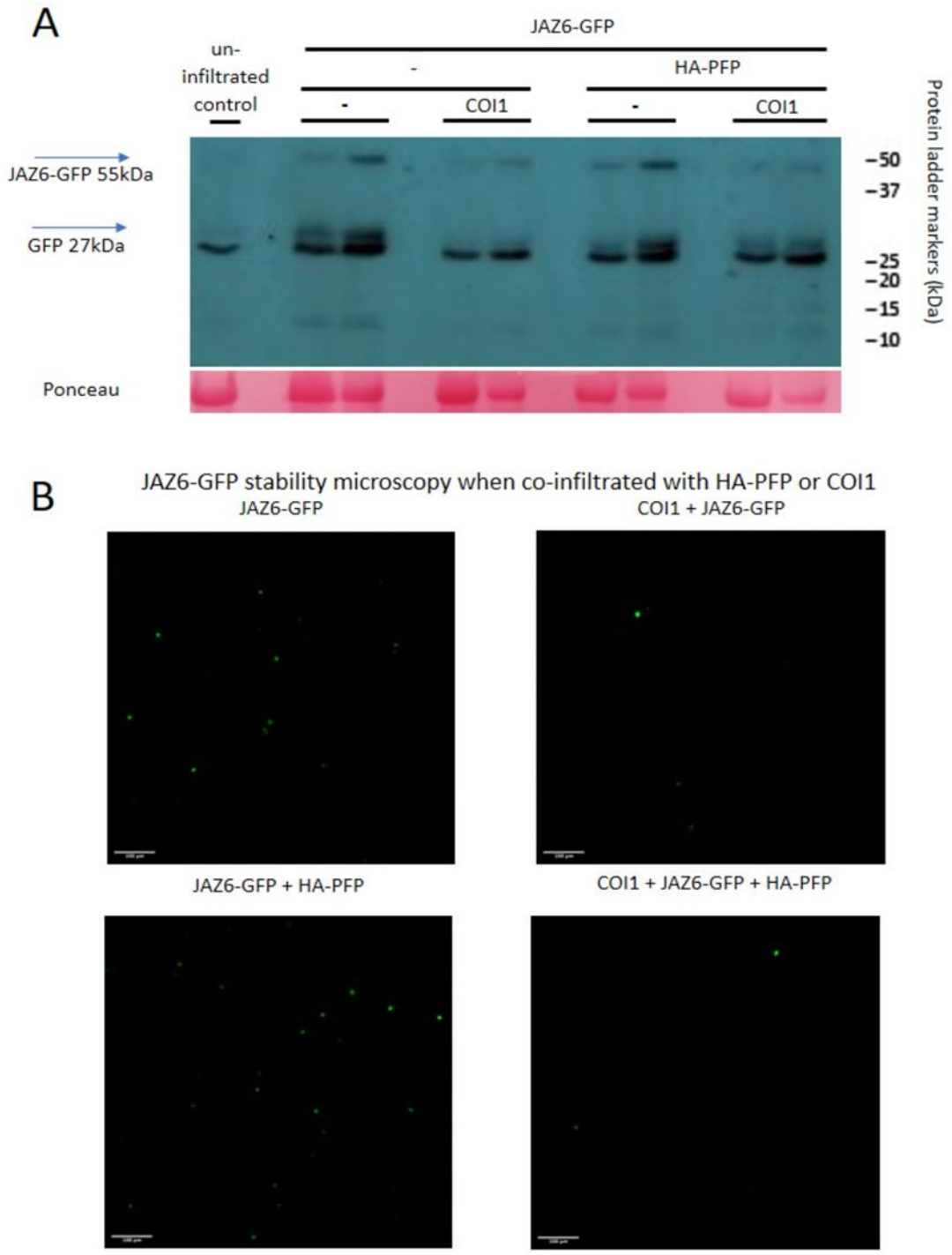


Figure 2.4.24: HA-PFP does not influence JAZ6-GFP levels.

A, JAZ6-GFP protein stability when co-infiltrated with HA-PFP or COI1. Immunoblot showing JAZ6-GFP levels in *N. benthamiana* leaves coinfiltrated with *JAZ6-GFP, COI1,* and/or *HA-PFP.* Each combination was coinfiltrated in duplicate. Below is Ponceau staining of the blotted membrane. A non-specific band is visible at 25kDa in all samples. The expected sizes for proteins of interest are: JAZ6-GFP 55kDa, HA-PFP 55kDa, GFP 27KDa.

B, *N. benthamiana* leaves were transiently transformed with *JAZ6-GFP, COI1,* and/or *HA-PFP,* and imaged using a confocal microscope. Green indicates GFP. Scale bars represent 100 µm.

2.5 Discussion

2.5.1 *JAZ6* is transcriptionally repressed by LUX

Predicting *JAZ6* expression regulation by *in silico* techniques gave a plausible and testable hypothesis for direct *JAZ6* transcriptional repression by the circadian clock transcription factor LUX (Figure 2.4.9), which is supported by *JAZ6* upregulation in the *lux-6* mutant (Y. Zhang *et al.*, 2018). It is an advantage of the simple circadian model (De Caluwé *et al.*, 2016) that a testable hypothesis can be produced with little kinematic data on the behaviour of JAZ6. It also covers many of the key central circadian clock genes, often combined into single nodes which significantly reduces the parameters involved. This makes the model less mathematically complex and less computationally intensive.

Considering the simplistic reduction of the clock in the circadian model (De Caluwé *et al.*, 2016), the prediction of transcriptional repression of *JAZ6* by LUX can be refined by further modelling and experimental data, particularly for transcription factors not included in the simplistic model. For example, it has been shown that JAZ6 is regulated in a diurnal fashion by genes outside the central circadian clock, like ABI5 (Ju *et al.*, 2019). However, adding complexity to the computational models comes with a cost, because new interactions and parameters will need to be defined and estimated (Avello *et al.*, 2019). More complex modelling work would benefit from experimental data on the kinematics of JAZ6 through biochemical assays, which would greatly refine the estimation of its abundance.

While JAZ6 gene expression in mutants for key circadian clock genes could identify further genes under circadian control, the timing of sampling is crucial to capture differential expression, and different clock gene mutants may behave differently. For example, Zhang *et al.* (2018) reported upregulation of *JAZ6* expression in *lux-6* compared to WT, while Ezer *et al*. (2017) did not observe deregulation of *JAZ6* expression in *lux-4* mutant plants. The reason for these conflicting reports is not clear.

I have used a combination of experimental data and quantitative techniques to predict *JAZ6* transcriptional regulation by LUX. The upregulation of JAZ6 in *lux-6* mutants is evidence in favour of this (Y. Zhang *et al.*, 2018), but the hypothesis could be further assessed by a more

complex model or experiments which demonstrate LUX repressing *JAZ6* directly such as ChIP-qPCR. The model is restricted by its simplicity, and the lack of experimental kinematic data for JAZ6 behaviour. Other circadian clock genes which are not present in the simplistic model may be responsible for regulating JAZ6, possibly in addition to LUX.

JAZ6 is also regulated at the post-translational level by jasmonic acid (Chini *et al.*, 2007; Sheard *et al.*, 2010). This post-translational regulation could be influenced by the clock, indirectly through central circadian clock regulator TOC1 transcriptional repression of jasmonic acid synthesis genes (Grundy, Stoker and Carré, 2015; Atamian and Harmer, 2016). The post-translational regulation of JAZ6 by jasmonic acid levels was represented by parameterising JA levels over the day (Figure 2.4.10), which vary diurnally (Goodspeed *et al.*, 2012). Surprisingly, diurnal fluctuations in JA did not appear to alter the pattern of JAZ6 protein abundance from the pattern of *JAZ6* expression (Figure 2.4.11). It is not yet established if the diurnal fluctuations in jasmonic acid significantly alter JAZ6 degradation experimentally, but it is still surprising that the model would not consider jasmonic acid to have a significant effect. This would likely be due to inaccurate parameters used for the repressive effect of jasmonic acid levels on JAZ6. Further experimental work would seek to revise the model by varying the strength of the repression of JAZ6 by jasmonic acid. Ideally this would be validated with experimental data on the levels of JAZ6 protein across the day.

During this modelling, it was assumed that the daily fluctuations in jasmonic acid were sufficient to degrade JAZ6 proportionally to their concentration. This may not be true, as it is stress-induced increases in jasmonic acid which are known to degrade JAZ6 (Wasternack and Hause, 2013), which may be significantly larger than circadian fluctuations in jasmonic acid. For example ozone stress in tomato plants induced the concentration of jasmonic acid to increase 13-fold (Zadra, Borgogni and Marucchini, 2006). Despite this induction, no change in the concentration of methyl-jasmonate was observed, which leads to the second point. The model was fitted to experimental data on the levels of jasmonates (Goodspeed *et al.*, 2012), which may not correspond to the levels of the bioactive jasmonate: (+)-7-iso-Jasmonoyl-L-isoleucine (Fonseca *et al.*, 2009).

2.5.2 Selected yeast-2-hybrid assay results were repeated in BiFC

Only one of the seven protein-protein binding partners of JAZ6 in Yeast-2-hybrid (Y2H) was shown to bind *in planta* with BiFC. While this may seem like a low number, several factors influence the repeatability of Y2H results in BiFC. While comparatively inexpensive and easy to perform, Y2H screens can produce false positives (Xing *et al.*, 2016). One type of false positive concerns binding which is possible but does not occur in plants due to spatial or temporal reasons. With yeast being a unicellular organism, during Y2H screening the bait and prey parts of the system are produced in the same cell, while *in planta* this may not be the case (i.e. expression at different times and/or different tissues). However, in the BiFC system, both putative interactors are located at the same time and in the same tissue under a constitutive promoter, and hence interactions observed in yeast should be also observed in this system. Also, an advantage of BiFC is that proteins would need to be localised to specific subcellular locations such as the nucleus, to interact so protein-protein binding seen in Y2H may not be repeated due to different subcellular localisation for the two proteins. In both systems (Y2H and BiFC), interactors are linked to different moieties, "reporter" proteins, which may allow or permit interactions in one but not the other system. These moieties may prevent protein-protein binding, possibly by steric inhibition and physically blocking the interaction site, or by causing larger conformational shifts in the protein structure which disrupt the binding site. Another explanation would be the different cellular environment, which will contain very different background proteins. BiFC is a technique prone to both false positives and false negatives (Horstman *et al.*, 2014), and so could produce false-negative results for proteins which bind in the native context. One potential reason for this is the steric inhibition the fragments of YFP cause when they are on proteins. Despite the multiple possible orientations of pBIFP vector combinations to prevent steric hindrance (Azimzadeh *et al.*, 2008), YFP fragments are large compared to JAZ6 and the interactors and may still prevent proteins from binding in particular orientations.

It is surprising that BiFC assays do not show JAZ6 and FHY3 binding (Figure 2.4.14), given this interaction was one of the strongest reported from Y2H screening (Stoker, 2016), passing a more stringent threshold than other protein binding results. This interaction has also been reported in the literature using BiFC assays with different vectors (Y. Liu *et al.*, 2019). The different vectors may result in different amounts of the protein made or change the

potential for steric inhibition from the reporter proteins. The pBIFP constructs used here have been validated though showing FHY3-EIN3 protein-protein binding (Figure 2.4.14), which has been previously described (Liu *et al.*, 2017). Therefore, FHY3 is still able to bind other proteins when tagged with YFP fragments in pBIFP constructs, and the negative result seen here is likely a false negative.

2.5.3 PFP binds to EAR domains

JAZ6 binding to PFP occurs in three of eight possible vector combinations (Table 2.4.1), which is likely restricted to those three orientations due to steric hindrance by the YFP fragment tags. This is supported by JAZ6 binding to PFP in Y2H (Figure 2.4.21) and co-immunoprecipitation (Figure 2.4.22). Multiple techniques validating the protein-protein binding in different systems with different constructs gives confidence that the interaction is real and not a false positive, which are common for Y2H and BiFC (Horstman *et al.*, 2014; Xing *et al.*, 2016). Further evidence for JAZ6 PFP binding in planta comes from co-immunoprecipitation assays with both anti-GFP and anti-HA pulldown (Figures 2.4.22 & 23).

However, it would be ideal to show that JAZ6 binds PFP in the native context in Arabidopsis. Co-immunoprecipitation on lines overexpressing *JAZ6* or *PFP* would be a possible approach to show JAZ6-PFP protein-protein binding in Arabidopsis, though the high amounts of protein may lead to false positives. Expressing tagged versions under native promoters would accurately match native protein levels, however the amount of protein acquired may be too small to detect easily. Attempts were made to measure native levels of JAZ6 with an anti-JAZ6 antibody. While transient overexpression of JAZ6-GFP in *N. benthamiana* was detectable, native protein levels were very difficult to detect. Complementing a *jaz6* mutant line with tagged JAZ6 would give confidence that the JAZ6 is functional and able to bind to its protein-protein binding partners, and such a line could be used in co-immunoprecipitation mass spectroscopy to identify proteins in multimeric complexes with JAZ6.

PFP also binds to JAZ5 (Figure 2.4.19), which was not seen in previous Y2H assays (Stoker, 2016). This could be due to the different cellular environment and different reporter moieties attached to proteins of interest in BiFC compared to Y2H, as above. However, it is

not surprising that JAZ5 binds PFP, as the ZIM, Jas, and EAR domains of JAZ5 and JAZ6 have extremely similar amino acid sequences (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). However, as *JAZ5* is not necessary for diurnal variation in susceptibility to *B. cinerea* (Ingle *et al.*, 2015), this implies that PFP is not specifically binding to JAZ6 to regulate circadian susceptibility. This is as we would expect JAZ5 to complement the role of JAZ6 in a *jaz6-3* mutant by binding to PFP.

PFP binds to JAZ5 and JAZ6 but not JAZ1 or JAZ8. These are all similar JAZ proteins, containing similar domains, though JAZ1 lacks EAR domains and JAZ8 lacks the DLNxxPT EAR domain (Figure 2.4.15). As such, the fact that PFP does not bind to JAZ1 or JAZ8 is suggestive of PFP binding to a domain unique to JAZ5 and JAZ6, which could be the DLNxxPT domain. This hypothesis is also supported by the BiFC of domain deletions and mutations of JAZ6 (Figure 2.4.18). These show that Jas and ZIM domains are dispensable for binding to PFP, while mutating both the EAR domains appears to abolish protein-protein binding. Future experiments would determine which EAR domain is important for both TPL binding and PFP.

2.5.4 PFP may be involved in the same protein complexes as JAZ6

To evaluate if PFP could be involved in the same multimeric protein complexes as JAZ6, BiFC was also used to test the interaction of PFP with known binding partners of JAZ6: FHY3 (Figure 2.4.14), and TPL (Figure 2.4.17). PFP did appear to bind to both proteins in BiFC, in multiple orientations, suggesting that it could be present in the same protein complexes as JAZ6. Evidence that PFP binds to FHY3 and TPL is also suggestive of a function for PFP in controlling gene expression by binding to these two proteins, as it is an epigenetic regulator of gene expression possibly through ubiquitinating histones like its orthologues in humans and rice (Adhikary *et al.*, 2019; Zheng *et al.*, 2022).

Validation using other protein-protein binding techniques could give more confidence that JAZ6 and PFP are present in the same protein complexes. Particularly co-immunoprecipitation mass spectrometry would be informative as to protein complexes JAZ6 and PFP are involved with. However, protein complexes are dynamic and PFP may not necessarily bind FHY3, TPL, and JAZ6 all at the same time, which may be elucidated by

further understanding of protein domains which are key for protein-protein binding and competitive binding assays.

2.5.5 PFP does not stabilise or destabilise JAZ6

The orthologues of PFP in rice and humans act as ubiquitin ligases (Adhikary *et al.*, 2019; Zheng *et al.*, 2022). JAZ6 is degraded by the E3 ubiquitin ligase COI1 (Sheard *et al.*, 2010), and another JAZ protein JAZ12 is stabilised by the E3 ubiquitin ligase KEEP ON GOING (KEG) (Pauwels *et al.*, 2015). Therefore, a stability assay was conducted to assess how PFP might affect JAZ6 protein stability. Here no significant stabilisation or destabilisation of JAZ6 by PFP was observed (Figure 2.4.24). This may reflect how PFP binds to an EAR domain in JAZ6 as shown above, leaving the Jas domain open for COI1. The lack of stabilisation or destabilisation implies the functional role of JAZ6-PFP interaction may not be the regulation of JAZ6 activity. Instead, it may be the repression of PFP by JAZ6, as with MYC2 (Fernández-Calvo *et al.*, 2011). This assay could be validated by precise *in vitro* assays with specific amounts of each protein, and concentration gradients, as a potential effect of PFP on JAZ6 stability may be too subtle for the *in planta* approach compared to COI1 degradation of JAZ6. This work will further elucidate the function of JAZ6 binding to PFP.

In summary, JAZ6 binds PFP via its EAR domain(s). However, binding to JAZ5 indicates that PFP may not be responsible for diurnal variation in susceptibility to *B. cinerea*. Evidence from BiFC thoroughly supports protein-protein interaction between JAZ6 and PFP, localised to the nucleus. Despite the caveats associated with this technique, this interaction is supported by Y2H and co-immunoprecipitation. The functional role of JAZ6-PFP protein-protein binding is explored in Chapter 3 of this thesis.

3: *JAZ6* and *PFP* have antagonistic roles in pathogen susceptibility

3.1 Introduction

The previous chapter validated the novel protein-protein binding partners of JAZ6, and the specificity of this binding. That work leads to further questions on identifying what the role of these binding partners in plant defence response is, and what is the mechanism to influence plant defence; objectives 3 and 4 (Section 1.7). In order to answer these questions a reverse genetics approach was used, as will be explained in this chapter.

The classic reverse genetics approach involves disrupting a gene of interest to infer its function (O'Malley and Ecker, 2010). This is achieved using knock down, knock out, or overexpression. By altering gene expression in one of these ways, phenotypic changes (compared to wildtype) in the mutants can imply the involvement of the gene in the phenotype of interest. However, even with changes in gene expression there are cases where phenotypic changes are not observed. There are several reasons for this apparent lack of phenotypic differences, including low precision of our observations and functional redundancy between genes, in addition to not knowing the phenotype to test for in the first place. For *JAZ* genes functional redundancy has previously been noted, with a high degree of complementation possible between *JAZ* genes (Q. Guo *et al.*, 2018; Liu *et al.*, 2021). To better elucidate the function of multiple genes with overlapping functions, or genes acting in the same pathway, plants with multiple mutations can be constructed by cross-breeding Arabidopsis (Li, Altschmied and Chory, 1994), which has been used to construct multiple *JAZ* gene Arabidopsis mutants (Q. Guo *et al.*, 2018; Liu *et al.*, 2021). An example of functional redundancy is the involvement of *JAZ* genes in pollen development - whereas single mutants possess mild or absent phenotypes, the decuple (10 out of 13 JAZ genes) mutant is almost sterile due to complications from the disruption of pollen development (Liu *et al.*, 2021).

For Arabidopsis, floral dipping with *Agrobacterium* for T-DNA insertion is a particularly useful method for generating mutant lines of interest (Clough and Bent, 1998), and libraries of T-DNA insertions provide useful sources of knockdown and knockout mutants (O'Malley, Barragan and Ecker, 2015). In rare cases, T-DNA insertions in regulatory regions can also

result in overexpression such as *jaz7* (Thatcher *et al.*, 2016), by preventing repression of transcription. Floral dipping can also be used to generate plants with overexpression of a gene or gene fragment of interest under strong, constitutive promoters (Clough and Bent, 1998). This allows for phenotypic assessment of the impact of overexpression in a target plant, which may be combined with data from knockdown or knockout mutants. However, overexpression may not represent the true properties of a gene of interest, as it can disrupt the balance of proteins in protein complexes and cause non-canonical protein interactions by saturating the cellular environment. T-DNA insertions may also be targeted for gene silencing (Gao and Zhao, 2013), or the target gene for overexpression degraded post-translationally. Despite these limitations, reverse genetics remains a thorough and detailed approach for inferring the activity of genes of interest. More recently, CRISPR editing has been used to develop mutant lines of interest (Gao *et al.*, 2016; Miki *et al.*, 2018). This editing technique allows for precise changes in DNA sequences, including specific amino acid deletions avoiding a frameshift, or single nucleotide insertions to cause a frameshift, or inserting an early stop into a sequence. Alternatively, CRISPR may be used to insert fluorescent proteins or tags onto native coding sequences for genes of interest (Miki *et al.*, 2018).

The JAZ proteins can influence susceptibility to *B. cinerea*, for example *jaz6* and *jaz8* mutants are more resistant to *B. cinerea* infection than WT plants (Li *et al.*, 2019). Quintuple *jazQ* mutants deficient in JAZ1,3,4,9,10 are more susceptible to *B. cinerea* (Q. Guo *et al.*, 2018), while decuple *jazD* mutants deficient in JAZ1-7,9,10,13 are more resistant (Q. Guo *et al.*, 2018). Arabidopsis is more susceptible to *B. cinerea* after inoculation at dusk compared to dawn (Ingle *et al.*, 2015), however *jaz6* mutants lack the circadian-driven increase in susceptibility to *B. cinerea*. Specifically, it was shown that *jaz6* mutants lack a difference in disease progression between plants infected at dawn compared to at night, while Col-0 wild type plants show a clear difference (Ingle *et al.*, 2015), which may be termed circadian susceptibility. JAZ6 is thus necessary for circadian susceptibility, in contrast to JAZ5, 7, and 10 which have been linked to general plant defence (Thatcher *et al.*, 2016). Inoculation of WT Arabidopsis results in differential expression of key plant defences against *B. cinerea*, plant defensins (Ingle *et al.*, 2015).

JAZ6 may selectively bind a transcription factor to regulate expression of plant defensins, causing differential expression of defence proteins at different times of day. One potential JAZ6 interactor is PFP (PHD finder domain containing protein). It is a member of the UBR (ubiquitin ligase N-recognin) protein family conserved across eukaryotes (Tasaki *et al.*, 2005), which are known for their role in N-end mediated protein degradation (Garzón *et al.*, 2007). The N-end rule determines proteins to be degraded on perception of external abiotic stresses (Vicente *et al.*, 2017), but has also been linked to pathogen responses (de Marchi *et al.*, 2016).

Despite containing a UBR domain PFP does not exhibit N-end target specificity (Garzón *et al.*, 2007), and the human HsUBR7 has instead been linked to chromatin regulation through histone monoubiquitination by its PHD (plant homeodomain) finger (Adhikary *et al.*, 2019). In tobacco, NbUBR7 has been demonstrated to be a negative regulator of virus defence through targeting the N protein for degradation (Yongliang Zhang *et al.*, 2019, p. 7). While in Arabidopsis, PFP has been shown to regulate flowering time through FLC (Yokoyama, Kobayashi and Kidou, 2019), hypothetically by monoubiquitinating histones (Cao *et al.*, 2008). PFP also contains a DLNxxP EAR domain (Kagale, Links and Rozwadowski, 2010), which suggests a possible role in ethylene-responsive transcriptional repression.

The most similar protein to PFP is another member of the UBR family, BIG (Tasaki *et al.*, 2005; Garzón *et al.*, 2007), which possesses a UBR domain like PFP but not a PHD domain. BIG has previously been established to be a regulator of the circadian clock (Hearn *et al.*, 2018). BIG has also been shown to influence plant defence (Ruo-Xi Zhang *et al.*, 2019), in particular through jasmonic acid production and control of stomata.

Another similar protein to PFP is EBS (Early bolting in short days) (Piñeiro *et al.*, 2003), which possesses a PHD domain but not a UBR domain. EBS regulates flowering through chromatin remodelling of key flowering genes (Yang *et al.*, 2018), which PFP may also direct (Yokoyama, Kobayashi and Kidou, 2019). However, PFP and its homologues in other species exhibit an atypical PHD domain with the key zinc binding amino acids CCHHCC instead of

CCHCCC (Adhikary *et al.*, 2019). This sequence is not seen in other PHD domain proteins in plants, including EBS.

Here we have elucidated the function of the interaction of JAZ6 with PFP, including selective control of JA responses during pathogen attack, presenting a target for manipulation in crop plants for increased pathogen resistance.

3.2 Aims

The main goal of this chapter is to understand how *JAZ6* and *PFP* regulate susceptibility to disease, with a focus on how *JAZ6* is promoting susceptibility to *B. cinerea*.
It has already been established that *JAZ6* promotes susceptibility to necrotrophic pathogens (Li *et al.*, 2019; Liu *et al.*, 2021), while *PFP* represses flowering and has no previously known role in disease in Arabidopsis (Yokoyama, Kobayashi and Kidou, 2019). Therefore, this chapter investigates more widely the roles of *JAZ6* and *PFP* in disease resistance against necrotrophic and also biotrophic pathogens, as well as flowering, including any synergistic or antagonistic effects. A reverse genetic approach was necessary in order to achieve these goals.

3.3 Methods

3.3.1 Plant material

Arabidopsis thaliana L. Heynh. (Arabidopsis) Columbia-0 accession (Col 0, WT, N1092) were used as wild type plants. The *jaz6-3* (SAIL_1156_C06) and *pfp-1* (SALK_034619) alleles were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The double *jaz6-3 pfp-1* (SAIL_1156_C06 and SALK_034619 insertions) mutant line was generated by crossing the single mutant lines in this work, as detailed further below. The *jaz6-31* CRISPR mutant line was described previously (Li *et al.*, 2019), and was provided by Professor Ying Fu. The PFP-Ox overexpression line, as described previously (Yokoyama, Kobayashi and Kidou, 2019), was provided by Professor Shin-Ichiro Kidou.

3.3.2 Plant growth

Plants were grown in F2 compost (Levington), stratified for 2 days at 4°C covered with tinfoil, and then placed in either growth cabinets (for infection and flowering time assays) or the glasshouse (for all other experiments), as detailed in Table 3.1. Arabidopsis was grown in 2cm^2 square pots (Desch plant-pak), *N. benthamiana* was grown in 7cm^2 (7K) square pots (Desch plant-pak). The University of York horticulture team assisted in pot filling and plant cultivation.

For sterile growth, as in root assays, Arabidopsis seeds were sterilized in 1% sodium hypochlorite. After vortexing for 3 minutes the sodium hypochlorite was removed and seeds were washed four times with milli-Q dH_2O (Millipore). Seeds were then sown onto ½ MS (0.5x Murashige and Skoog) solid media (Duchefa Biochemie) 1cm apart on 11cm^2 square Petri dishes (Murashige and Skoog, 1962). Specifically: 1% (w/v) phytoagar (Nacalai Tesque), 1% (w/v) sucrose, 0.46% (w/v) Murashige and Skoog salts including vitamins, 0.05% (w/v) 2-(N-morpholino)ethane sulphonic acid at pH 5.7. These were placed in growth cabinets as described in Table 3.1.

Table 3.1: Plant growth conditions

Parameter	Growth cabinet	Glasshouse
Photoperiod	16h (8h for short-day)	Ambient
Temperature	20°C	20°C (day), 17°C (night)
Relative Humidity	60%	Ambient
Light Intensity	100 µmol.m^2.s^{-1}	Ambient, with supplementary light from sodium lamps when weather conditions reduced light intensity during the day
CO_2 concentration	350 ppm	Ambient

3.3.3 Microbial growth and subculturing

Botrytis cinerea pathovar Pepper (Denby, Kumar and Kliebenstein, 2004) was cultured on sterile tinned apricot halves in sealed petri dishes in constant dark at 25°C. Every two weeks, spores were collected by washing and then scraping spores off the apricots with pipette tips into 1mL of sterile water in a class II flow cabinet. The spore suspension was then filtered through Miracloth (Merk) and used to inoculate fresh apricot halves or in infection assays as detailed below. This was performed with the assistance of Dr Gillian Higgins.

Sclerotinia sclerotiorum isolate L6 (Taylor *et al.*, 2018) was cultured on soil which was treated with *S. sclerotiorum,* as described previously (Clarkson *et al.*, 2014). Briefly, sclerotia were produced by inoculation of wheat germ. Sclerotia were then buried in F2 compost for one month to produce apothecia. Ascospores were collected from apothecia by vacuuming spores onto filter paper. Filter papers were then agitated in distilled water and filtered through Miracloth (Merk) to isolate ascospores for use in infection assays as detailed below. This was performed with the assistance of Dr Gillian Higgins.

Hyaloperonospora arabidopsidis isolate Noks-1 was revived from -80°C frozen stock of infected Wassilewskija Ws-0 ecotype *eds-1* mutant Arabidopsis (Parker *et al.*, 1996), as described (Rehmany *et al.*, 2005). *H. arabidopsidis* was cultured on Arabidopsis Col0 in sealed propagator trays for one week, after which the infected above ground parts of seedlings were cut. The infected plant parts were agitated in distilled water which was then filtered through Miracloth (Merk) and used to inoculate fresh Arabidopsis seedlings or in infection assays as detailed below. This was performed with the assistance of Dr Sarah Harvey and Dr Theresa Catania.

3.3.4 Genotyping plant lines

Arabidopsis SALK and SAIL T-DNA insertion lines were genotyped using PCR to ensure homozygosity. Genomic DNA was extracted using 5% sucrose solution as previously described (Berendzen *et al.*, 2005). Primers were supplied by Integrated DNA Technologies and are detailed in the Appendix. Three week old plants were used for genotyping.

3.3.5 Gel electrophoresis and sequencing

Gel electrophoresis for DNA was performed at 100V for 1 hour. Gels consisted of 1% (w/v) agarose and 1xTris-Borate-EDTA, with 0.00002% (w/v) ethidium bromide (Sigma-Aldrich). Unless otherwise stated Quick-Load® Purple 1 kb DNA Ladder (New England Biolabs) was used as a size marker. Sanger sequencing was performed by Eurofins Genomics GATC.

3.3.6 Generating a double mutant

The *pfp-1* SALK and *jaz6-3* SAIL T-DNA insertion lines detailed above were crossed, by the application of pollen from the SAIL line onto the flowers of the SALK line for 6-week-old

Arabidopsis plants. The offspring were selected on BASTA for two generations to identify lines carrying the *jaz6-3* SAIL T-DNA insertion, as this T-DNA insertion confers resistance to BASTA. F2 seed was collected from F1 plants which had survived BASTA selection, indicating these F1 plants were heterozygous for both T-DNA insertions, termed double-heterozygous mutants. The remaining surviving plants conformed to a segregation ratio of 1:15 (double-homozygous mutants : non-double-homozygous mutants), as in the segregation Table 3.2 below. Confirmation of homozygosity was performed as with genotyping the single T-DNA insertion lines above by PCR. Three week old plants were used for genotyping.

Table 3.2 Two loci cross F_2 generation segregation

Gametes from heterozygous parent	*PFP, JAZ6*	*pfp-1, JAZ6*	*PFP, jaz6-3*	*pfp-1, jaz6-3*
PFP, JAZ6	*PFP, JAZ6*	Heterozygous *pfp-1, JAZ6*	*PFP,* heterozygous *jaz6-3*	Heterozygous *pfp-1* and *jaz6-3*
pfp-1, JAZ6	Heterozygous *pfp-1, JAZ6*	Homozygous *pfp-1, JAZ6*	Heterozygous *pfp-1* and *jaz6-3*	Homozygous *pfp-1*, heterozygous *jaz6-3*
PFP, jaz6-3	*PFP,* heterozygous *jaz6-3*	Heterozygous *pfp-1* and *jaz6-3*	PFP, homozygous *jaz6-3*	Heterozygous *pfp-1,* homozygous *jaz6-3*
pfp-1, jaz6-3	Heterozygous *pfp-1* and *jaz6-3*	Homozygous *pfp-1*, heterozygous *jaz6-3*	Heterozygous *pfp-1,* homozygous *jaz6-3*	Homozygous *pfp-1* and *jaz6-3*

Yellow highlight indicates double-homozygous mutants.

3.3.7 Root growth inhibition assays

Root growth inhibition assays with Arabidopsis plants grown on vertical Petri dishes were performed as described previously (Li *et al.*, 2019). Arabidopsis primary root length growth under 25 µM methyl-jasmonic acid or DMSO mock treatment imbued into the growth agar was measured after 14 days using FiJi ImageJ (Schindelin *et al.*, 2012). Seeds were placed onto the Petri dishes, and grew for the 14 days until root length was measured.

3.3.8 Flowering time assays

Flowering time assays with Arabidopsis plants grown in short day (8:16 hours in light:dark conditions) were performed as described previously (Sharma *et al.*, 2016). The number of rosette leaves was measured when the bolting stem extended past 10 mm. Plants were grown for several months, until bolting was observed as detailed above in all plant lines.

3.3.9 Pathogen infection assays

B. cinerea infection assays were performed as described previously (Ingle *et al.*, 2015). Spore suspensions were prepared from *B. cinerea* culture as in Section 2.2, then the number of spores was calculated using a haemocytometer. The spore concentration was then set to 50,000 spores per mL in 50% grape juice, made up to 5 mL with sterile water. Per leaf, one 10 µL drop of inoculum was placed on the centre of detached leaves from four-week-old Arabidopsis, which were arrayed on 0.8% agar in propagation trays along with a 5cm scale bar. Inoculation took place 15 hours into the 16-hour light period, except for infections at subjective dawn which took place 2 hours into the light period. Lesions were photographed 72 hours post inoculation, and Fiji ImageJ was used to measure the lesion area (Schindelin *et al.*, 2012). Plants were grown for exactly four weeks and then infected, phenotypes measured in the next 72 hours.

Alternatively, *B. cinerea* infection trays were placed in a Navautron box with a camera linked to a Raspberry Pi set to take images every 10 minutes, as detailed in a previous publication (Barbacci *et al.*, 2020), from which lesion size growth rate calculation was automated as described. As above, plants were grown for exactly four weeks and then infected, phenotypes measured in the next 72 hours.

S. sclerotiorum infection assays were conducted in a similar manner to *B. cinerea* infection assays, as per a previously described protocol (Clarkson *et al.*, 2014). Spores were isolated as in Section 2.2, then the number of spores was calculated using a haemocytometer. The spore concentration was then set to 150,000 spores per mL in 50% potato dextrose broth guar, made up to 5 mL with sterile water. Per leaf, one 10 µL drop of inoculum was placed on the centre of detached leaves from four-week-old Arabidopsis, which were arrayed on 0.8% agar in propagation trays along with a 5cm scale bar. Inoculation took place on four-week-old Arabidopsis detached leaves 15 hours into the 16-hour light period. As for *B. cinerea* infection, lesions were photographed 72 hours post inoculation, and Fiji ImageJ was used to measure the lesion area (Schindelin *et al.*, 2012). As above, plants were grown for exactly four weeks and then infected, phenotypes measured in the next 72 hours.
H. arabidopsidis infection assays were performed as previously described (Rehmany *et al.*, 2005). Spores were isolated as in Section 2.3, then the number of spores was calculated using a haemocytometer. The spore suspension was set to 30,000 spores per mL with sterile water. Inoculation took place on one-week old Arabidopsis using an airbrush as previously described (Tomé, Steinbrenner and Beynon, 2014). Spores from pools of 20 Arabidopsis seedlings were counted 7 days post-inoculation using a haemocytometer on a light microscope. Plants were grown for one week and then infected, phenotypes measured after one further week.

3.3.10 RNA extraction

Plant material was collected in 1.5 mL Eppendorf tubes and frozen in liquid nitrogen. Samples were ground to a homogenous paste using sterile ceramic mortars and pestles while frozen. RNA was extracted using Machary-Nagal plant RNA extraction kits (Machary-Nagal) with on-column DNAse digestion (Machary-Nagal) as per the manufacturer's instructions. For samples to be sequenced, this was followed by lithium chloride clean-up (Walker and Lorsch, 2013). The resultant RNA was stored at -80°C until analysis. Plant material for sequencing was from an infection assay, as above plants were grown for exactly four weeks and then infected, phenotypes measured in the next 72 hours. For expression after wounding plants were also grown for four weeks.

3.3.11 cDNA synthesis

RNA concentration was measured using a NanoDrop 2000 microvolume spectrophotometer (Fisher), using 1 µL of RNA solution in wavelength absorption spectra analysis. 1µg of total RNA per sample was used as a template for reverse transcription using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific), with anchored-oligo$(dT)_{18}$ primer. The resultant 20 µL of cDNA was diluted 10-fold in 80µl TE buffer and 100 µL milli-Q dH_2O, the concentration was measured using a NanoDrop (Fisher), and then the cDNA stored at -20°C.

3.3.12 Quantitative PCR

cDNA samples were used as templates for quantitative RT-PCR (qPCR) analysis with SYBR™ Green PCR Master Mix (Applied Biosystems), in a CFX96 Touch™ Real-Time PCR Detection System machine (BioRad) with the assistance of Dr Fabian Vaistij. Primers were supplied by Integrated DNA Technologies and are detailed in the Appendix.

Biological samples were analysed in triplicate (n=3), and relative gene expression was quantified using the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001), with *UBIQUITIN 11 (UBQ11, AT4G05050)* as the housekeeping gene for normalisation (Tyler *et al.*, 2004). Primers were assembled by Integrated DNA Technologies, according to sequences given later. Stock primer concentrations were held at 100µM, with 10X dilutions made for using 10µM primers in qPCR reactions.

3.3.13 RNA sequencing

A bioanalyser (Aglient) was used to confirm RNA integrity and concentration by the York Technology Facility. Aliquots were then taken to create solutions with RNA at a concentration of 0.5 mg per mL. Library preparation and subsequent sequencing were performed by Novogene, using 150bp paired end reads.

FastQC was used to verify quality of the raw reads (Andrews, 2019). Following this, the Galaxy platform was used to trim adapters and low quality reads (Q<30) using TrimGalore (Afgan *et al.*, 2016; Krueger, 2019). Pseudoalignment of reads to the Arabidopsis AtRTD3 reference transcriptome (R. Zhang *et al.*, 2022), or *B. cinerea* ASM83294v1 assembly reference transcriptome (Amselem *et al.*, 2011; Van Kan *et al.*, 2017), was then performed

on Galaxy using Salmon (Patro *et al.*, 2017). 3DRNAseq was then used to filter and normalize reads (Guo *et al.*, 2019), before using limmaVoom with Benjamini and Hochberg correction to identify differential expression (adjusted p-value <0.05, 2-fold change minimum) between genotypes and stages of infection (Benjamini and Hochberg, 1995; Law *et al.*, 2014; Ritchie *et al.*, 2015). ShinyGO was used to quantify and graph GO (gene ontology) term enrichment for sets of differentially expressed genes (Ge, Jung and Yao, 2020).

3.3.14 Data processing and statistics

Data processing and statistics were done where appropriate using a combination of Excel and R Studio (RStudio Team, 2020; R Core Team, 2022), with ggplot2 (Wickham, 2009). Where performed, statistical analysis tests were ANOVAs and, where appropriate, Tukey's HSD (honest significant difference) tests (Tukey, 1949). Estimation graphics were also used to produce figures and perform statistical analysis (Ho *et al.*, 2019). Error bars on graphs represent the standard deviation, unless otherwise noted.

3.4 Results

3.4.1 *JAZ6* and *PFP* mutants display altered target gene expression

A reverse genetics approach was taken to characterise the function of *JAZ6*, characterising a T-DNA insertion mutant and supporting results with a CRISPR mutant. Firstly, loss of function *jaz6* mutant lines were obtained from Nottingham Arabidopsis Stock Centre and Professor Fu (China Agricultural University, Beijing). The SAIL1156_C06 mutant (*jaz6-3*) was identified in the T-DNA insertion mutant library (Figure 3.4.1A) and genotyped by PCR to verify homozygosity of the seed stock for the T-DNA insertion interrupting *JAZ6* (Figure 3.4.1B).

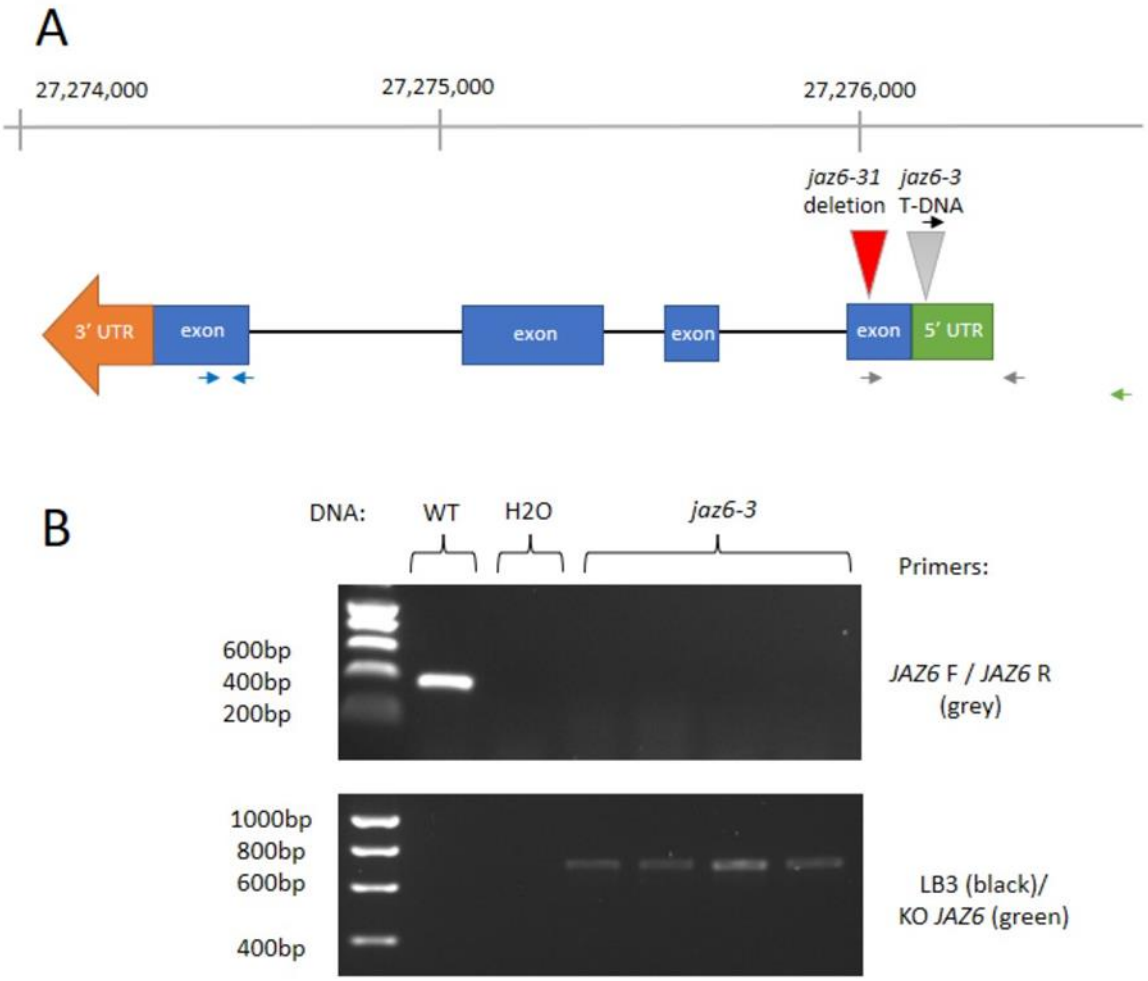


Figure 3.4.1: *JAZ6* gene structure and T-DNA location

(A) Schematic of the genetic map of *JAZ6* including the exons, 3' UTR (untranslated region), 5' UTR, T-DNA insertion site, and primers used for mutant identification and quantitative RT-PCR. Ruler indicates the Arabidopsis chromosome 1 location and total length of the unspliced JAZ6 transcript. Grey arrows indicate positions of the primer pair for genotyping WT JAZ6, green for genotyping *jaz6-3*, blue arrows indicate positions of the primer pair for qRT-PCR.

(B) Representative PCR verification of the T-DNA insert in *jaz6-3*. Expected band size for WT amplification (upper) is 350bp with genomic primers (grey), T-DNA amplification (lower) with the SAIL T-DNA LB3 (black) primer and KO JAZ6 (green) is 700bp. Hyperladder 1kb ladders were used to indicate size.

While *jaz6-1* (SALK_136462) and *jaz6-2* (SALK_038013) T-DNA insertion mutants were previously reported (Yu *et al.*, 2015), they were not used as neither possess T-DNA

insertions in the *JAZ6* gene. The T-DNA insertion for *jaz6-1* is approximately 300bp downstream of *JAZ6* while for *jaz6-2* it is approximately 900bp upstream of *JAZ6*. *jaz6-3* is named for being the third published mutant of *JAZ6* (Ingle *et al.*, 2015). Recently, *jaz6-4* (CSHL_ET30) and *jaz6-5* (AY198742) were backcrossed into a Columbia ecotype background from *Landsberg erecta* (Q. Guo *et al.*, 2018; Hu *et al.*, 2021; Liu *et al.*, 2021).

CRISPR *jaz6-31* mutants provided by Professor Fu were previously described (Li *et al.*, 2019), and verified by sequencing. These mutants possess a 24-nucleotide deletion after the 125th bp of the JAZ6 sequence (Figure 3.4.1A), which results in the deletion of 8 amino acids after the S40 residue and does not result in a frameshift of the open reading frame. With no change in the open reading frame, this mutation would not be predicted to change gene expression.

Previous data on the *jaz6-3* mutant suggested *JAZ6* expression was unchanged in mock treated *jaz6-3* mutant plants compared to WT (Ingle *et al.*, 2015). In order to confirm or otherwise this observation, in this study *JAZ6* expression in the *jaz6-3* mutants was measured to verify whether the T-DNA insertion was impacting *JAZ6*. Real time quantitative RT-PCR measured *JAZ6* expression in WT and *jaz6-3* mutant plants using primers in the 4th exon of *JAZ6* (Figure 3.4.1A), with the expression of housekeeping gene ubiquitin 11 (*UBQ11*) used as a control for relative expression. As previous data on this *jaz6-3* mutant suggested *JAZ6* expression was unaltered in unstressed conditions (Ingle *et al.*, 2015), both WT and *jaz6-3* mutant plants were stressed with wounding, which induces *JAZ6* expression (Chung *et al.*, 2008). The results show a 10-fold decrease in *JAZ6* expression in *jaz6-3* mutants compared to WT plants grown in unstressed conditions (Figure 3.4.2), and a 4-fold decrease in *JAZ6* expression in wounded *jaz6-3* mutants compared to wounded WT plants (Figure 3.4.2). Wounding induces a 5-fold increase in *JAZ6* expression in WT plants, and a 10-fold increase in JAZ6 expression in *jaz6-3* mutant plants. The expression of *JAZ6* in wounded *jaz6-3* mutant plants is like *JAZ6* expression in unwounded WT plants. This data is further supported by RNAseq data showing lower expression of *JAZ6* in mock treated *jaz6-3* mutants than in WT (Figure 3.4.5). Here we also see that *JAZ6* is upregulated following *B. cinerea* attack, in both WT and *jaz6-3* mutants.

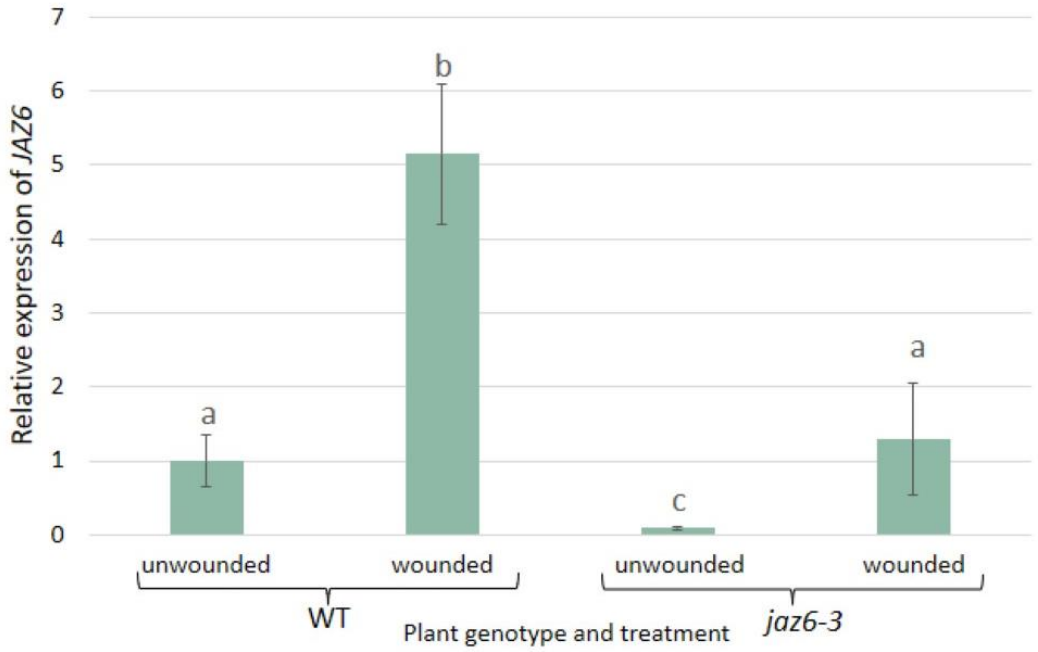


Figure 3.4.2: *jaz6-3* mutants exhibit reduced expression of *JAZ6* compared to wild-type (WT) plants, both in unwounded and wounded conditions.
3 replicates of pooled samples of 3 leaves taken from different 4-week-old Arabidopsis plants were used. Values shown are mean expression values (normalised to *UBQ11,* relative to *JAZ6* expression in unwounded WT plants), and error bars represent standard deviations. ANOVA for DCt values indicated a significant effect of both plant genotype ($P < 0.001$) and wounding treatment ($P < 0.001$) on JAZ6 relative expression. Different letters indicate a significant difference in DCt values, by Tukey's HSD. N=3.

These results differ from Ingle et al., 2015, which did not show differential expression of *JAZ6* in *jaz6-3* mutants in unstressed conditions, or the induction of JAZ6 by jasmonic acid-inductive stress in the form of *B. cinerea* infection. One reason for this would be that mock inoculated leaves used by Ingle et al. were wounded when they were cut from the plant, which induces *JAZ6* expression (Chung *et al.*, 2008). This could explain the seeming lack of difference between mock treated WT and *jaz6-3* plants, as *JAZ6* expression could have been upregulated to similar levels by wounding in both types of plants. Another explanation may be the use of actin (*ACT2*) as a reference housekeeping gene in the previous report, which is downregulated during *B. cinerea* infection (Windram *et al.*, 2012). Together with the

previous point, this could explain the apparent non-responsiveness of *JAZ6* to *B. cinerea* infection in *jaz6-3* mutants, as *JAZ6* could be upregulated by *B. cinerea* to the same degree that *ACT2* is downregulated by *B. cinerea*.

And so, two mutant alleles have been validated for *JAZ6*, for confirming future mutant phenotypes are due to disruption of *JAZ6*. I might predict variation in JAZ6 expression between *jaz6-3* and *jaz6-31* as *jaz6-31* is a small deletion which does not change the reading frame, so may not alter transcription of *JAZ6*.

Similarly, a reverse genetics approach was taken to characterise *PFP*, with a T-DNA mutant line and supporting results from a line overexpressing *PFP* (Yokoyama, Kobayashi and Kidou, 2019). There are multiple T-DNA insertion SALK lines identified with the same apparent insertion site in *PFP*: SALK_034281, SALK_034317, and SALK_034619 (Figure 3.4.3A). The insertion site for these lines is the result of a sequencing error (O'Malley, Barragan and Ecker, 2015), only one of these lines would contain an insertion in *PFP*, so PCR was used to investigate which line contained this insertion (Figure 3.4.3B). PCR showed that the SALK_034619 line contained the insertion (Figure 3.4.3B). This line has been independently verified and characterised as a knock-down mutant called *pfp-1* (Yokoyama, Kobayashi and Kidou, 2019), affecting flowering time. RNAseq data shows lower expression of *PFP* in *pfp-1* mutants than in WT (Figure 3.4.5). Here we also see that *PFP* is upregulated in response to *B. cinerea* infection.

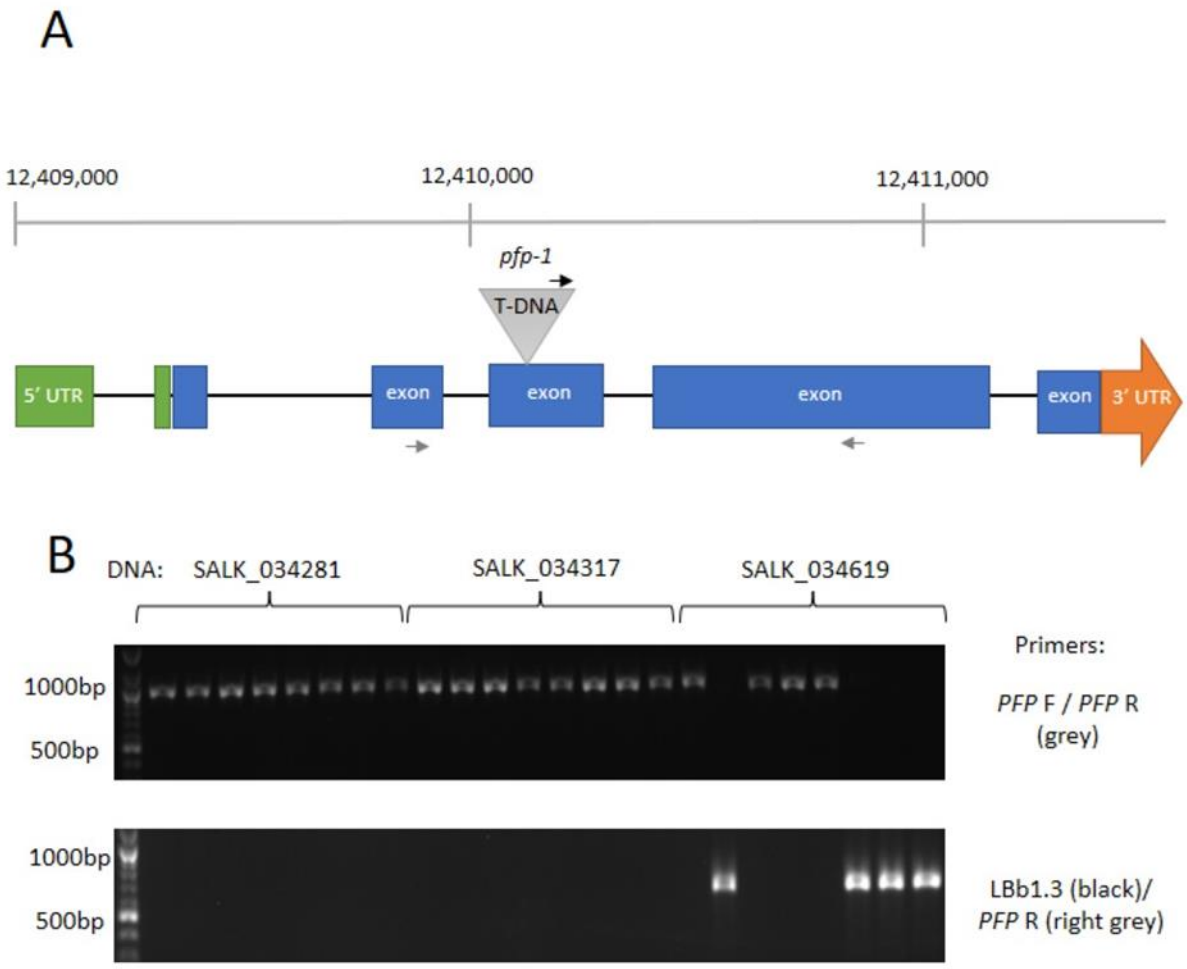


Figure 3.4.3: *PFP* gene structure and T-DNA location

(A) Schematic of the genetic map of *PFP* including the exons, 3' UTR (untranslated region), 5' UTR, T-DNA insertion site, and primers used for mutant identification and quantitative RT-PCR. Ruler indicates the Arabidopsis chromosome 4 location and total length of the unspliced *PFP* transcript. Grey arrows indicate positions of the primer pair for genotyping.

(B) Representative PCR verification of the T-DNA insert in *pfp-1*. Expected band size for WT amplification (top) is 1000bp, T-DNA amplification (bottom) is 750bp. Quick-load 100 bp purple ladder was used to indicate size.

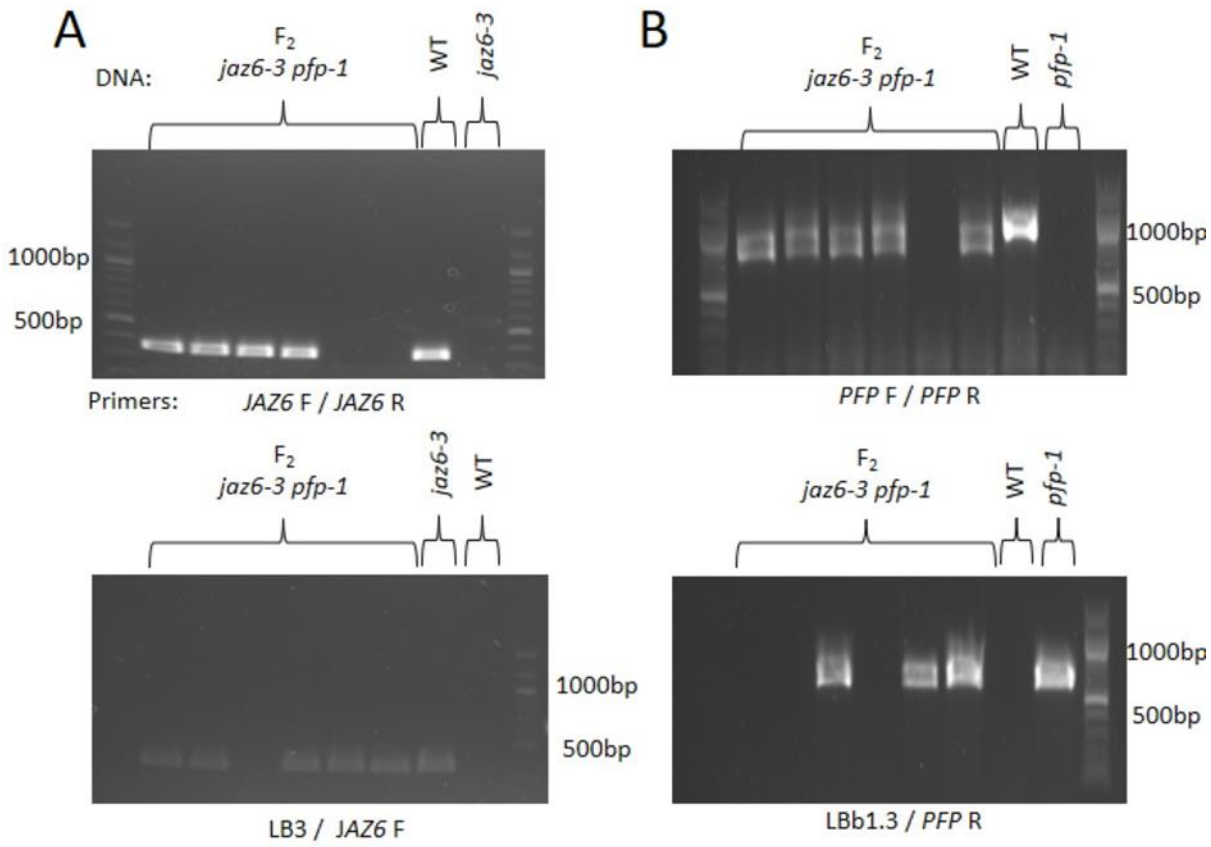


Figure 3.4.4: *JAZ6 PFP* double mutant characterisation

(A) Representative PCR verification of *jaz6-3* T-DNA inserts in F2 *jaz6-3 pfp-1* double mutants. Primers used as previously described (Figure 3.4.1). Expected band size for WT *JAZ6* amplification (top) is 350bp, *jaz6-3* T-DNA amplification (bottom) is 200bp as JAZ6 R instead of KO JAZ6 was used in the previous slide. Of the six plants tested here, the fifth and sixth plants are homozygous for a *jaz6-3* insertion. Quick-load 100 bp purple ladder was used to indicate size.

(B) Representative PCR verification of *pfp-1* T-DNA inserts in F2 *jaz6-3 pfp-1* double mutants. Primers used as previously described (Figure 3.4.3). Expected band size for WT *PFP* amplification (top) is 1000bp, *pfp-1* T-DNA amplification (bottom) is 750bp. Of the six plants tested here, the fifth plant is homozygous for a *pfp-1* insertion. This sample corresponds to the fifth sample on the left, making it a double mutant. Quick-load 100 bp purple ladder was used to indicate size.

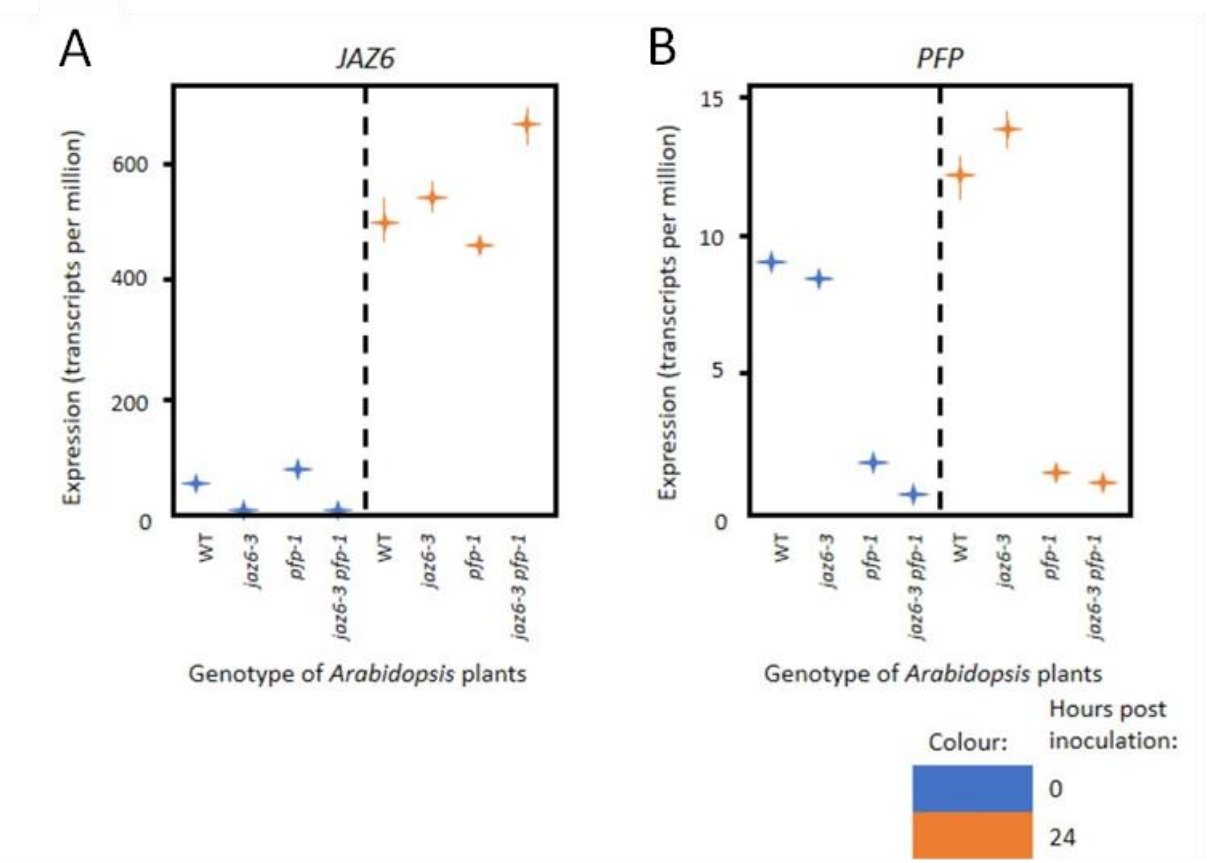


Figure 3.4.5: *JAZ6* and *PFP* gene expression is reduced in the *jaz6-3 pfp-1* double mutant.

Expression in transcripts per million for *JAZ6* (A) and *PFP* (B). Arabidopsis leaf samples were taken before (0 hpi) and during (24 hpi) *B. cinerea* infection. There are significant differences and a larger than 2-fold change in gene expression for these genes in in 3DRNAseq, between WT and the relevant single or double mutant.

To investigate possible synergistic regulatory interactions between *JAZ6* and *PFP*, the double mutant line of *jaz6-3 pfp-1* was created. Double mutant *jaz6-3 pfp-1* plants were constructed by manual cross-pollination (Li, Altschmied and Chory, 1994), with a maternal *pfp-1* and paternal *jaz6-3*. After self-pollination of F_1 plants selected for resistance to BASTA, indicative of the presence of the *jaz6-3* T-DNA insertion, homozygous double mutants were identified from the F_2 generation by PCR (Figure 3.4.4). RNAseq data shows lower expression of both *JAZ6* and *PFP* in *jaz6-3 pfp-1* mutants than in WT (Figure 3.4.5).

3.4.2 *JAZ6* prepresses resistance to necrotrophic pathogens

Previous publications have reported the involvement of *JAZ6* in susceptibility to *B. cinerea* (Ingle *et al.*, 2015; Li *et al.*, 2019; Liu *et al.*, 2021). The *jaz6-3* mutant was previously characterised for a role in diurnally oscillating resistance to *B. cinerea* infection (Ingle *et al.*, 2015). It was shown that WT plants exhibited higher susceptibility to *B. cinerea* after inoculation after dusk (ZT18) compared to dawn (ZT0), while *jaz6-3* mutant plants did not display a difference in *B. cinerea* susceptibility between dusk and dawn (Ingle *et al.*, 2015). However, the susceptibility to *B. cinerea* of *jaz6-3* mutants was not compared to WT susceptibility (Ingle *et al.*, 2015). Recently, other *JAZ6* mutants have been characterised in direct comparisons to WT plants (Li *et al.*, 2019; Liu *et al.*, 2021). It was shown that *jaz6-18* and *jaz6-31* CRISPR mutants were more resistant to *B. cinerea* infection (Li *et al.*, 2019), while *jaz6-5* mutants were more resistant to *B. cinerea* and *P. syringae* infection (Liu *et al.*, 2021). However, the time-of-day of inoculation was not stated.

To characterise the role of *JAZ6* in disease susceptibility specifically at dusk, experiments were conducted to investigate the susceptibility of *jaz6-3* and *jaz6-31* mutants. These experiments involved application of virulent fungal spore suspension to cut leaves embedded into an agar tray. To measure pathogen growth, the spread of lesions on leaves was captured in images taken between 55 and 88 hours after inoculation, depending on the speed of infection for the inoculum used.

Initially, an experiment was performed to verify the role of *JAZ6* in diurnal variation in susceptibility to *B. cinerea* infection (Figure 3.4.7), which also aimed to verify the susceptibility of the *jaz6-3* mutant to *B. cinerea*. This assay was performed with the assistance of Dr Gillian Higgins. *JAZ6* was previously shown to be essential for diurnal variation in susceptibility in *B. cinerea* infection (Ingle *et al.*, 2015). However, the susceptibility of *jaz6-3* mutants was not directly compared to the susceptibility of WT plants. Therefore, this diurnal infection assay was performed. One difference between this diurnal infection assay and the published work is the timing of the dusk inoculation, which is ZT16 here instead of ZT18. This change is because infection at ZT18 would be during the subjective night, as the plants experience 16 hours of light in a photoperiod. The change removes a potential effect on disease susceptibility from circadian stress (Nitschke *et al.*,

2016), which could cause jasmonic acid production at ZT18. With inoculation at either ZT16 or ZT18 WT plants are more susceptible than inoculation at dawn, ZT0 (Ingle *et al.*, 2015). The dawn inoculation is moved to ZT2 for similar reasons, as significant amounts of time would be taken to detach leaves and so starting to cut leaves before ZT0 would disrupt their circadian clock with a sudden period of light. The previous report indicates that with inoculation at ZT2 plants were not more susceptible than inoculation at dawn, ZT0 (Ingle *et al.*, 2015). Here we see that WT plants inoculated at dawn were less susceptible to *B. cinerea* infection than after inoculation at dusk (Figure 3.4.7B), as previously reported (Li *et al.*, 2019). We also see that *jaz6-3* mutants lack this phenotype with no difference in susceptibility due to inoculation at dawn or dusk (Figure 3.4.7B), also previously shown (Ingle *et al.*, 2015).

It was previously reported that CRISPR *jaz6-31* mutant lines were more resistant to *B. cinerea* infection than WT plants (Li *et al.*, 2019). Here CRISPR *jaz6-31* mutants were resistant to *B. cinerea* (Figure 3.4.8), the same resistance phenotype as the *jaz6-3* T-DNA insertion mutant line. Verifying these independent mutant lines share the same phenotype demonstrates that the disruption of *JAZ6* causes increased resistance to *B. cinerea* compared to WT plants, as any potential off target effects will be different in these independent lines.

To assess the involvement of JAZ6 in necrotrophic pathogen resistance more broadly, infection was conducted with the pathogen *S. sclerotiorum* (Figure 3.4.9). *S. sclerotiorum* is a necrotrophic fungal pathogen like *B. cinerea* (Bolton, Thomma and Nelson, 2006; Williamson *et al.*, 2007). They are taxonomically closely related, and are both within the Sclerotiniaceae family of fungi (Amselem *et al.*, 2011). Both have a broad host range of plant species of more than 200 species as opportunistic pathogens colonising fruit and wounds (Blancard, 2012), causing diseases after crops have been harvested such as postharvest rot in carrots (Hocking, 2014). Infections with *S. sclerotiorum* showed *jaz6-3* mutants less susceptible to infection compared to wildtype (Figure 3.4.9), like jaz6-3 mutants less susceptible to *B. cinerea* (Figure 3.4.8).

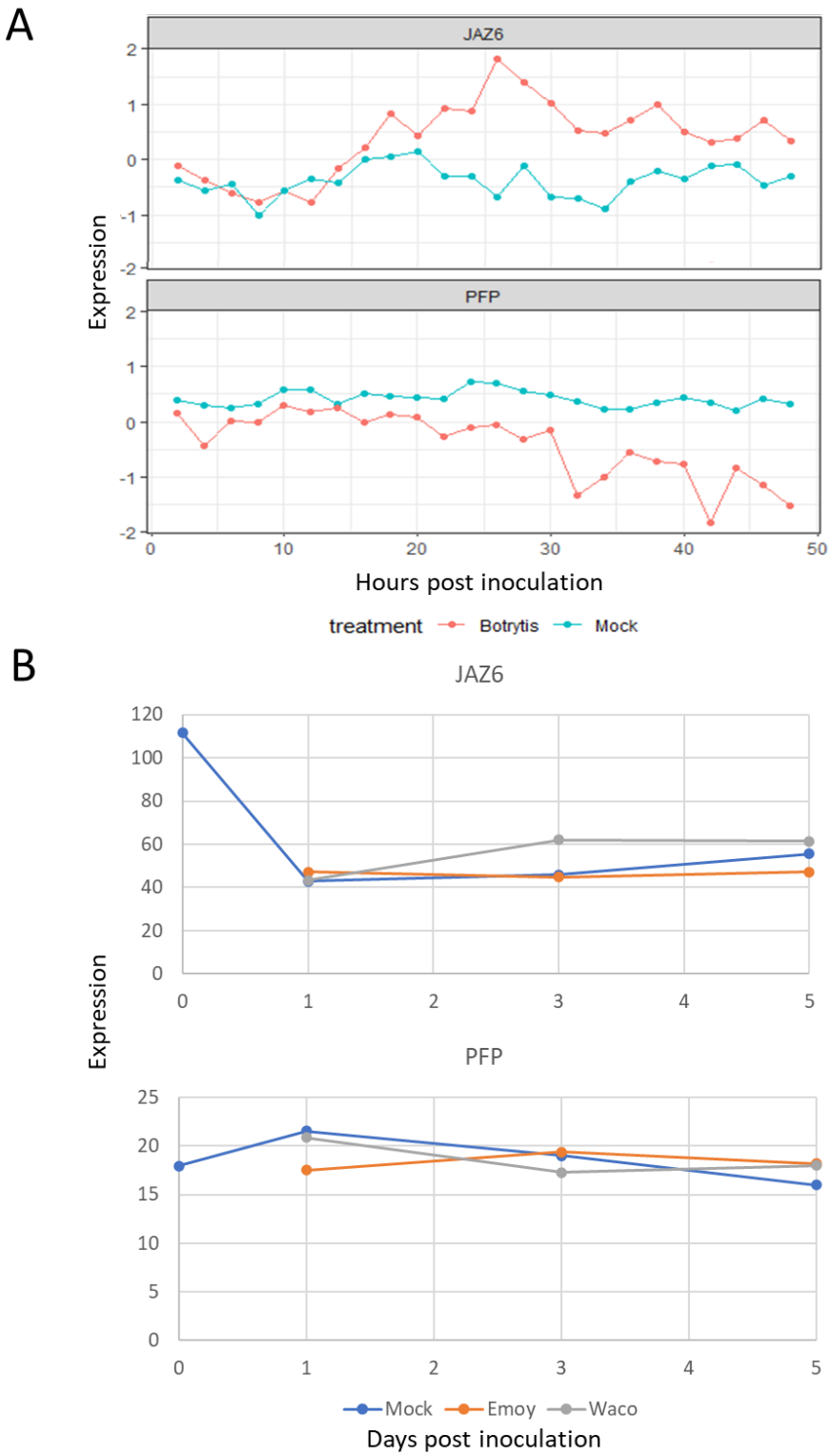


Figure 3.4.6: *PFP* and *JAZ6* expression responds to biotic stress.

(A) *PFP* and *JAZ6* expression in response to *B. cinerea* infection of Arabidopsis, from 2 to 48 hpi. Data and analysis from Windram et al., (2012).

(B) *PFP* and *JAZ6* expression in response to *H. arabidopsidis* infection of Arabidopsis, from 0 to 5 days post inoculation. Data and analysis from Asai et al. (2014).

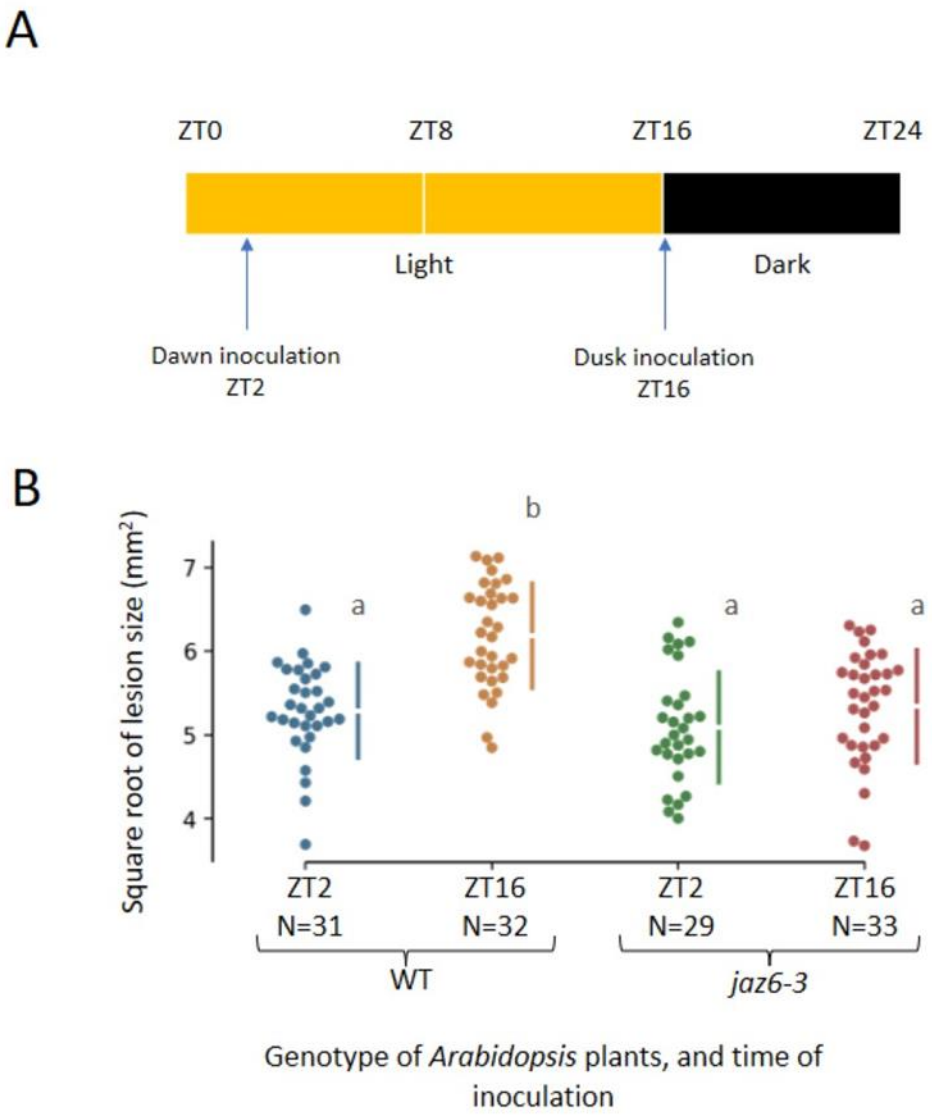


Figure 3.4.7: *JAZ6* is necessary for diurnal variation in susceptibility to *B. cinerea*

(A) Schematic diagram of photoperiod and inoculation times of day. Dawn (ZT2) and dusk (ZT16) inoculation times are labelled.

(B) Susceptibility of WT, and *jaz6-3* Arabidopsis plants to *B. cinerea*, inoculated at dawn and dusk. The square root of *B. cinerea* lesion size (mm^2) was measured on leaves 60 hours post-inoculation (hpi).

Bars alongside each group of values represent the upper and lower quartiles around the mean. "N" is the number of leaves assayed for each set of inoculations. Different letters indicate groups with a significant ($p<0.05$) difference between them in lesion size, by Tukey's HSD. This experiment was repeated 3 times.

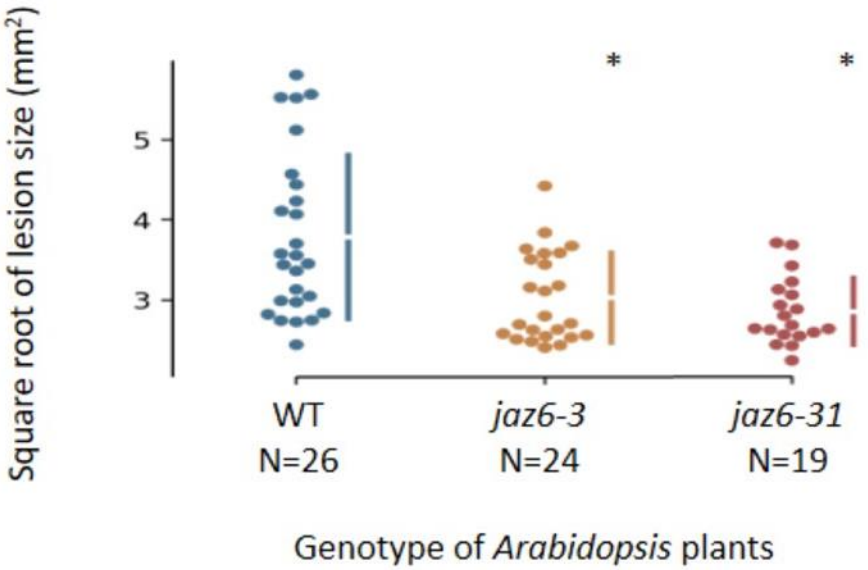


Figure 3.4.8: *JAZ6* represses resistance to *B. cinerea*

Susceptibility of WT, *jaz6-3*, and *jaz6-31* Arabidopsis plants to *B. cinerea*, inoculated at dusk. The square root of *B. cinerea* lesion size (mm^2) was measured on leaves 72 hours post-inoculation (hpi).

Bars alongside each group of values represent the upper and lower quartiles around the mean. "N" is the number of leaves assayed for each set of inoculations. Different letters indicate groups with a significant ($p<0.05$) difference between them in lesion size, by Tukey's HSD. This experiment was repeated three times.

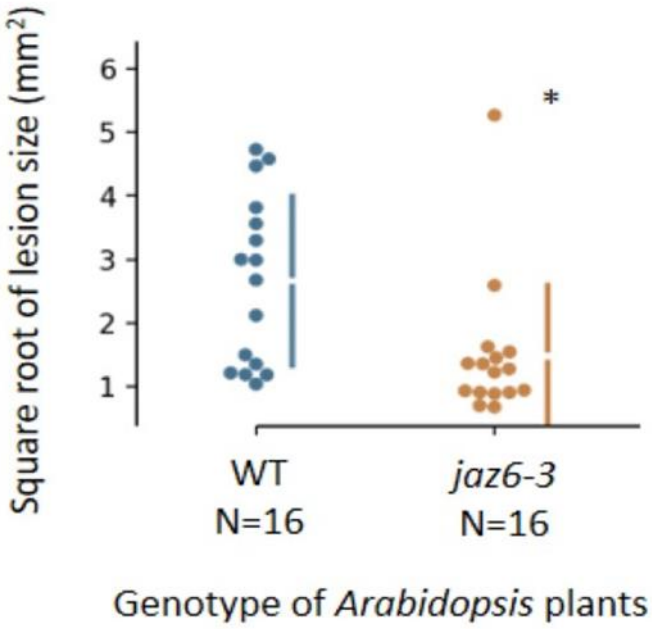


Figure 3.4.9: *JAZ6* represses resistance to *Sclerotinia sclerotiorum*

Susceptibility of WT, and *jaz6-3* Arabidopsis plants to *Sclerotinia sclerotiorum*, inoculated at dusk. The square root of *Sclerotinia sclerotiorum* lesion size (mm^2) was measured on leaves 88 hours post-inoculation (hpi).

Bars alongside each group of values represent the upper and lower quartiles around the mean. "N" is the number of leaves assayed for each set of inoculations. Asterisks represent statistically significant ($P<0.05$) differences from WT, by ANOVA. This experiment was conducted once.

3.4.3 *PFP* promotes resistance to *B. cinerea*

As JAZ6 protein-protein binds PFP, the possibility of *PFP* affecting disease susceptibility was investigated. Experiments were carried out to investigate the susceptibility of *pfp-1* mutants and PFP-Ox overexpressors to *B. cinerea.*

As *JAZ6* is upregulated in response to *B. cinerea* infection (Ingle *et al.*, 2015), we looked at *PFP* expression in Arabidopsis in response to the biotic stresses *B. cinerea* and *H. arabidopsidis* in publicly available time series transcriptome datasets (Windram *et al.*, 2012; Asai *et al.*, 2014) (Figure 3.4.6). These two pathogens use contrasting necrotrophic and biotrophic strategies respectively to cause disease in plants. *PFP* expression is responsive to *B. cinerea* inoculation, but contrasts to *JAZ6* (Figure 3.4.6A). *PFP* is significantly downregulated in response to *B. cinerea* infection (Figure 3.4.6A), while *JAZ6* is upregulated. However, this contrasts to transcriptomic analysis of WT plants during *B. cinerea* infection (Figure 3.4.5), where *PFP* expression in WT plants is not downregulated in response to *B. cinerea* inoculation. This may be due to the different inoculation time in these experiments, 6 hours after dawn for Windram et al. (2012), and 16 hours after dawn here. In contrast, neither *PFP* nor *JAZ6* are differentially expressed in response to *H. arabidopsidis* infection (Figure 3.4.6B).

As JAZ6 regulates resistance to biotic stress at dusk and JAZ6 interacts with PFP, we investigated whether *PFP* mutants and overexpressors were also disrupted in plant defence (Figure 3.4.10). *pfp-1* mutant and PFP-Ox plants were inoculated with *B. cinerea* (Figure 3.4.10A), with WT and *jaz6-3* mutant plants used as controls. Plants were inoculated one hour before the end of the light period in 16h light / 8 h dark days. We observed, as previously described (Li *et al.*, 2019), that *jaz6-3* mutants are more resistant to infection than WT plants at dusk, with a smaller *B. cinerea* lesion area 65 hours post inoculation (hpi). Similarly, PFP-Ox plants were more resistant than the WT control (Figure 3.4.10A), with a smaller *B. cinerea* lesion area 72 hpi, like *jaz6-3* mutant plants. However, *pfp-1* mutant plants were more susceptible than the WT control (Figure 3.4.10A & B), with a larger *B. cinerea* lesion area 55-65 hpi. This indicates that *PFP* acts as a positive regulator of defence against necrotrophic pathogens.

To investigate potential genetic interaction between *JAZ6* and *PFP*, the T-DNA insertion mutant lines *jaz6-3* and *pfp-1* were crossed. This double T-DNA insertion mutant of both *jaz6-3* and *pfp-1* exhibited a *B. cinerea* resistance phenotype in-between the two parental lines (Figure 3.4.10B), more susceptible than *jaz6-3* mutants, but less susceptible than *pfp-1* mutants. This indicates there is a genetic interaction between the two mutants, possibly as they act in different pathways to affect defence, or that the other JAZ proteins are compensating for the loss of JAZ6 and the two genes act on the same pathway.

As *JAZ6* and *PFP* appeared to regulate the severity of disease symptoms, the dynamics of lesion growth over time were estimated on WT, *jaz6-3, pfp-1,* and double *jaz6-3 pfp-1* mutant lines. A modified *B. cinerea* infection assay was designed to capture the speed at which *B. cinerea* lesions spread over time. While the infection protocol remained the same, infection trays were placed into a lightbox with a Raspberry Pi computer programmed to take images every 10 minutes. Using an adapted form of software originally designed to estimate *S. sclerotiorum* lesion growth (Barbacci *et al.*, 2020), *B. cinerea* lesion growth was estimated (Figure 3.4.11). Here it is evident that lesion growth speed is faster on *pfp-1* mutants than WT, and slower on *jaz6-3* mutants than WT. The double *jaz6-3 pfp-1* mutant exhibited similar dynamics to WT but seemed to approach a growth limit sooner.

To elucidate the mechanism of this genetic interaction further, RNA-seq transcriptomic analysis of the double mutant, together with parental lines and WT plants, before and during infection, was performed.

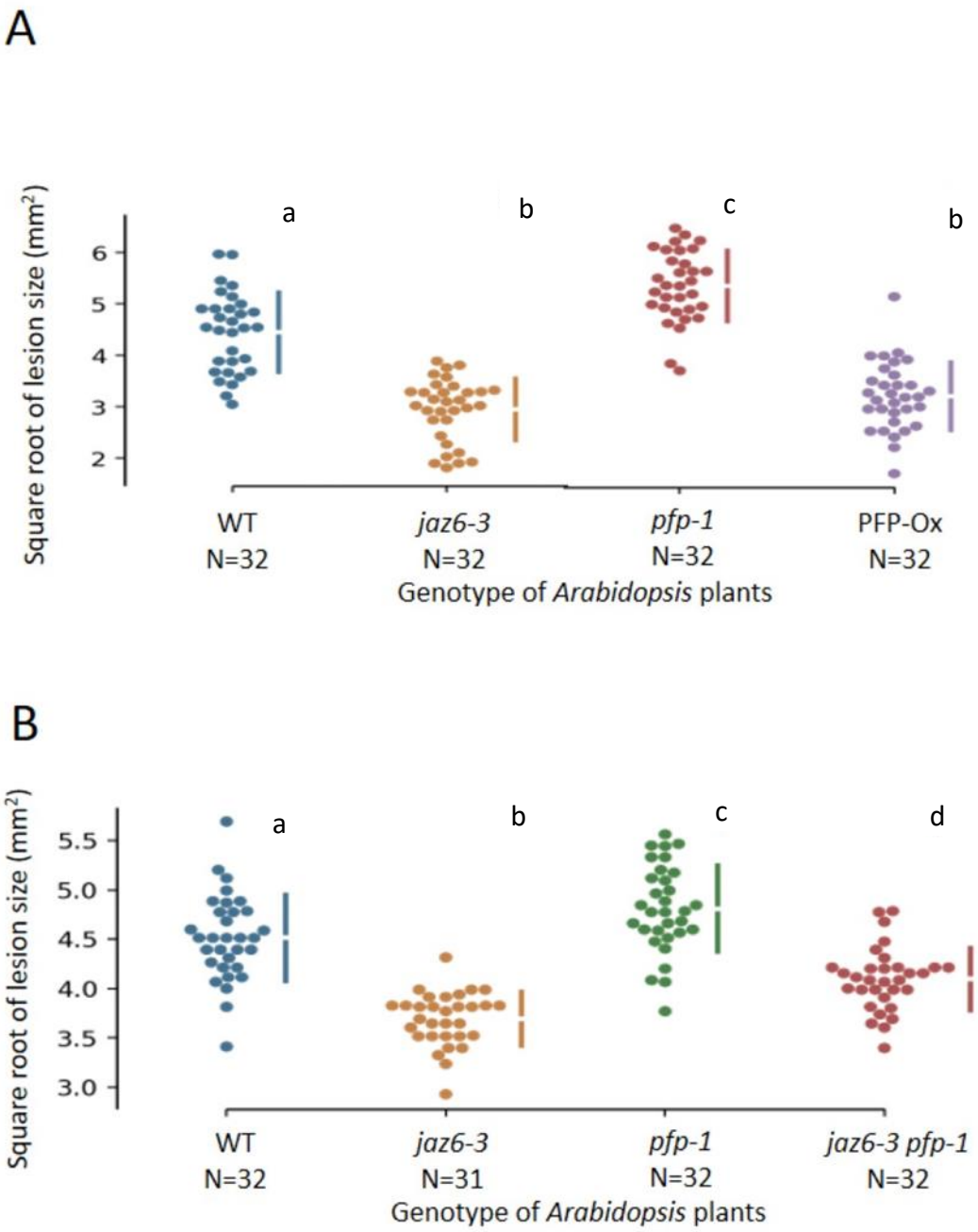


Figure 3.4.10: *PFP* promotes resistance to *B. cinerea*

Susceptibility of WT, *jaz6-3, pfp-1*, and (A) PFP-Ox (B) *jaz6-3 pfp-1* Arabidopsis plants to *B. cinerea*, inoculated at dusk. The square root of *B. cinerea* lesion size (mm^2) was measured on leaves 60 hours post-inoculation (hpi).

Bars alongside each group of values represent the upper and lower quartiles around the mean. "N" is the number of leaves assayed for each set of inoculations. Different letters indicate groups with a significant ($p<0.05$) difference between them in lesion size, by Tukey's HSD. These experiments were conducted twice.

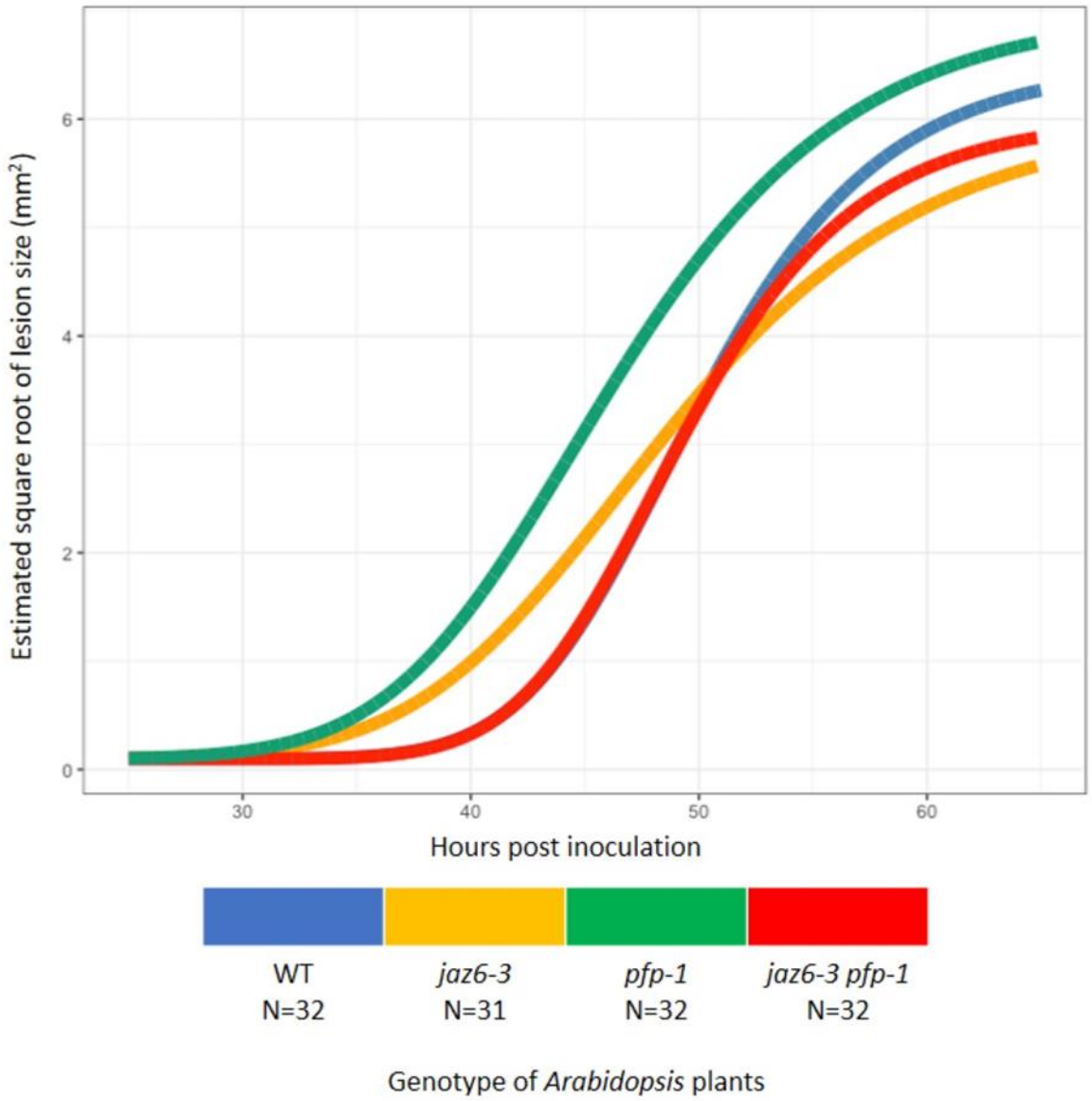


Figure 3.4.11: *JAZ6* and *PFP* affect the rate at which infection spreads

Susceptibility of WT (blue), *jaz6-3 (yellow), pfp-1 (green)*, and *jaz6-3 pfp-1* double mutant (red) Arabidopsis plants to *B. cinerea*, inoculated at dusk. Images were taken every 10 minutes, cross-referenced with known lesion sizes, and fitted to Hill functions for logistic growth. This analysis was conducted twice.

3.4.4 *JAZ6* suppresses susceptibility to *H. arabidopsidis, whereas PFP* promotes susceptibility to *H. arabidopsidis*

Biotrophic pathogens contrast to necrotrophic pathogens, both in their modes of attack and the host plant response (Pieterse *et al.*, 2012). Genes which activate susceptibility to biotrophic pathogens can activate immunity to necrotrophic pathogens, such as GOLDEN2-LIKE transcription factors (Murmu *et al.*, 2014). This can be driven by antagonism between salicylic acid and jasmonic acid response pathways (Takahashi *et al.*, 2004; Van der Does *et al.*, 2013), but salicylic acid and jasmonic acid can otherwise operate synergistically depending on the pathogen (Mur *et al.*, 2006). Because of this, we considered if *JAZ6* and *PFP* affect susceptibility to the biotrophic oomycete pathogen *H. arabidopsidis*.

To understand how defence against biotrophic pathogens is affected by *JAZ6* and *PFP* WT, *pfp-1, jaz6-3, jaz6-31,* and *jaz6-3 pfp-1* mutant plants were inoculated with *H. arabidopsidis (*Figure 3.4.12). Here we see that *pfp-1* mutants are less susceptible to infection than WT plants (Figure 3.4.12), while *jaz6-3* and *jaz6-31* mutants are more susceptible. This shows that *JAZ6* regulates defence against biotrophic pathogens in addition to necrotrophic pathogens, by suppressing susceptibility to *H. arabidopsidis*. This is a novel role for *JAZ6.* Such activity has been described for wild grapevine *VqJAZ4* where suppressed susceptibility to the biotroph *Golovinomyces cichoracearum* was reported (Guofeng Zhang *et al.*, 2019). My results also indicate that *PFP* acts as a negative regulator of defence against biotrophic pathogens, as suggested by the role of its tobacco homologue *NbUBR7* in defence against viruses (Yongliang Zhang *et al.*, 2019). As seen in *B. cinerea* infection (Figure 3.4.7), there was an intermediate phenotype between the two parental phenotypes for *pfp-1* and *jaz6-3* double mutants (Figure 3.4.12A).

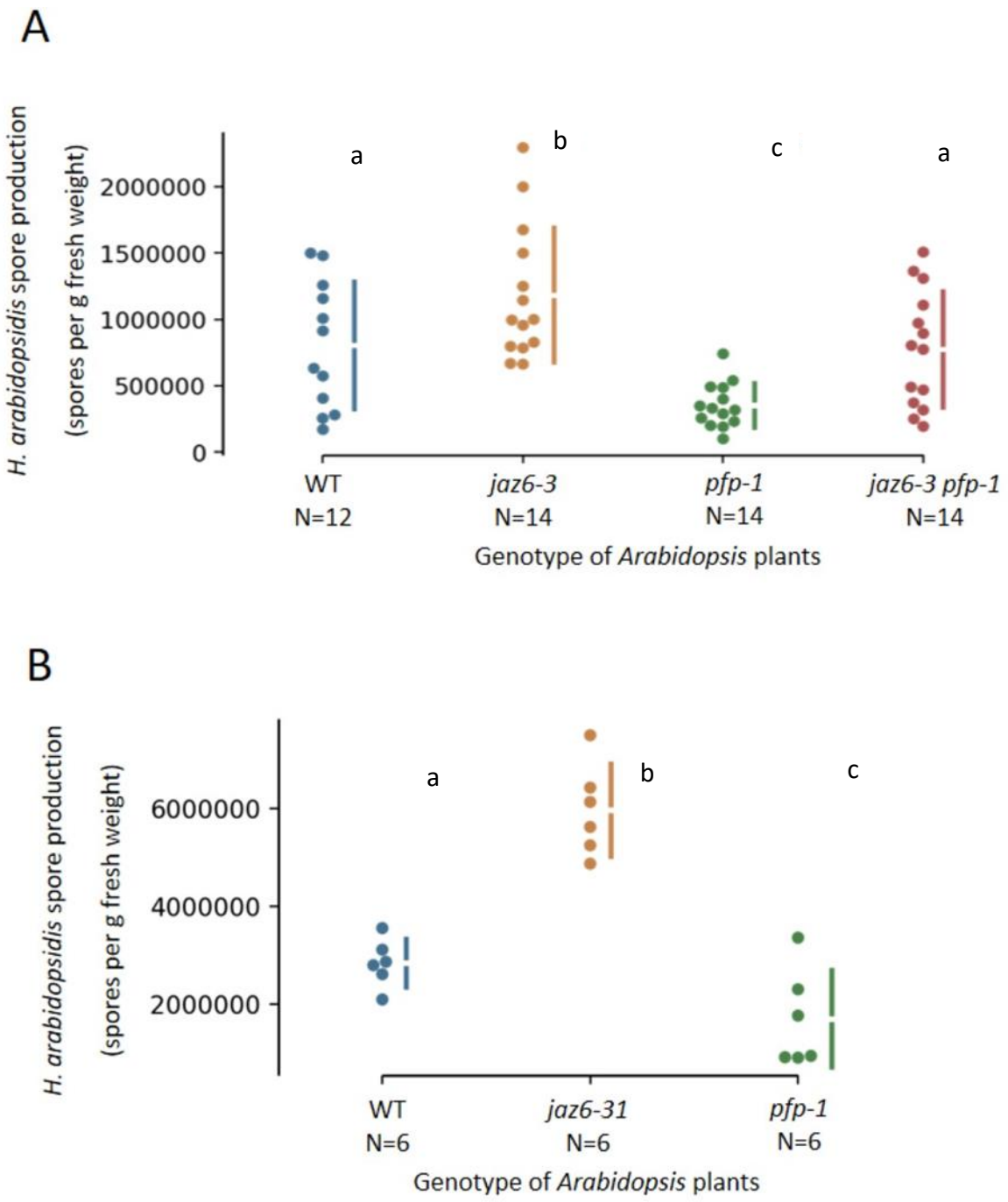


Figure 3.4.12: *JAZ6* represses, and *PFP* promotes, susceptibility to *H. arabidopsidis.* Susceptibility of wild-type (WT), *pfp-1*, (A) *jaz6-3* and double *jaz6-3 pfp-1* (B) *jaz6-31* mutant Arabidopsis plants to *H. arabidopsidis*, inoculated at dusk. The number of *H. arabidopsidis* spores on leaves was measured 7 days post-inoculation (hpi) and divided by the fresh weight of those seedlings. Bars alongside each group of values represent the upper and lower quartiles around the mean. "N" is the number of leaves assayed for each set of inoculations. Different letters indicate groups with a significant ($p<0.05$) difference between them in lesion size, by Tukey's HSD. These experiments were repeated twice.

3.4.5 *JAZ6* affects defence and is mostly independent of *PFP*

Considering that a *B. cienrea* infection assay phenotype was seen for both *jaz6-3* and *pfp-1* mutant lines, which would be expected to reflect differential gene expression due to the molecular mechanisms controlled by these mutations, RNAseq analysis was conducted to identify the molecular mechanisms behind susceptibility in *jaz6-3* and *pfp-1* mutant lines, as well as the genetic interaction between them. Specifically, RNAseq analysis was conducted to investigate gene expression both prior to (time 0h) and during (time 24h) *B. cinerea* infection, to gain a better understanding of how *JAZ6* and *PFP* regulate gene expression independently of infection, as well as during the course of infection.

RNA was extracted from pooled samples of four-week-old Arabidopsis plant leaves. As in the botrytis infection assays, WT*, jaz6-3, pfp-1,* and double *jaz6-3 pfp-1* mutant plants were used. Samples were taken immediately before inoculation, termed 0hpi (hours post inoculation). Leaves were inoculated with four 10 µL droplets of inoculum with 50,000 spores per mL, which is more than the single droplet used in phenotyping assays. 24 hours after inoculation, to avoid any circadian effects, the second sample was taken, termed 24hpi. RNA quality was verified by an Aglient Bioanalyser, then Novogene processed samples for library preparation and sequencing.

The quality of RNAseq reads which were returned by Novogene was checked with Fast QC, with most read at quality over Q30 and an average of Q36 (Figure 3.4.13). The reads were then trimmed and adapters were removed by TrimGalore. Then, they were aligned to a combined Arabidopsis-Botrytis transcriptome index by Salmon. Further processing of the samples took place in 3D-RNAseq platform. To show sample replicability, PCA analysis was performed as in Figure 3.4.14, showing clear separation across PC1 due to the timepoint of sampling, with infected samples further to the right. In PC2, the initial small separation between genotypes grows larger over the course of infection. The *jaz6-3* mutants and double *jaz6-3 pfp-1* mutants do not appear to cluster separately.

Expression data was then filtered and normalised to remove low expression genes. This 1 count per million cut-off removes the drop effect seen when plotting the mean-variance

trend as in Figure 3.4.16A. Data was then normalised to account for variation in library sizes, to ensure comparability of gene expression between samples, as in Figure 3.4.16B.

Differential expression analysis was performed with limmaVoom in 3DRNAseq, with a critical adjusted p-value of 0.05, and a minimum of a 2-fold change in expression. This means that genes identified as differentially expressed are more likely to be meaningful as the threshold for significance is higher. At the same time, this is a trade-off as real effects with a smaller change in expression will not be identified as differential expression.

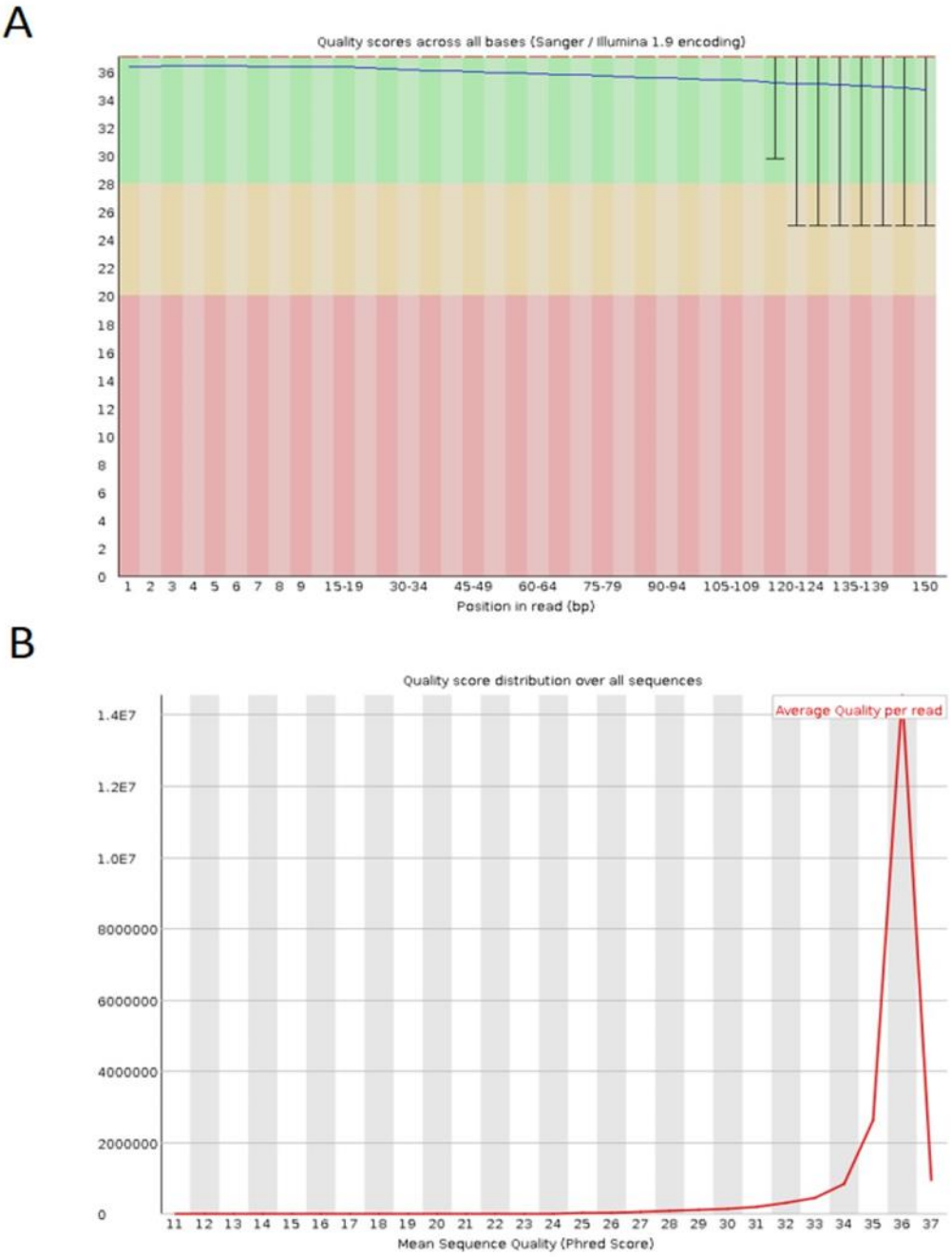
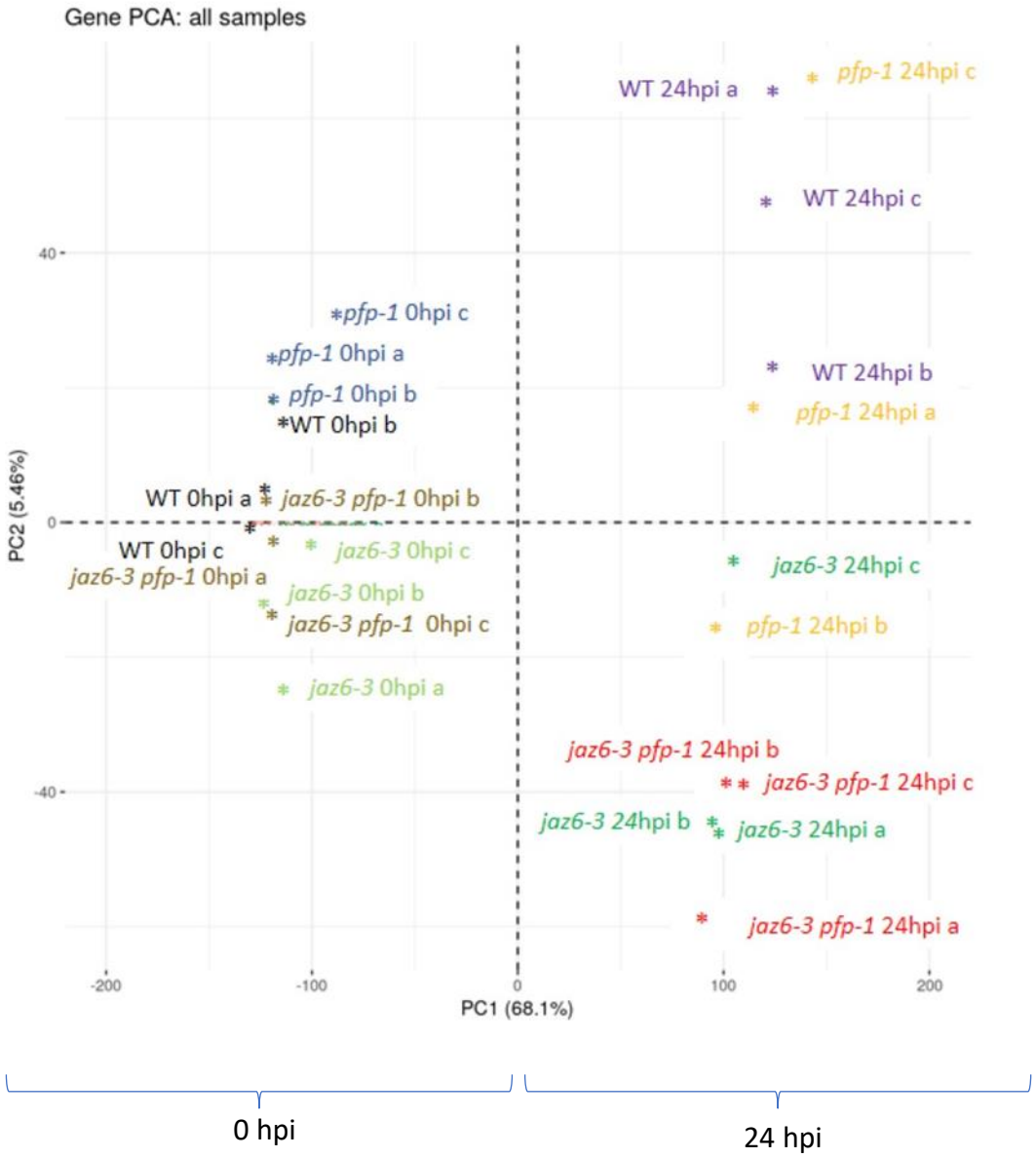


Figure 3.4.13: RNA read data was of good quality.

Representative sample quality from FastQC prior to TrimGalore removal of adapters and reads below Q30.

A. Position dependent quality scores across the length of reads. The quality score decreased slightly over the length of reads, but average quality remained over Q30.
B. Histogram of average quality per read. Most of the mean read quality scores were above Q30, the majority at a mean quality of Q36.



Genotype of *Arabidopsis* plants, and sample time post-inoculation

Figure 3.4.14: RNAseq samples group together based on the sampling time.
Gene level principal component analysis (PCA) of variance in gene expression. The main component corresponds with the majority of the variance in gene expression, and separates samples based on if they were taken before (0hpi) or during (24hpi) infection.

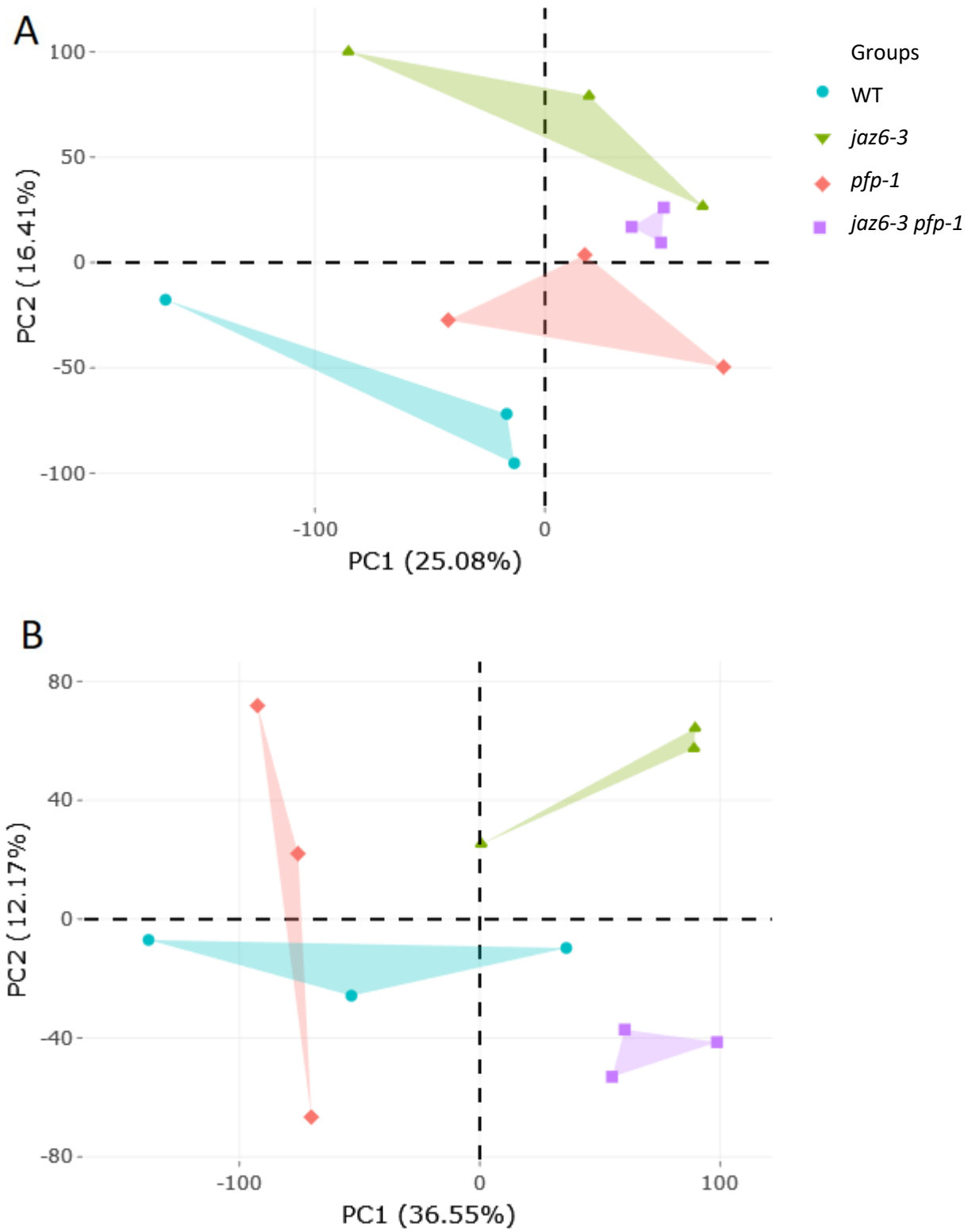


Figure 3.4.15: RNAseq samples from the same sampling time group together based on genotype. Gene level principal component analysis (PCA) of variance in gene expression. Polygons connect the different genotypes of Arabidopsis plants. Pre-infection, the different genotypes are delineated by both axes. During infection, there is still division between most groups, but WT and *pfp-1* mutants are more similar than before infection.

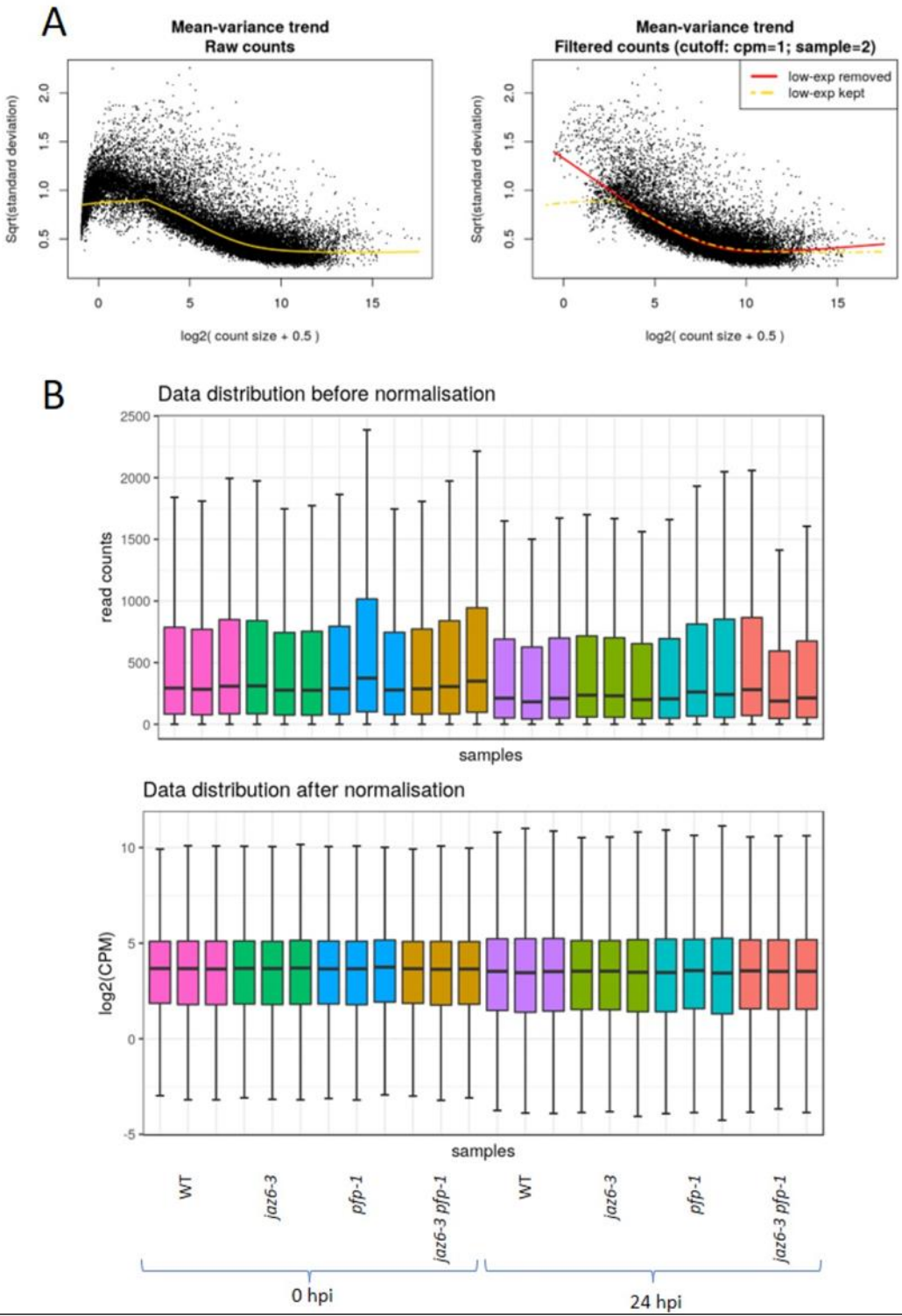


Figure 3.4.16: Filtering and normalising data in 3DRNAseq.

Gene level analysis of trends in gene expression to be filtered and corrected for comparison between samples.

A. Voom plot showing the removal of the drop effect with low expression genes by filtering

B. Normalisation of gene expression in 3DRNAseq showing the distribution of read counts before and after normalisation.

In order to determine what genes were differentially regulated due to the *jaz6-3* and *pfp-1* mutations, expression in the mutant lines was compared to WT. This was calculated for expression prior to and during infection separately.

However, it was also considered that such simple analysis could not accurately capture the interaction effects of *JAZ6* and *PFP*. For example, while the commonly used technique of Venn diagram overlap could, to an extent, indicate genes differentially expressed compared to WT in different conditions, such overlaps are not informative as to the potential for significant interaction effects of *JAZ6* and *PFP*. As an example, if gene expression of genes A and B doubles in the *jaz6-3* mutant compared to WT, and also doubles in the *pfp-1* mutant compared to WT, such genes are subject to control by both JAZ6 and PFP in some manner. However, in the double mutant compared to WT, gene A expression doubles and gene B expression quaduples. From such differences in gene expression it may be inferred that gene A is regulated by *JAZ6* and *PFP* differently to gene B, as expression of A is dependent on either one of *JAZ6* and *PFP* but expression of gene B is dependent on both. However, in the overlap on the Venn diagram of significant differences in gene expression, genes A and B are indistinguishable. Therefore, a mathematical method of discovering genes where control of gene expression depended on both *JAZ6* and *PFP* was conceived.

As explained above, for the effect of the double mutant, an additional comparison was made to quantify the effect the single *jaz6-3* mutant had in the *pfp-1* background, compared to the WT background. This is equal to the effect of the *pfp-1* mutation in the *jaz6-3* background, compared to WT. This contrast is termed interaction effects of *jaz6-3* and *pfp-1*, and is encoded like so: (WTΔ*jaz6-3)Δ(pfp-1Δjaz6-3 pfp-1).*

In total 891 genes were found to be differentially expressed prior to or during infection, between WT and *jaz6-3* or *pfp-1* or regulated by synergistic or antagonistic effects of the genetic interaction between *JAZ6* and *PFP* (Figure 3.4.17). The number of genes differentially expressed varied significantly between the main contrasts of interest.

Few genes were differentially expressed commonly across multiple comparisons (Figure 3.4.18), with most genes (514) differentially expressed exclusively between *jaz6-3 pfp-1* mutants and WT (the *jaz6-3 pfp-1*ΔWT contrast) during infection (Table 3.4.1).

To investigate genes regulated by *JAZ6* and *PFP*, the contrasts were considered in uninfected and infected plants. In general, for all contrasts, the differentially expressed genes during infection had only a small amount of overlap with the differentially expressed genes prior to infection (Figure 3.4.19).

GO analysis of functional enrichment within differentially expressed sets of genes is indicative of involvement of *JAZ6* in defence and hormone responses, as may be expected (Figure 3.4.20). For *PFP*, there is also some indication that differentially expressed genes are related to defence (Figure 3.4.21). For both *JAZ6* and *PFP,* there is some indication of involvement in defence, and also hypoxia (Figures 3.4.22, 23). From the above functional analysis, genes potentially thought to be involved in defence or the interaction of *JAZ6* and *PFP* were investigated further.

Genes which are positively associated with defence and upregulated in *jaz6-3* mutants compared to WT notably include plant defensins *PDF1.2a* (*AT5G44420*), PDF1.2c (*AT5G44430*) and *PDF1.3 (AT2G26010)*, which impact plant defence (Thomma, Cammue and Thevissen, 2002). We show clear upregulation of these plant defensin defence genes in *jaz6-3* mutants compared to WT, both prior to and during infection (Figure 3.4.24).

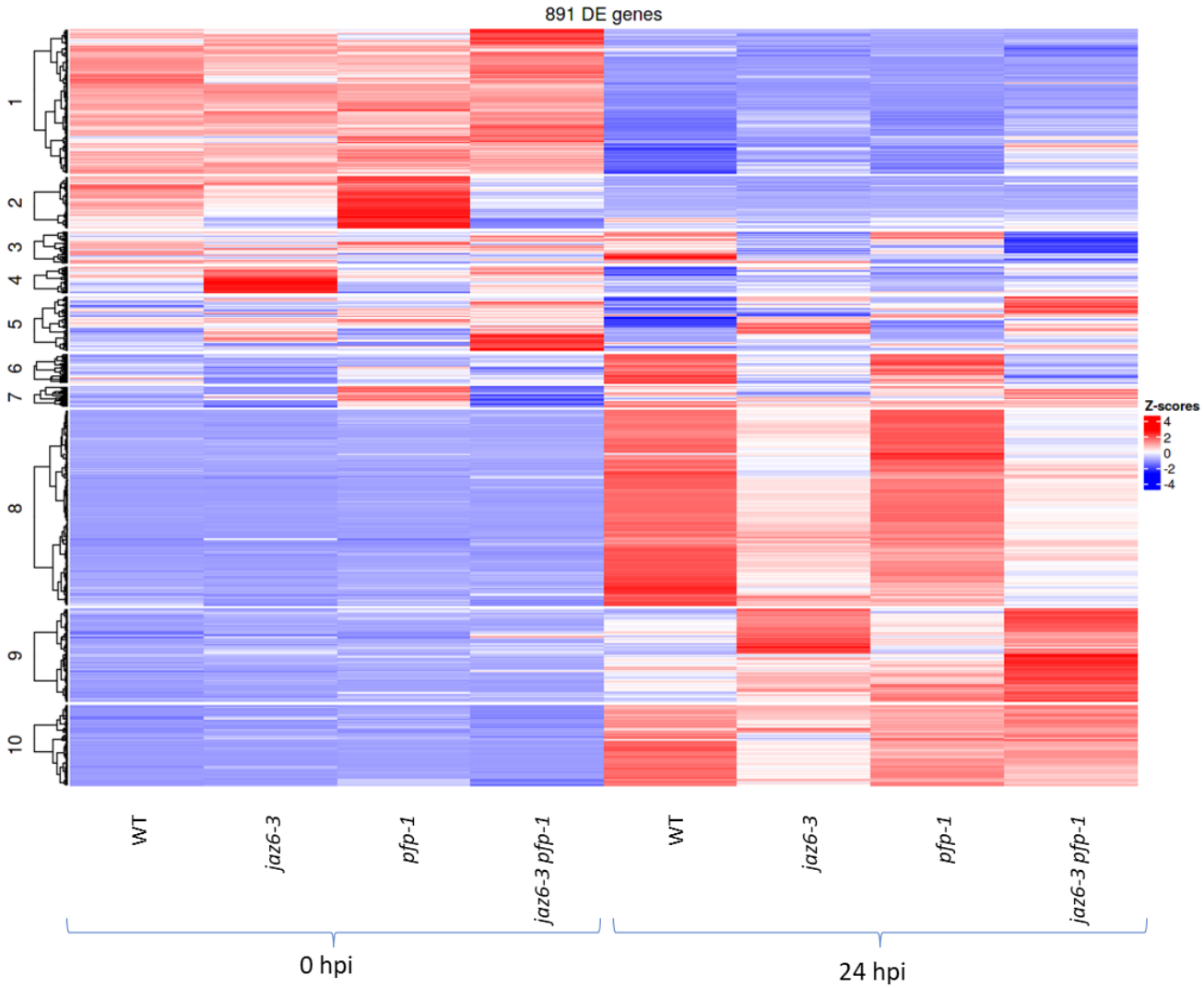


Figure 3.4.17: Transcriptomic analysis of *B. cinerea* infection reveals 891 genes regulated by *JAZ6, PFP*, or an interaction effect of *JAZ6* and *PFP.*

Heatmap of differentially expressed genes from the contrasts WTΔ*jaz6-3*, WTΔ*pfp-1* and the interaction contrast of (WTΔ*jaz6-3)Δ(pfp-1Δjaz6-3 pfp-1)* both before and during *B. cinerea* infection. Z-scores represent the relative average gene expression across all samples. Genes are clustered into 10 clusters of genes with similar expression patterns.

Table 3.3.1: Transcriptomic analysis of *B. cinerea* infection reveals different numbers of genes regulated by *JAZ6, PFP*, or an interaction effect of *JAZ6* and *PFP.*
The number of significantly 2-fold differentially expressed genes for each of the 8 main comparisons is listed, as well as how many genes are upregulated or downregulated.

Hours post inoculation	contrast	DE genes	Upregulated	Downregulated
0	*jaz6-3* vs. WT	50	40	10
0	*pfp-1* vs. WT	38	29	9
0	*jaz6-3 pfp-1* vs. WT	124	65	59
24	*jaz6-3* vs. WT	337	111	226
24	*pfp-1* vs. WT	11	8	3
24	*jaz6-3 pfp-1* vs. WT	514	227	287
0	*JAZ6 PFP* interaction (*jaz6-3* vs. WT) Vs. (*jaz6-3 pfp-1* vs. *pfp-1*)	137	97	40
24	*JAZ6 PFP* interaction (*jaz6-3* vs. WT) Vs. (*jaz6-3 pfp-1* vs. *pfp-1*)	15	1	14

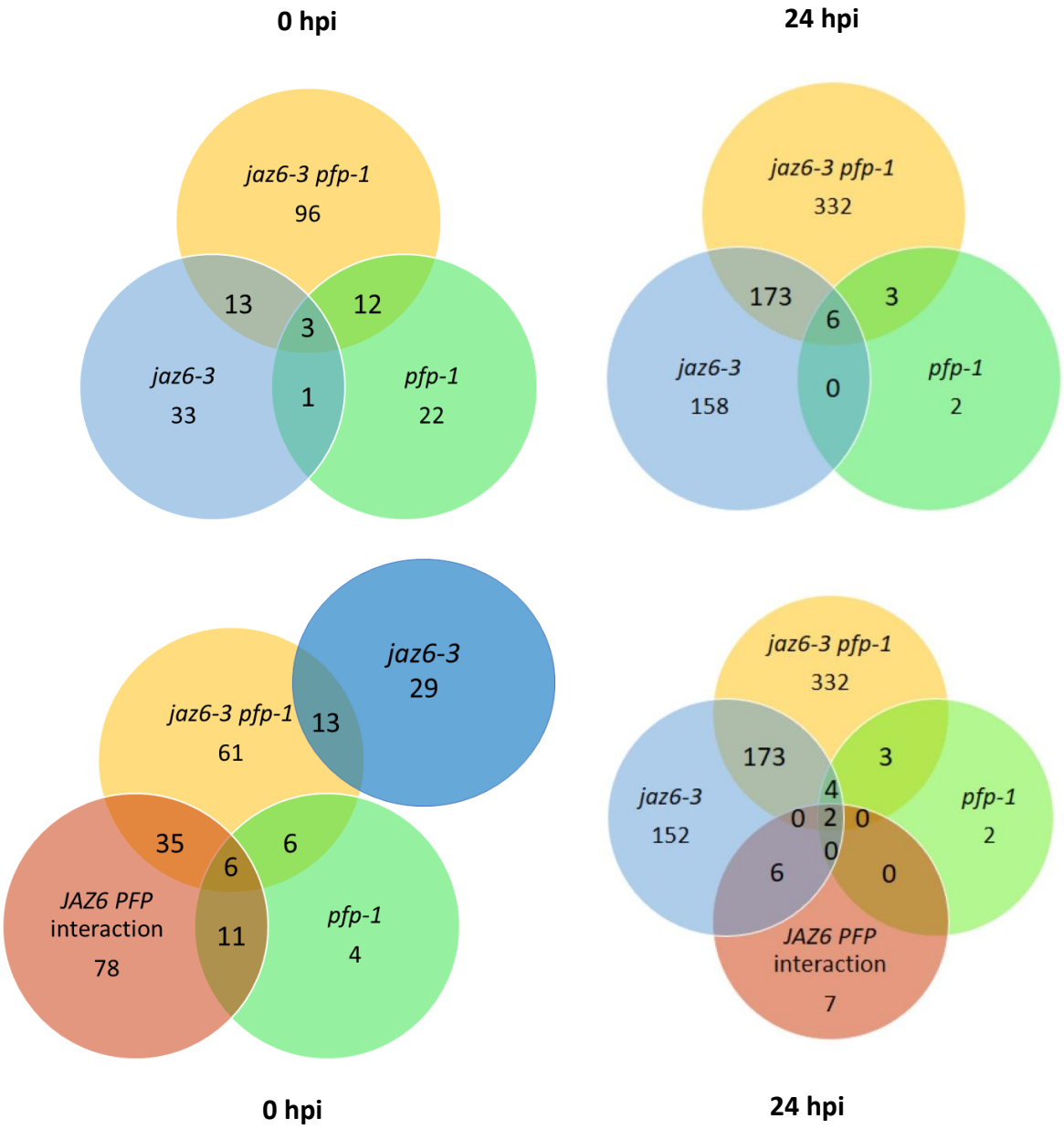


Figure 3.4.18: Many differentially expressed genes compared to WT both before and during infection are unique to the double *jaz6-3 pfp-1* mutant.

Venn diagrams of differentially expressed genes both before (left) and during (right) infection show numbers of significantly differentially expressed genes and how they overlap between contrasts.

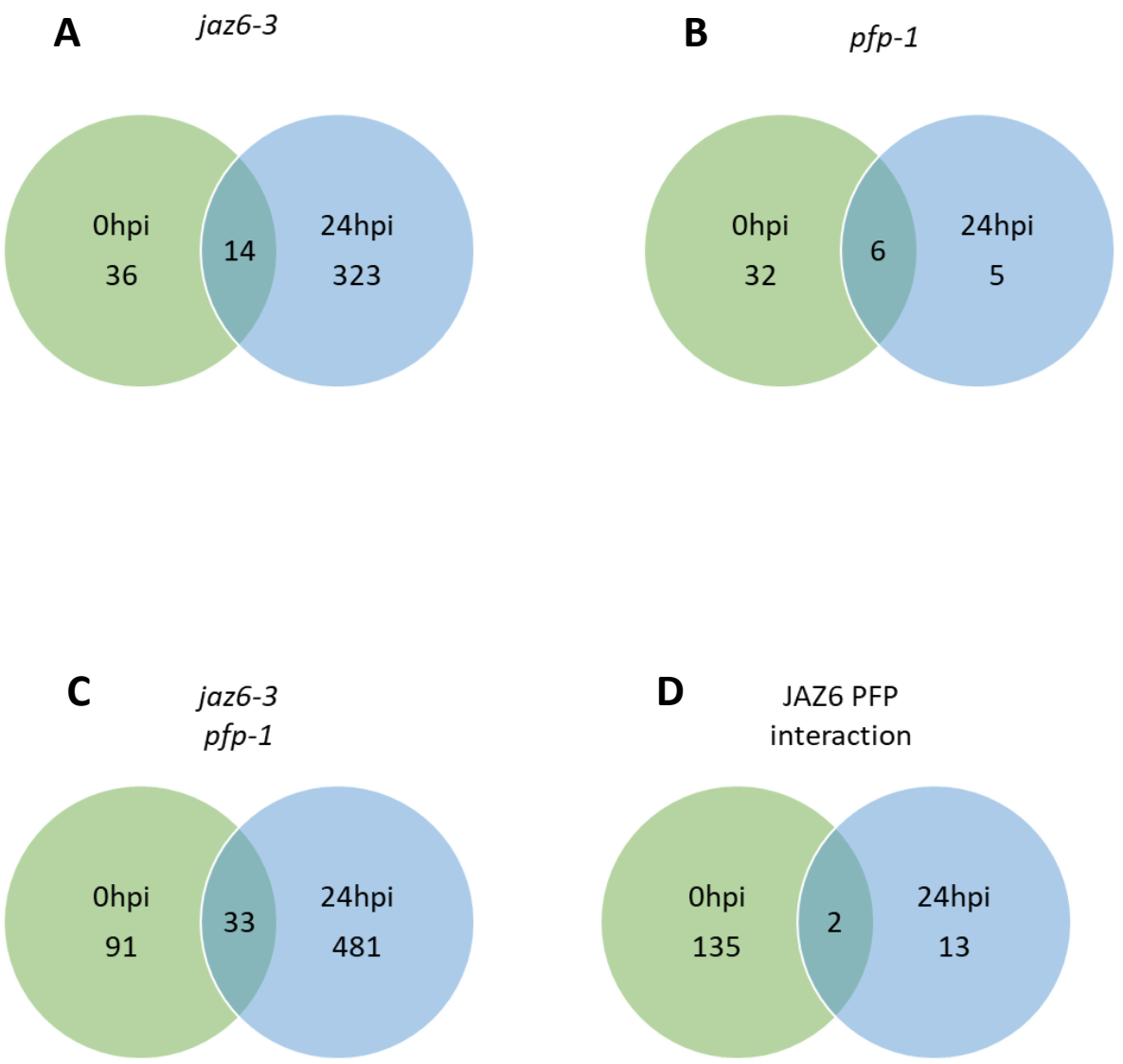


Figure 3.4.19: There is low crossover between Arabidopsis genes differentially expressed before and after infection.

Venn diagrams showing overlap in differentially expressed genes before and during infection for (A) *jaz6* to WT (B) *pfp-1* to WT (C) *jaz6-3 pfp-1* to WT (D) *JAZ6 PFP* interaction.

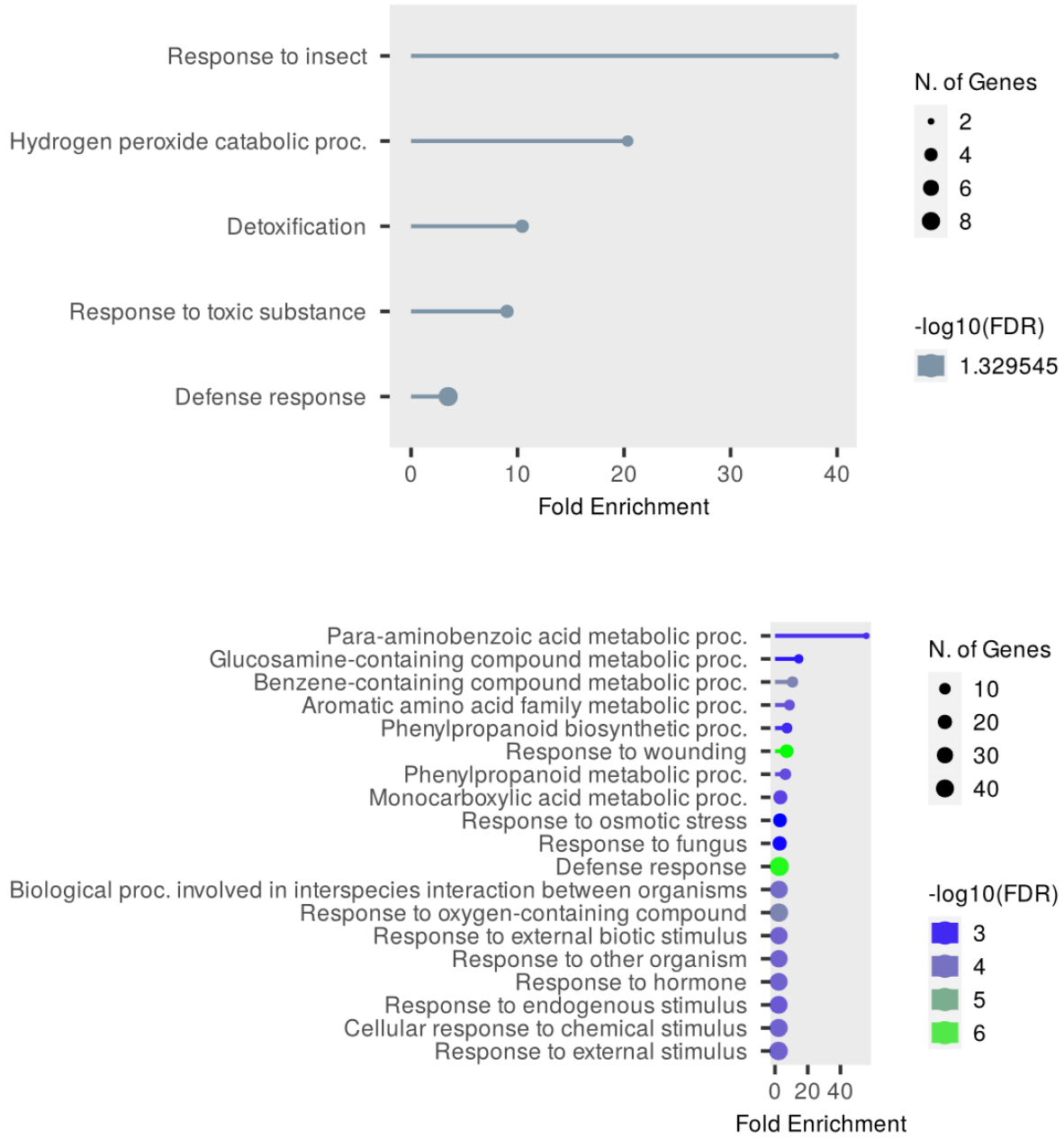


Figure 3.4.20: Genes differentially expressed in Arabidopsis in *jaz6-3* mutants have distinct roles before and during infection.

GO enrichment shows that in *jaz6-3* mutants prior to infection (upper) DE genes are related to defence. During infection (lower), DE genes are related to defence, hormone response, and metabolism.

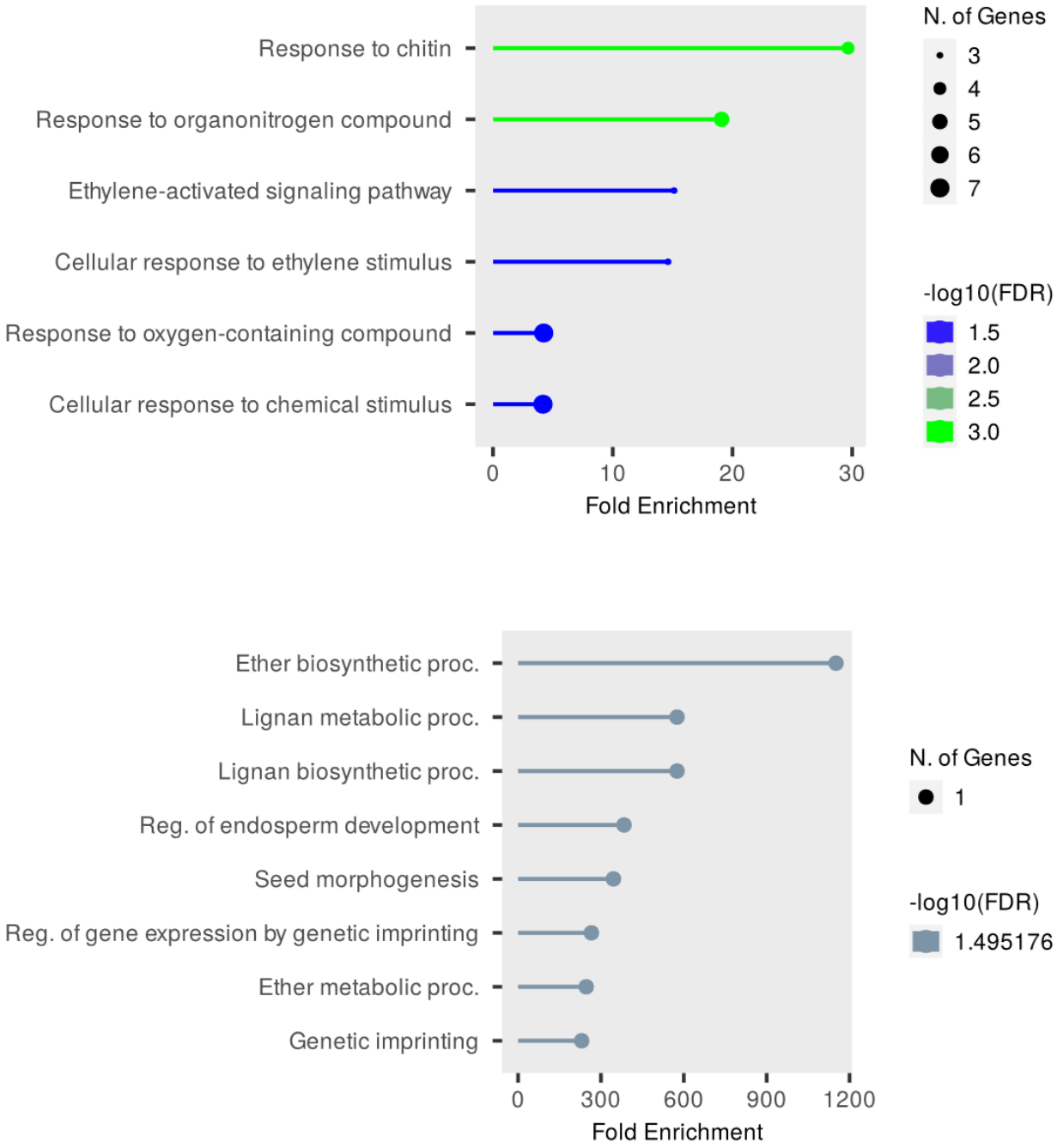


Figure 3.4.21: Genes differentially expressed in Arabidopsis in *pfp-1* mutants have distinct roles before and during infection.

GO enrichment shows that in *pfp-1* mutants prior to infection (upper) DE genes are related to fungal detection, ethylene signalling, and hypoxia. During infection (lower) DE genes are related to imprinting and metabolism.

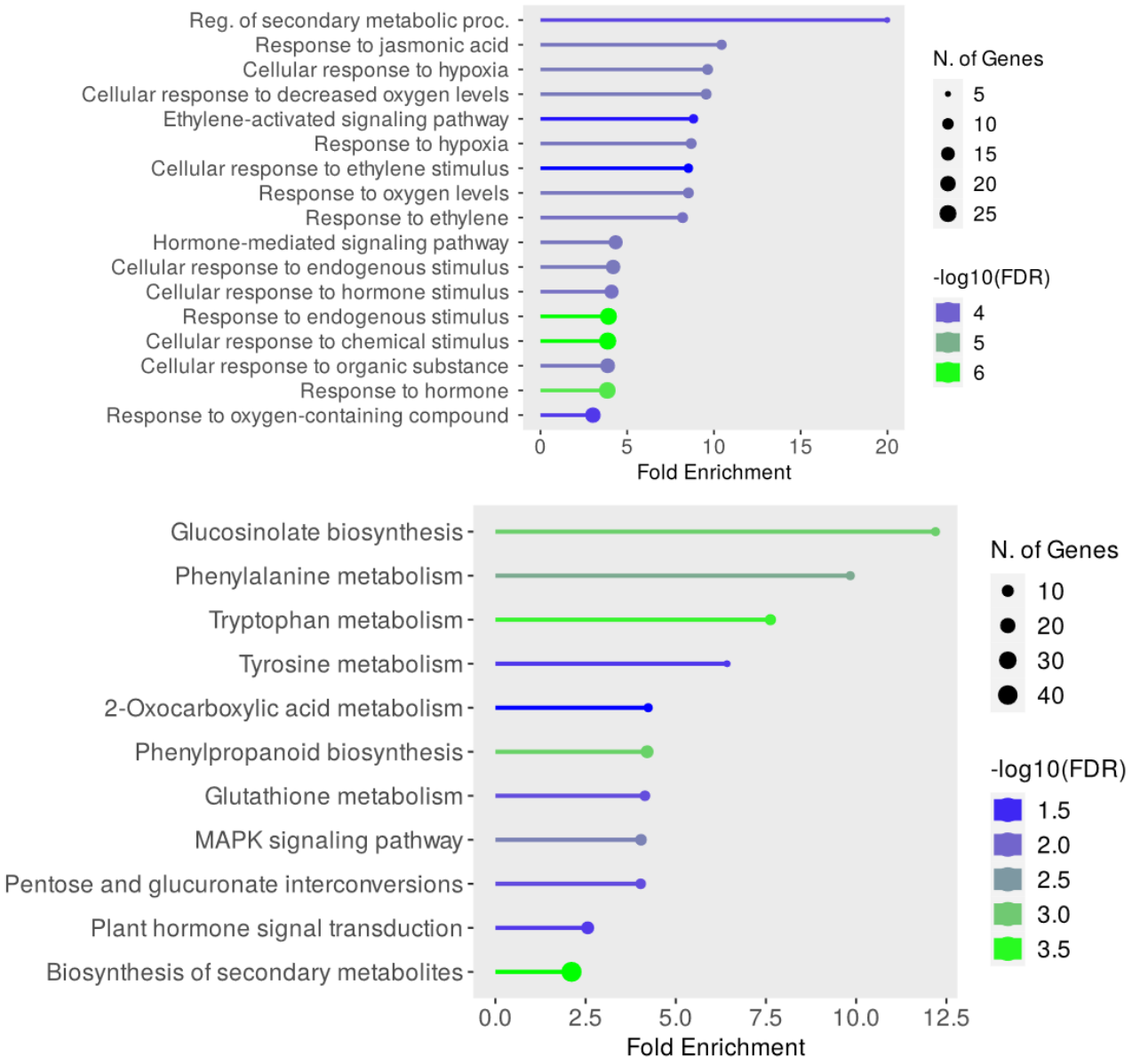


Figure 3.4.22: Genes differentially expressed in Arabidopsis in *jaz6-3 pfp-1* double mutants have distinct roles before and during infection.

GO enrichment shows that in *jaz6-3 pfp-1* mutants prior to infection (upper) DE genes are related to hypoxia and hormones. During infection (lower) DE genes are related to metabolism and hormones.

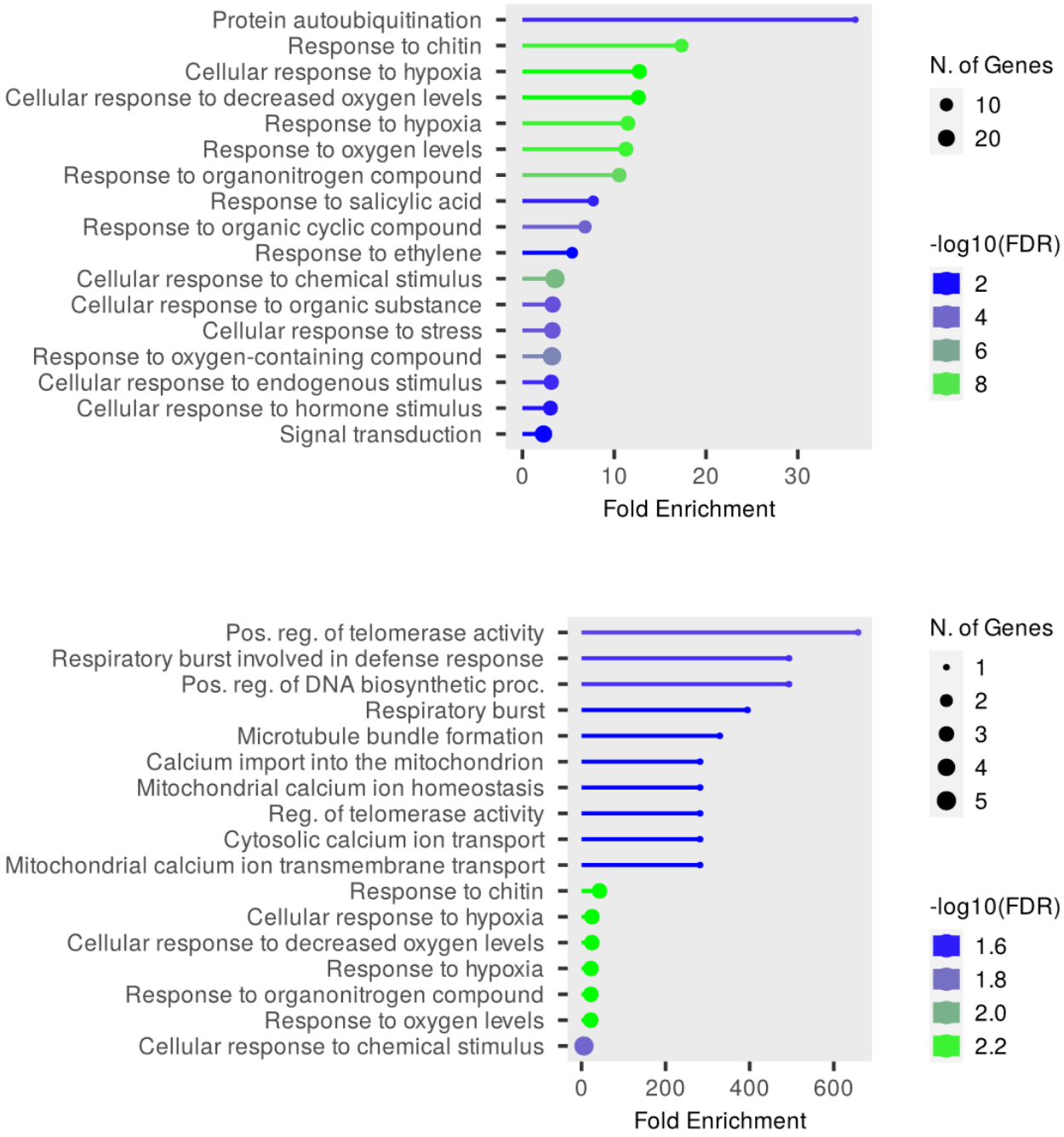


Figure 3.4.23: Genes differentially expressed in Arabidopsis due to interaction effects between *JAZ6* and *PFP* have distinct roles before and during infection.

GO enrichment shows that due to interaction effects between *JAZ6* and *PFP* prior to infection (upper) DE genes are related to ubiquitination, fungi responses, hypoxia, and hormones. During infection (lower) DE genes are related to hypoxia, fungal responses, and defence.

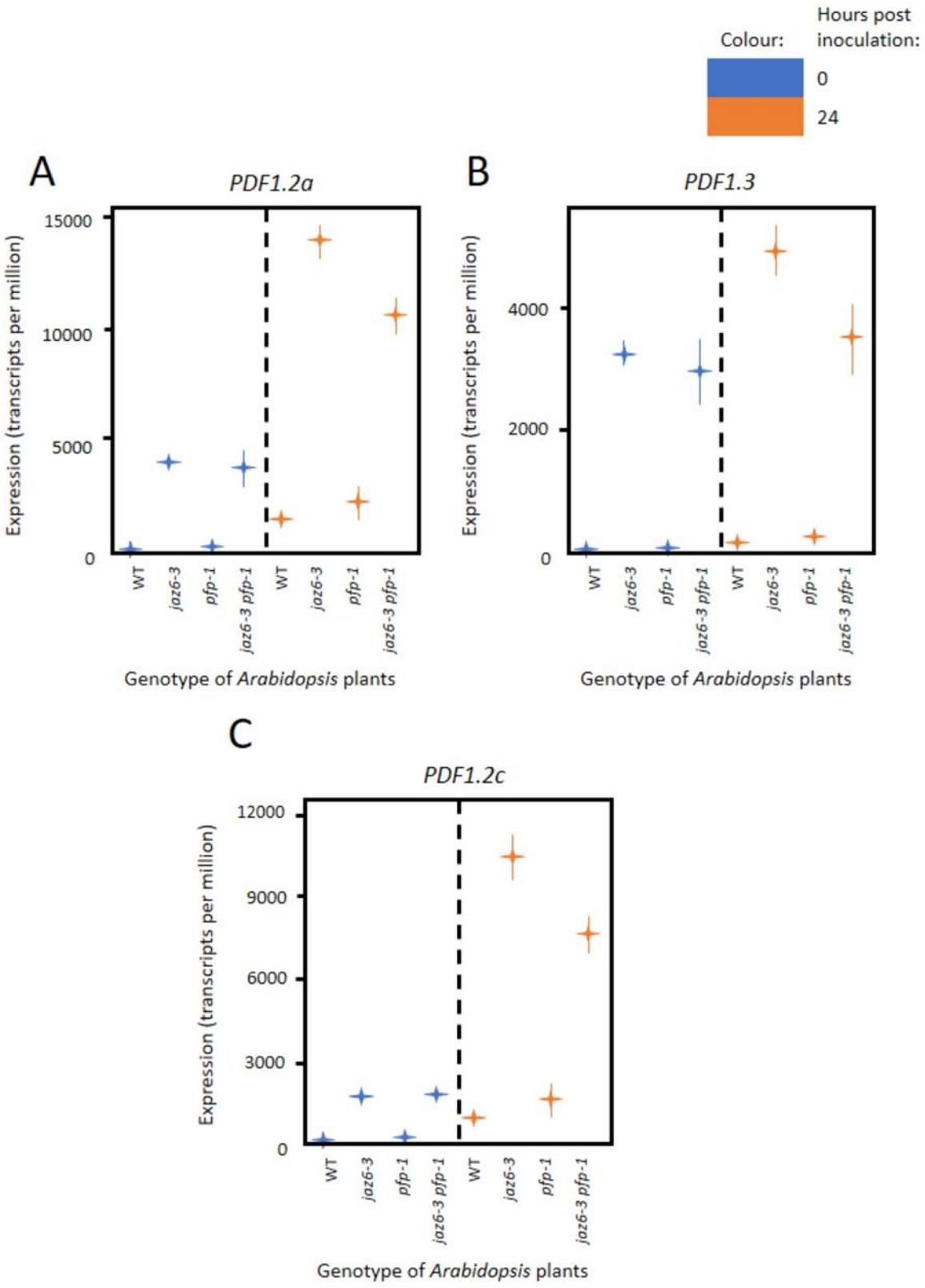


Figure 3.4.24: *JAZ6* regulates plant defensin expression independently of *PFP* and *B. cinerea*. Expression in transcripts per million for plant defensins *PDF1.2a* (A) *PDF1.3* (B) and *PDF1.2c* (C). Arabidopsis leaf samples were taken before (0 hpi) and during (24 hpi) *B. cinerea* infection. There are significant differences and a larger than 2-fold change in gene expression for these genes in in 3DRNAseq, in the WTΔ*jaz6-3* 0hpi contrast and the WTΔ*jaz6-3* 24hpi contrast.

Prior to infection, we see *PA2 (AT2G22420)* and *LBD11 (AT2G28500)* upregulated in *jaz6-3* compared to WT (Figure 3.4.25A & B), both of which could impact defence (Matsumura *et al.*, 2009; Shigeto *et al.*, 2013). During infection, we see *PATHOGEN CIRCADIAN CLOCK 1 (PCC1, AT3G22231)* downregulated in *jaz6-3* compared to WT (Figure 3.4.25C), which could feed into pathogen resistance or flowering time control (Mir *et al.*, 2013; Mir and León, 2014). Also, upregulation of *ERF12 (AT1G28360)* impacting flowering (Chandler and Werr, 2020) (Figure 3.4.25D).

Genes regulated by PFP were investigated for links to pathogen defence. To look at which genes are regulated by *PFP*, considering the *pfp-1*ΔWT contrast in uninfected and infected plants. Here we see 36 genes differentially expressed prior to infection, and 12 genes after, with an overlap of 6 genes which are differentially expressed before and during infection. Prior to infection, PUB23 and ERF5 are upregulated in pfp-1 compared to WT plants. These have negative impacts on *B. cinerea* defence *ERF5 (AT5G47230)* and E3 UBIQUITIN-PROTEIN LIGASE 23 (*PUB23, AT2G35930)* (Trujillo *et al.*, 2008; Moffat *et al.*, 2012).

Among the genes upregulated in *pfp-1* compared to WT which have a negative impact on *B. cinerea* defence are *KISS ME DEADLY 1 (KMD1, AT1G80440)* and the polycomb group repressor *MEDEA (MEA, AT1G02580)* (Kim *et al.*, 2013; Roy *et al.*, 2018) (Figure 3.4.26C & D). *KMD1* is differentially expressed prior to infection only (Figure 3.4.26C), while *MEA* is differentially expressed during infection only (Figure 3.4.26D). Expression of *KMD1* is induced by infection, while this is less apparent for *MEA*. The higher expression of *KMD1* and *MEA* in *pfp-1* correlates with higher susceptibility to *B. cinerea* infection (Figure 3.4.9).

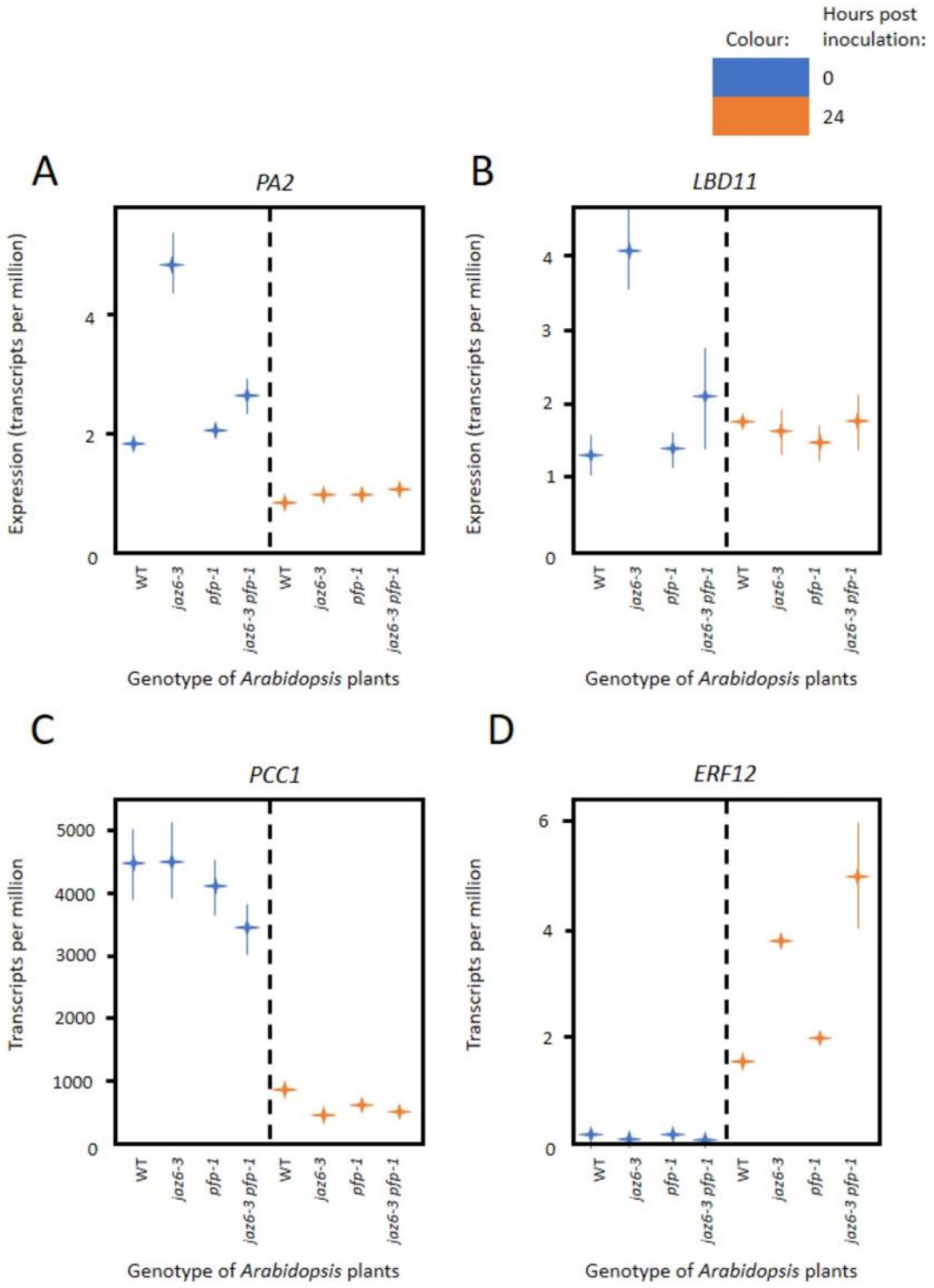


Figure 3.4.25: *JAZ6* regulates some defence related genes before, and others during, *B. cinerea* infection.

Expression in transcripts per million for *PA2* (A), *LBD11* (B), *PCC1* (C), and *ERF12* (D). Arabidopsis leaf samples were taken before (0 hpi) and during (24 hpi) *B. cinerea* infection. There are significant differences and a larger than 2-fold change in gene expression for these genes in in 3DRNAseq, in the WTΔ*jaz6-3* 0hpi contrast (A, B) and WTΔ*jaz6-3* 24hpi contrast (C, D).

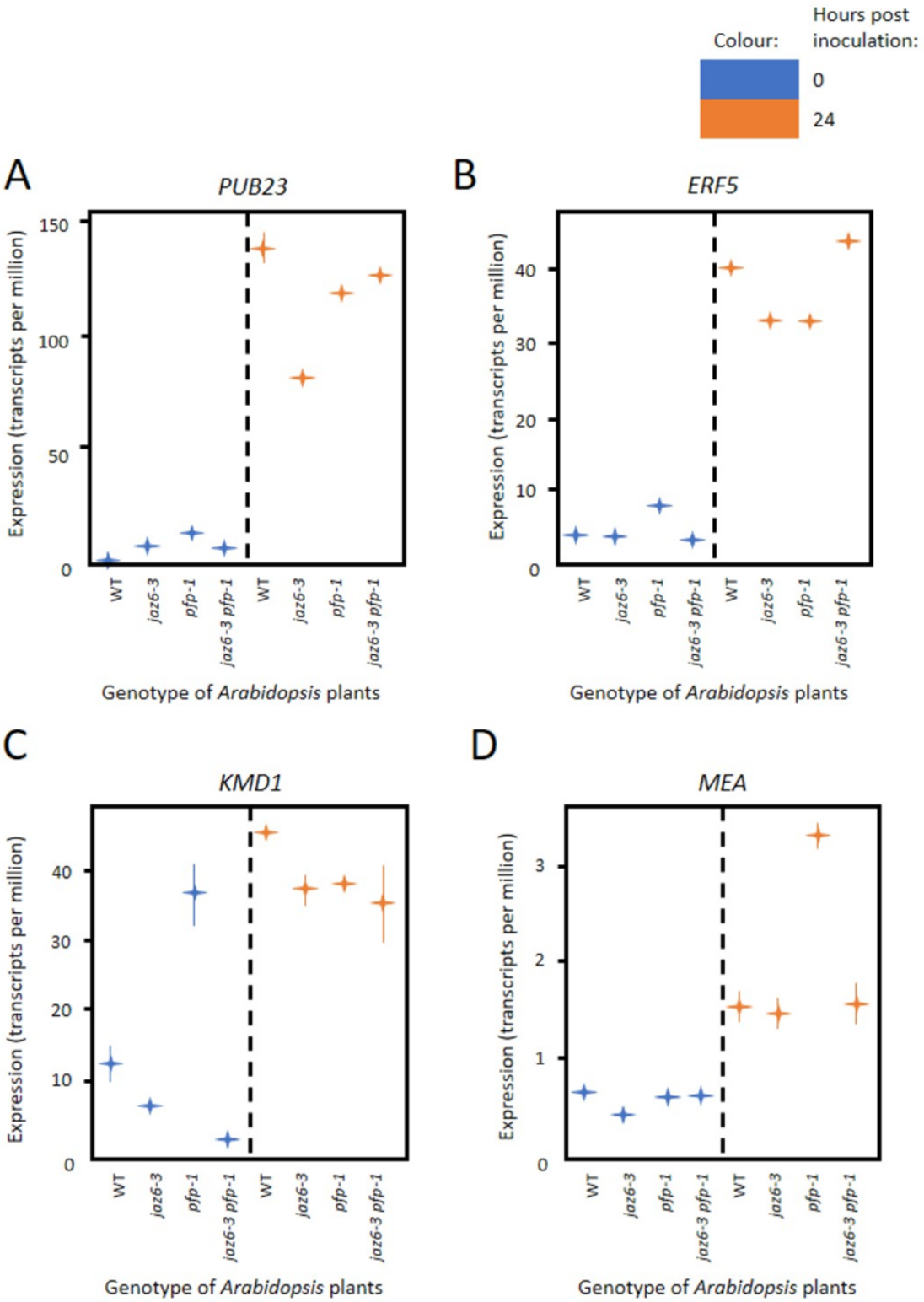


Figure 3.4.26: *PFP* regulates some defence related genes before, and others during, *B. cinerea* infection. Expression in transcripts per million for *PUB23* (A), *ERF5* (B), *KISS ME DEADLY 1* (C), and *MEDEA* (D). Arabidopsis leaf samples were taken before (0 hpi) and during (24 hpi) *B. cinerea* infection. There are significant differences and a larger than 2-fold change in gene expression for these genes in in 3DRNAseq, in the WTΔ*pfp-1* 0hpi contrast (A, B) and WTΔ*pfp-1* 24hpi contrast (C, D).

With the double *jaz6-3 pfp-1* mutant we can investigate the effects of the genetic interaction between *JAZ6* and *PFP*. Both flowering and infection assay phenotypes suggest an additive interaction, so we compared *jaz6-3*ΔWT and *jaz6-3 pfp-1*Δ*pfp-1* contrasts to look at synergistic or antagonistic interaction effects the mutations may be having. Genes significantly differentially expressed in this contrast of contrasts are reacting differently to the *jaz6-3* mutation depending on the presence of the *pfp-1* mutation. The inverse is also true, differentially expressed genes are reacting differently to the *pfp-1* mutation depending on the presence of the *jaz6-3* mutation. Here we see 139 genes differentially expressed prior to infection, and 17 genes after, with an overlap of 2 genes which are differentially expressed before and during infection.

Many of the genes regulated by interaction effects between *JAZ6* and *PFP* are also regulated by *PFP* prior to infection (Figure 3.4.26A & B), including the defence related *ERF5 (AT5G47230)* and E3 UBIQUITIN-PROTEIN LIGASE 23 (*PUB23, AT2G35930)* (Trujillo *et al.*, 2008; Moffat *et al.*, 2012).

Prior to infection, the interaction between *JAZ6* and *PFP* affects expression of *TIR-NBS-LRR* genes (including *AT1G63860)*and WRKY53 *(AT4G23810)* which could impact defence (Miao *et al.*, 2004; Faigón-Soverna *et al.*, 2006) (Figure 3.4.26A & C). *RAV (RELATED TO ABI3 and VP1)* 1 and 2 *(AT1G13260, AT1G68840)* (Figure 3.4.26B), were also regulated by the interaction, and could affect flowering (Matías-Hernández *et al.*, 2014) (Figure 3.4.26B). However, during infection *ERF98 (AT3G23230)* expression is regulated by interaction effects (Figure 3.4.26D), which may impact general stress tolerance (Zhang *et al.*, 2012).

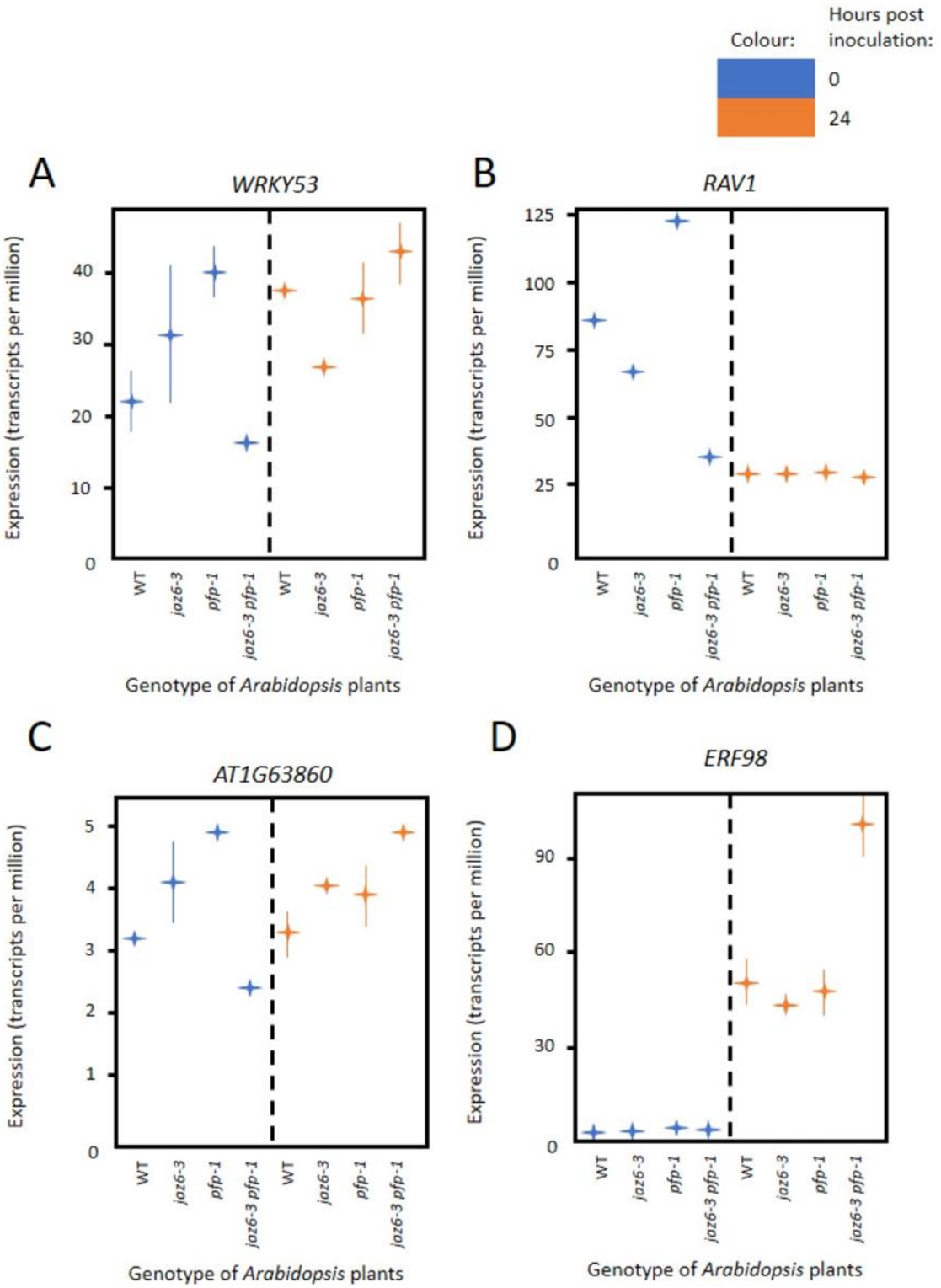


Figure 3.4.27: The genetic interaction between *JAZ6* and *PFP* regulates some defence related genes before, and others during, *B. cinerea* infection.

Expression in transcripts per million for *WRKY53* (A), *RAV1* (B), *AT1G63860* (C), and *ERF98* (D). Arabidopsis leaf samples were taken before (0 hpi) and during (24 hpi) *B. cinerea* infection. There are significant differences and a larger than 2-fold change in gene expression for these genes in in 3DRNAseq, in the (WTΔ*jaz6-3)Δ(pfp-1Δjaz6-3 pfp-1)* 0hpi contrast (A, B, C) and (WTΔ*jaz6-3)Δ(pfp-1Δjaz6-3 pfp-1)* 24hpi contrast (D).

3.4.6 Neither *JAZ6* nor *PFP* are essential for jasmonic acid signalling

Jasmonic acid represses primary root growth in Arabidopsis (Wasternack and Hause, 2013). Therefore, mutants of genes essential for jasmonic acid signalling such as *coi1-2* are unable to respond to jasmonic acid and so root growth is not inhibited (Xu et al., 2002). The *coi1-2* mutant would be an ideal negative control for root growth assays as their root growth is not inhibited by jasmonic acid as noted above. However, mutants of *coi1* can be difficult to cultivate due to the severe impact on fertility (Xie *et al.*, 1998). Additionally, as demonstrated with *NINJA* and *ECAP* the physiological role of a gene hypothesised to be involved in root jasmonic acid signalling can be inferred without using *coi1-2* mutants as a negative control (Acosta *et al.*, 2013; Li *et al.*, 2019).

As PFP binds JAZ6, which is involved in jasmonic acid signalling, jasmonic acid induced root growth inhibition was tested in *pfp-1* mutant and *PFP* overexpressing Arabidopsis lines (Yokoyama, Kobayashi and Kidou, 2019) compared to wildtype and *jaz6-3,* by growing plants in sterile vertical Petri dishes with methyl-jasmonic acid or a DMSO mock treatment (Figure 3.4.28).

jaz6-3 mutants did not have a root growth inhibition phenotype (Figure 3.4.28), which is in agreement with *jaz6-18* and *jaz6-31* mutant analysis (Li *et al.*, 2019). This is not surprising as there are many other JAZ proteins to complement the role of *JAZ6* in JA signalling in a *jaz6-3* mutant. *pfp-1* mutants also did not have a root growth phenotype (Figure 3.4.28), and while we saw that root growth in *PFP* overexpressing lines was higher prior to JA treatment they also lacked a significant change in JA induced root growth inhibition. This suggests that *PFP* is not an essential part of JA signalling networks.

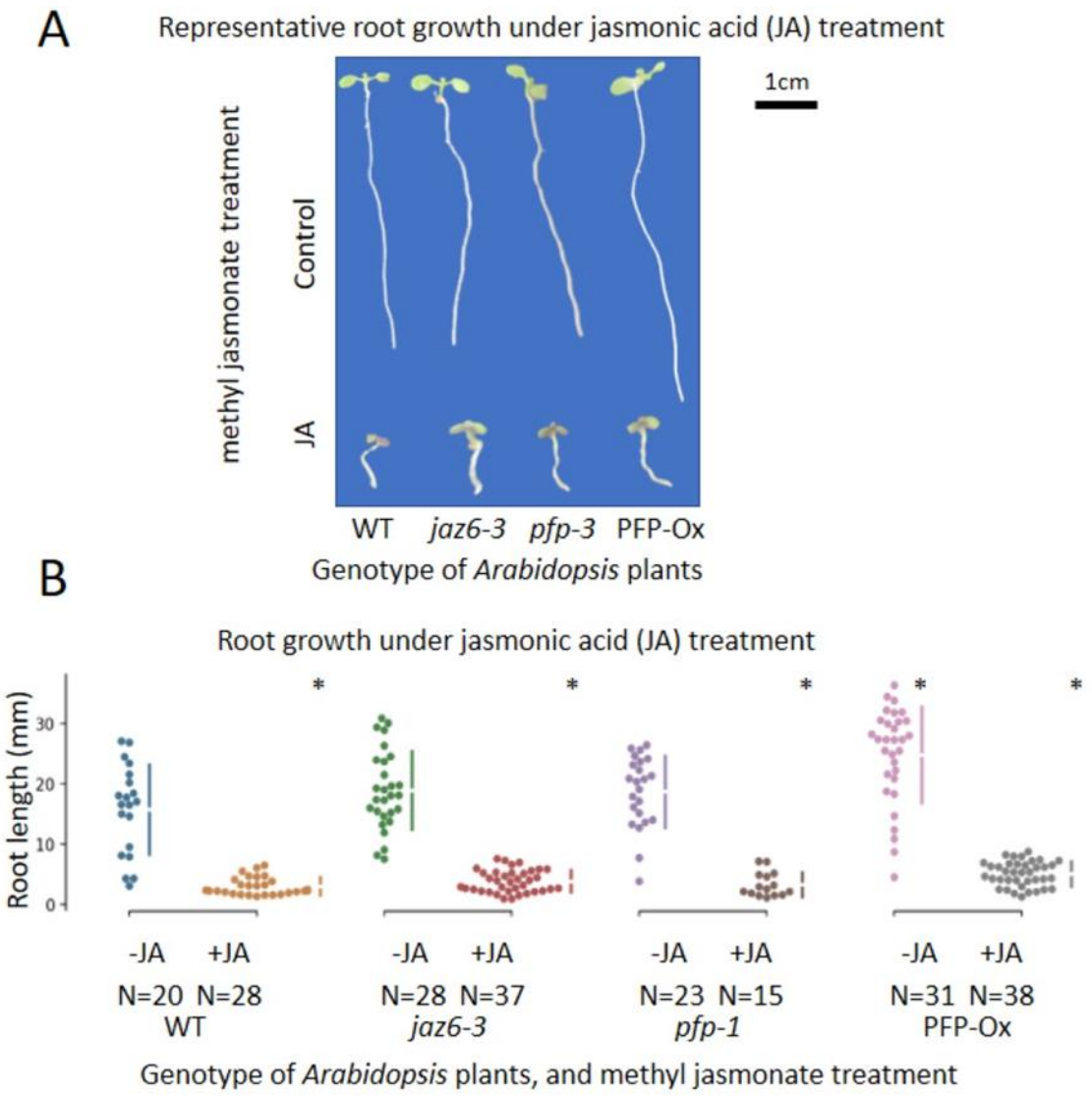


Figure 3.4.28: *PFP* is not essential for jasmonic acid root growth inhibition.

A and B, Root growth inhibition assays, imaged and measured after 14 days of growth on 25 µM methyl-jasmonic acid or mock DMSO imbued ½ MS media. (A) Representative seedlings. (B) Growth of primary root length. Scale bar represents 1cm.

Bars alongside each group of values represent the upper and lower quartiles around the mean. "N" is the number of seedlings assayed for each set of inoculations. Asterisks represent statistically significant differences from WT, from 95% confidence intervals produced using Estimation Stats (Ho et al., 2019). This experiment was repeated three times.

3.4.7 Botrytis gene expression reflects the host plant genotype

Concurrently with aligning RNA reads to the Arabidopsis transcriptome, reads were also aligned to the *B. cinerea* transcriptome. While this approach discounted *B. cinerea* reads from being mistakenly assigned to Arabidopsis genome, it also enabled investigation of *B. cinerea* co-expression dynamics which reflect the changes in host genotype. The importance of plant and plant pathogen co-expression dynamics has recently been highlighted, as *B. cinerea* gene expression is reactive to the genetics and gene expression of its host.

The delineation of *B. cinerea* gene expression when on different hosts is clearly shown by PCA analysis (Figure 3.4.29). As for Arabidopsis, reads were processed, filtered, and normalised to give gene expression for *B. cinerea* (Figure 3.4.30). In total 518 genes were differentially expressed due to host genotype (Figure 3.4.31), however, the number of genes differentially expressed greatly varies depending on the host (Table 3.3.2). In addition, as shown for Arabidopsis, differential expression is mostly unique to a particular genotype, when compared to infection of WT plants (Figure 3.4.19).

Functional analysis of differentially expressed *B. cinerea* genes using GO analysis shows that in response to the different host genotypes, *B. cinerea* gene expression may reflect changes in metabolic production in response (Figures 3.4.33-34). Only a handful of genes such as *Bcin15g02380* are differentially expressed in all genotypes compared to WT (Figure 3.4.35). However, a few *B. cinerea* genes appear to respond to interaction effects between JAZ6 and PFP, including *Bcin10g05230* and the CAZyme *Bcin14g02510* (Figure 3.4.35B & C).

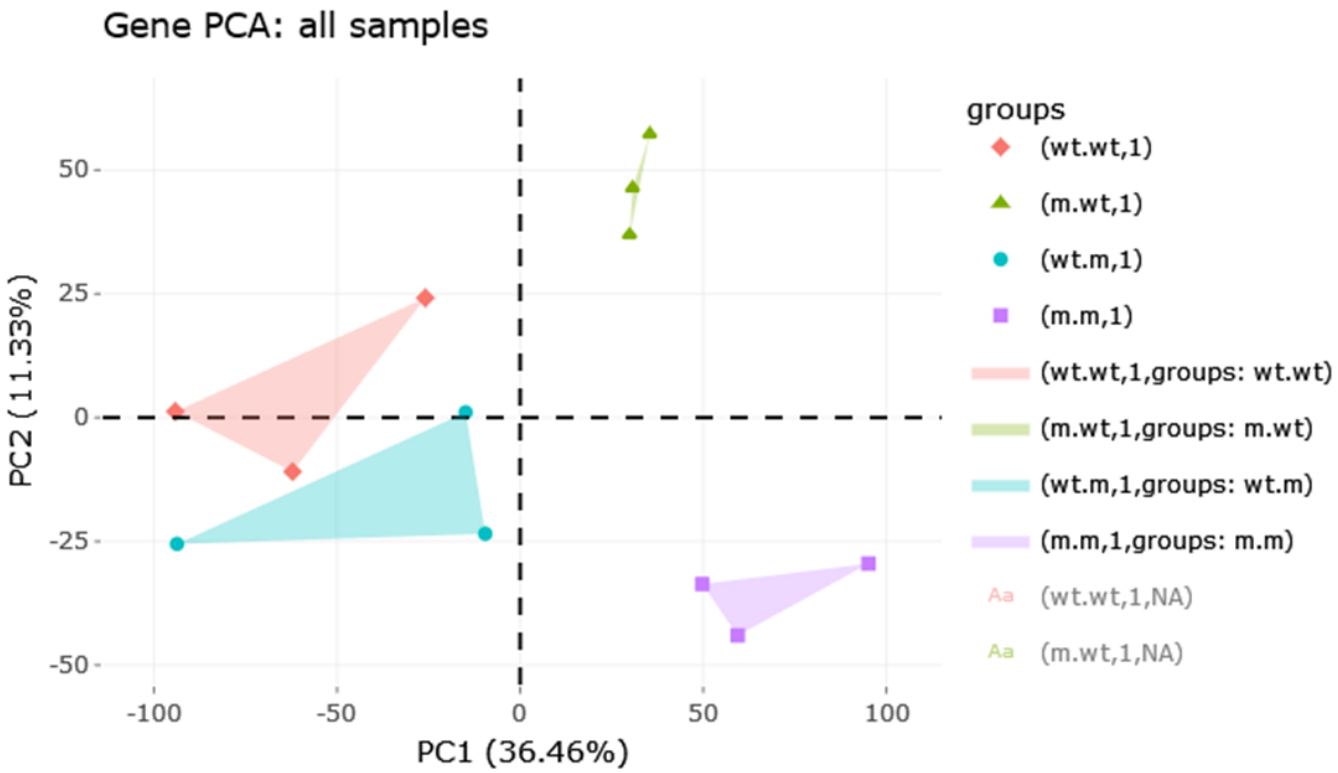
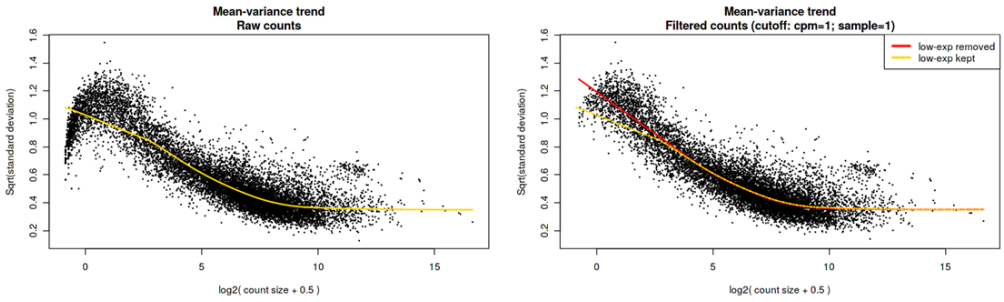
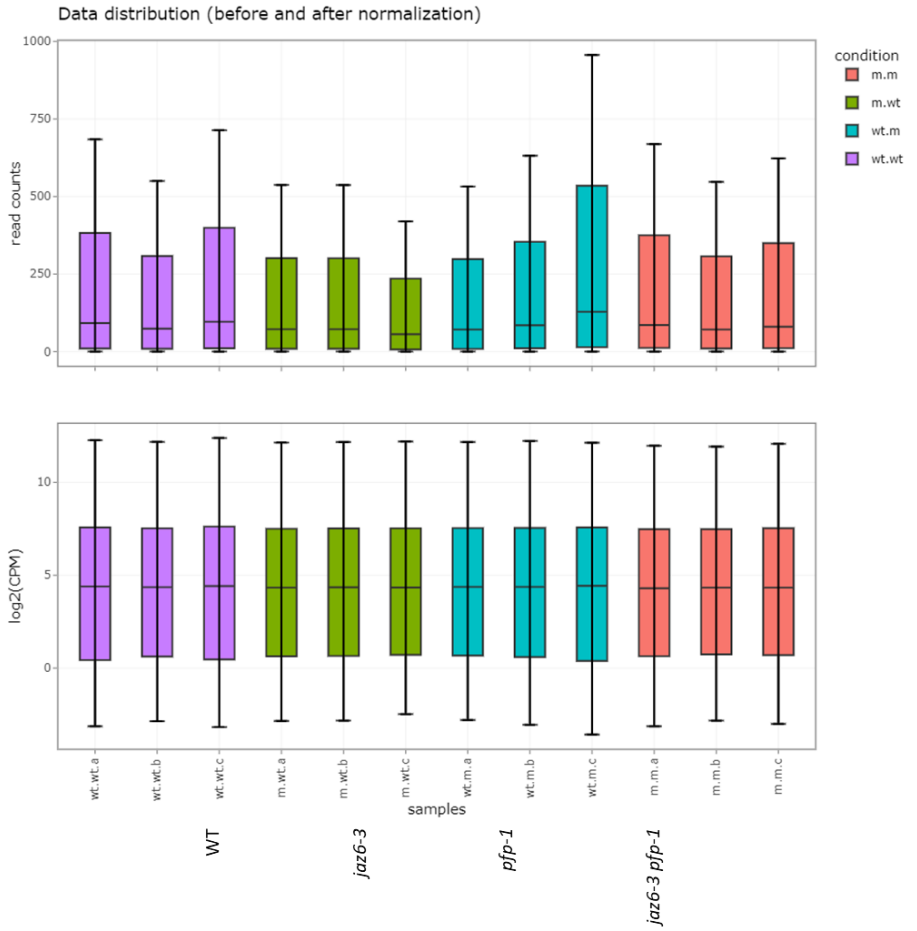


Figure 3.4.29: RNAseq samples aligned to *B. cinerea* group together based on plant genotype
Only 24hpi samples used.

A



B



Genotype of *Arabidopsis* plants, and sample time post-inoculation

Figure 3.4.30: Filtering and normalising *B. cinerea* data in 3DRNAseq.
Gene level analysis of trends in gene expression to be filtered and corrected for comparison between samples.
A. Voom plot showing the removal of the drop effect with low expression genes by filtering
B. Normalisation of gene expression in 3DRNAseq showing the distribution of read counts before and after normalisation.

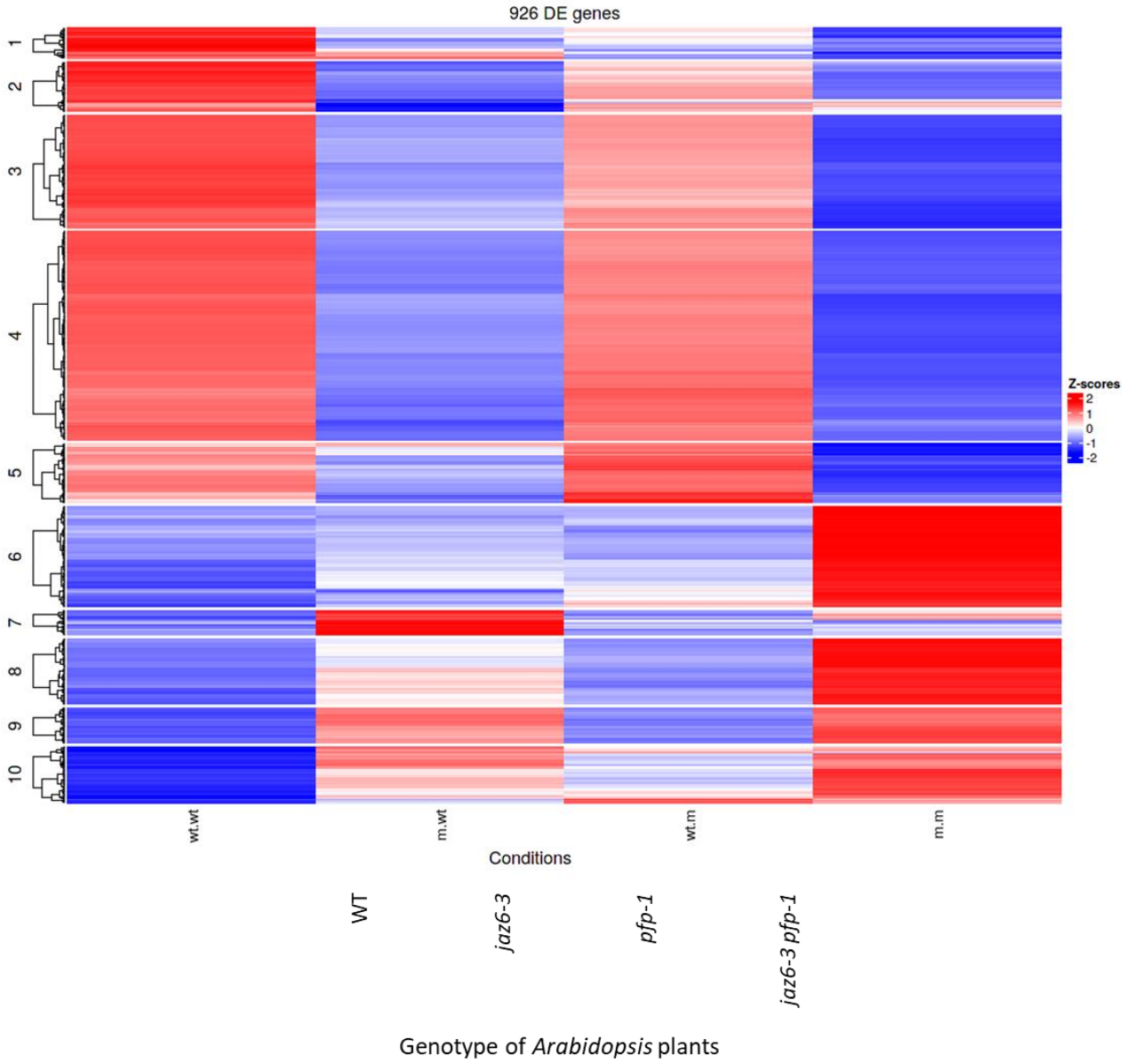


Figure 3.4.31: Transcriptomic analysis of *B. cinerea* infection reveals 926 genes differentially regulated in *B. cinerea* by *JAZ6, PFP*, or an interaction effect of *JAZ6* and *PFP.*
Heatmap of differentially expressed genes from the contrasts WTΔ*jaz6-3*, WTΔ*pfp-1* and the interaction contrast of (WTΔ*jaz6-3)Δ(pfp-1Δjaz6-3 pfp-1)* both before and during *B. cinerea* infection. Z-scores represent the relative average gene expression across all samples. Genes are clustered into 10 clusters of genes with similar expression patterns.

Table 3.3.2: Transcriptomic analysis of *B. cinerea* infection reveals 518 genes regulated by *JAZ6, PFP*, or an interaction effect of *JAZ6* and *PFP.*
Heatmap of differentially expressed genes from the contrasts WTΔ*jaz6-3*, WTΔ*pfp-1* and the interaction contrast of (WTΔ*jaz6-3)Δ(pfp-1Δjaz6-3 pfp-1)* both before and during *B. cinerea* infection. Z-scores represent the relative average gene expression across all samples. Genes are clustered into 10 clusters of genes with similar expression patterns.

Contrast	DE genes	Upregulated	Downregulated
jaz6-3 vs. WT	373	93	280
pfp-1 vs. WT	7	6	1
jaz6-3 pfp-1 vs. WT	853	302	551
JAZ6 PFP interaction (*jaz6-3* vs. WT) vs. (*jaz6-3 pfp-1* vs. *pfp-1*)	9	4	5

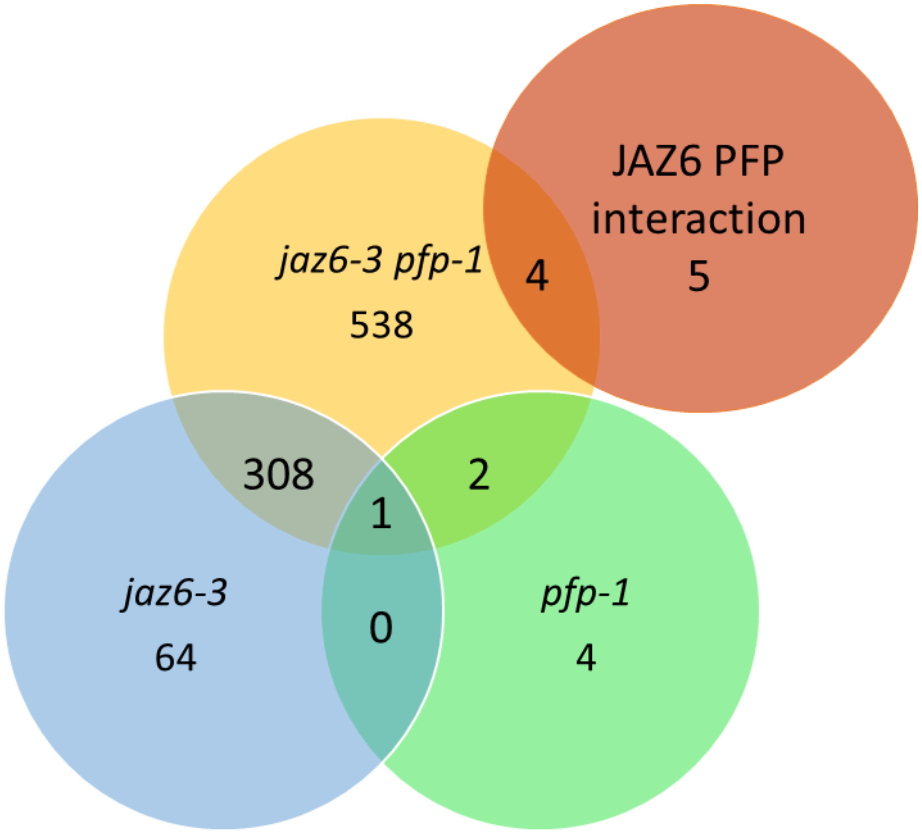


Figure 3.4.32: There is limited overlap between the genes differentially expressed in *B. cinerea* on different plant genotypes. Particularly for the double mutant *jaz6-3 pfp-1*.

Venn diagram of overlap in DE genes between plant genotypes.

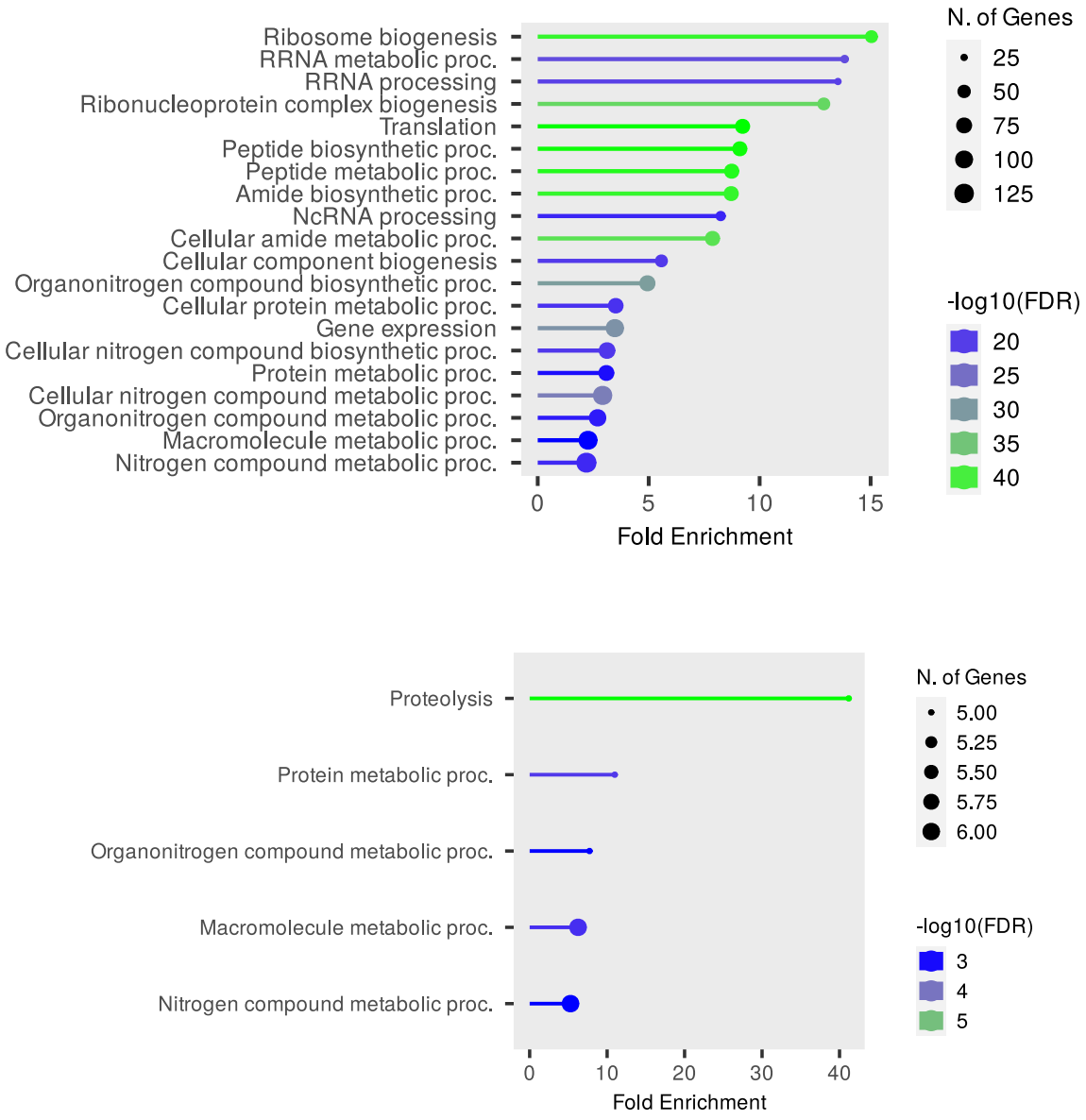


Figure 3.4.33: *B. cienrea* differentially expressed genes are related to metabolism.

Genes DE in *B. cinerea* on *jaz6-3* mutants (upper) and *pfp-1* mutants (lower) DE express genes related to metabolism and biosynthesis.

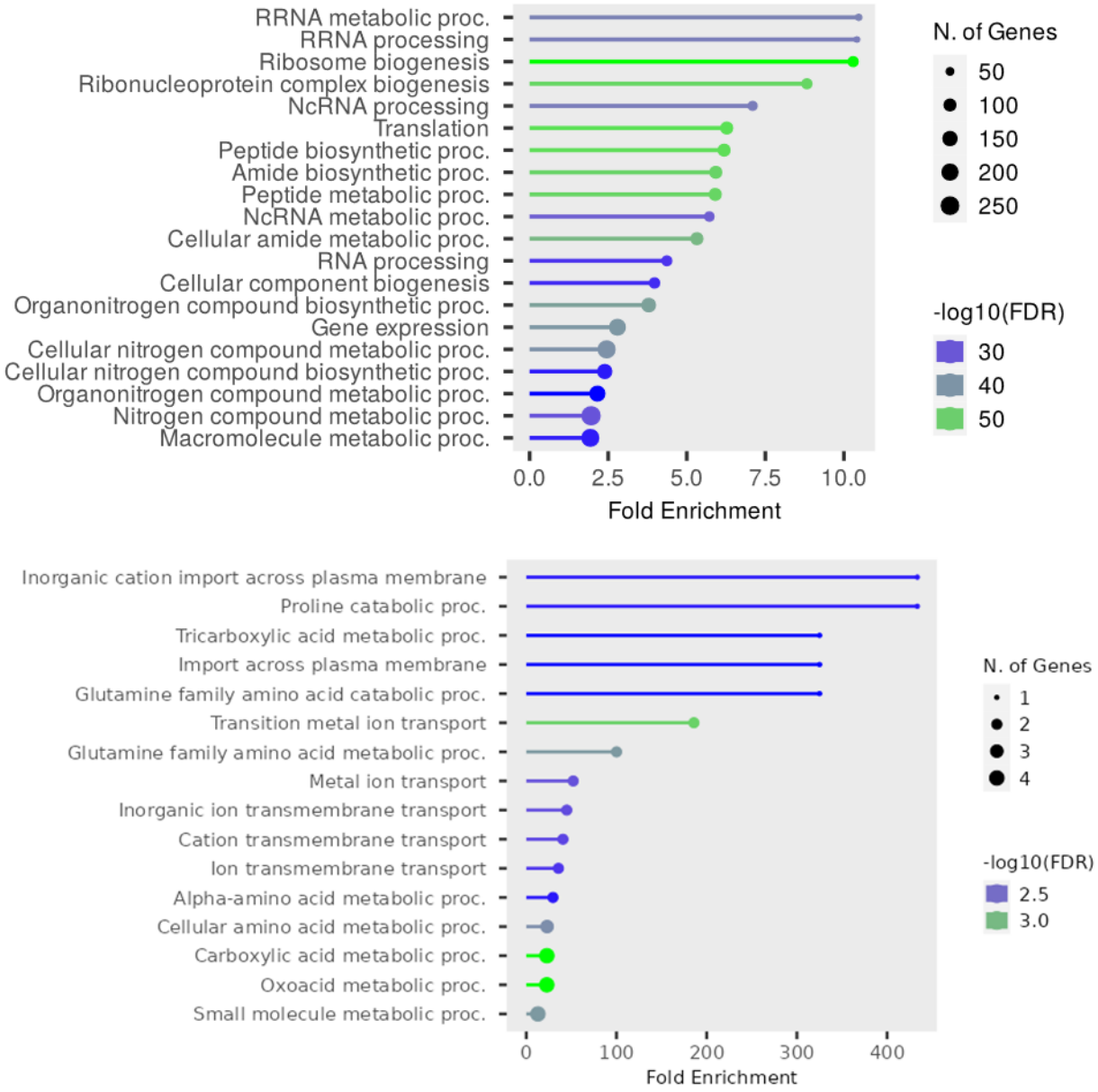


Figure 3.4.34: *B. cienrea* differentially expressed genes on *jaz6-3 pfp-1* are related to metabolism. Genes DE in *B. cinerea* on *B. cinerea* on *jaz6-3 pfp-1* mutants (upper) and subject to interaction effects between JAZ6 and PFP (lower) DE genes are related to metabolism and biosynthesis.

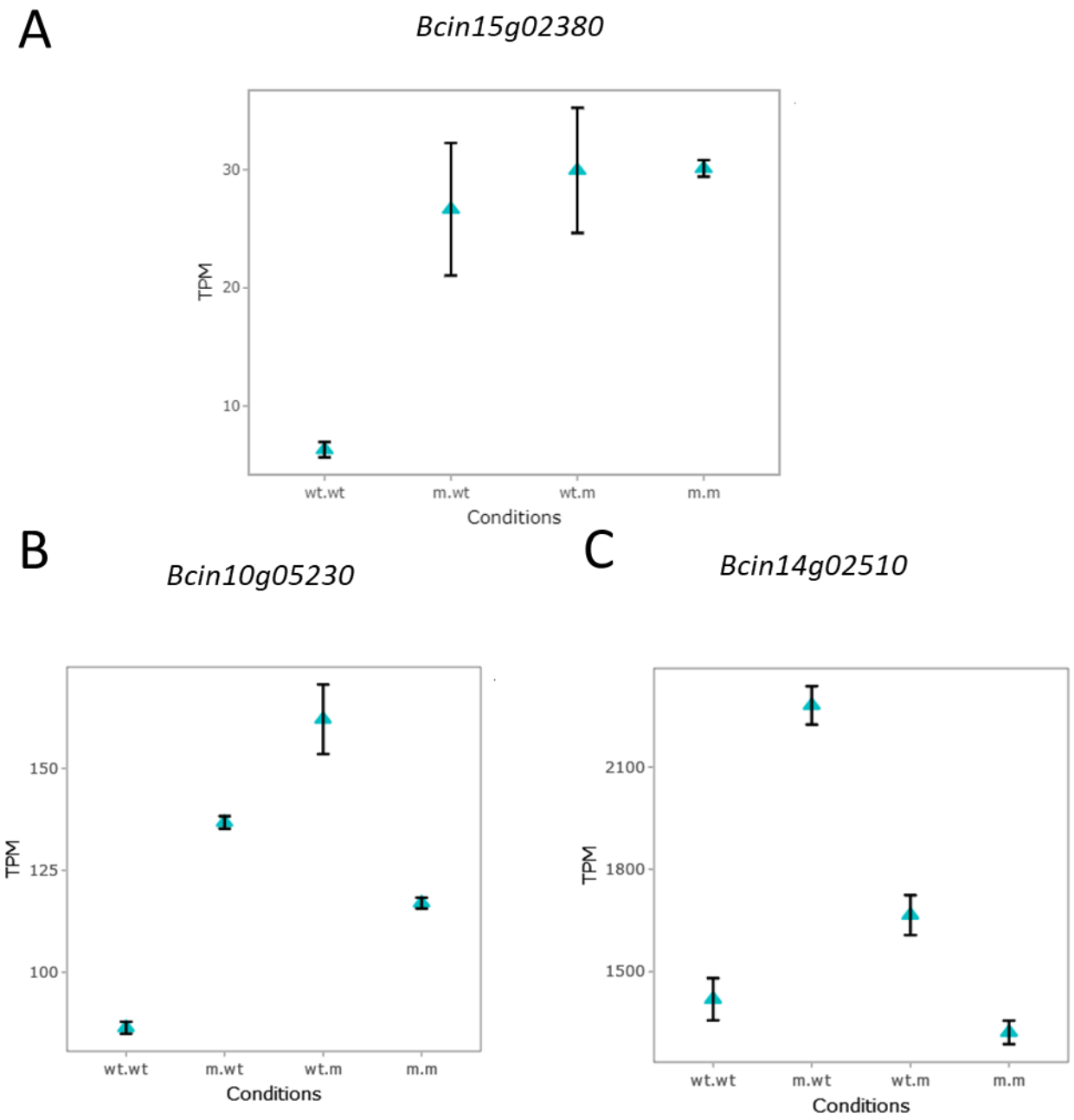


Figure 3.4.35: The genetic interaction between *JAZ6* and *PFP* regulates some defence related genes before, and others during *B. cinerea* infection.
Expression in transcripts per million for pH regulator *Bcin15go2380* (A), *Bcin10g05230* (B), *Bcin14g02510* (C). Arabidopsis leaf samples were taken during (24 hpi) *B. cinerea* infection.

3.4.8 *JAZ6* represses flowering in short days, while *PFP* promotes flowering

PFP has an established role in repressing the transition from vegetative growth to flowering. *pfp-1* mutants have been noted to show an early flowering phenotype in both long day (16 hour light, 8 hour dark) and short day (8 hour light, 16 hour dark) growth conditions (Yokoyama, Kobayashi and Kidou, 2019). However, a chance observation suggested this phenotype was not present in the double *jaz6-3 pfp-1* mutant (Figure 3.4.25) for plants grown in long day photoperiods. This suggested that the genetic interaction between *PFP* and *JAZ6* affects aspects of plant physiology like flowering.

Previous work has established that in long day conditions the *JAZ6* T-DNA insertion mutant *jaz6-5* does not display an altered flowering time phenotype in long day growth conditions compared to WT plants (Liu *et al.*, 2021). However, genes affecting the autonomous and non-photoperiodic flowering pathways in Arabidopsis may not have a significant effect on flowering time in long day growth conditions (Sharma *et al.*, 2016), and so the mutants of these genes may only show a phenotype in short day conditions. Therefore, a flowering time assay was performed with the *jaz6-3, jaz6-31,* and *pfp-1* single mutants and the double *jaz6-3 pfp-1* mutant under short day conditions (Figure 3.4.26).

pfp-1 mutants flower earlier than WT in short day conditions, as reported previously (Yokoyama, Kobayashi and Kidou, 2019). As suggested by the flowering phenotypes in long day conditions, the double mutant flowered later than the single mutant, at an intermediate leaf number between the *pfp-1* and *jaz6-3* or *jaz6-31* single mutants (Figure 3.4.26). In addition, *jaz6-3* and *jaz6-31* mutants flower later than WT plants (Figure 3.4.26), which was not evident in long day photoperiods (Figure 3.4.25). This suggests that *JAZ6* represses flowering in short day conditions, a novel physiological role for *JAZ6*.

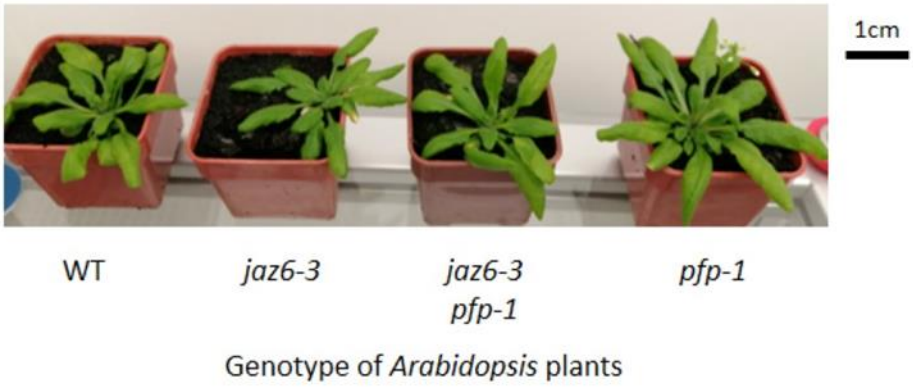


Figure 3.4.36: The genetic interaction between *JAZ6* and *PFP* affects flowering time.

Flowering time observation under long day growth conditions, representative plants. Scale bar represents 1cm.

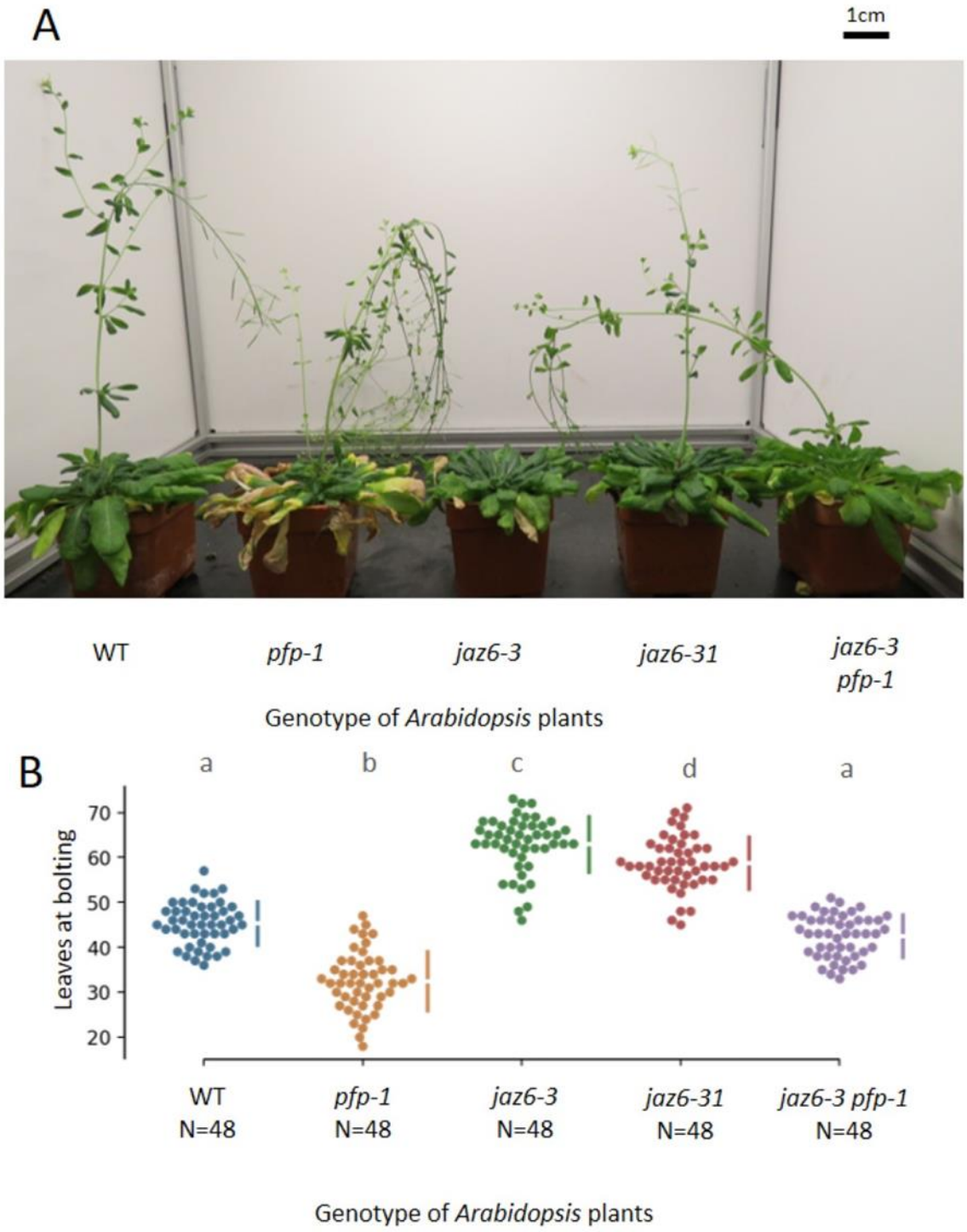


Figure 3.4.37: *PFP* and *JAZ6* control flowering in antagonistic ways.

Flowering time assays under short day conditions. (A) Representative plants. (B) Number of rosette leaves at flowering for plants in short day growth conditions. Scale bar represents 1cm. Bars alongside each group of values represent the upper and lower quartiles around the mean. "N" is the number of plants assayed. Asterisks represent statistically significant differences from WT, from 95% confidence intervals produced using Estimation Stats (Ho et al., 2019).

ANOVA indicated a significant effect of plant genotype ($P < 0.00001$) on the number of leaves at bolting. Different letters indicate a significant difference in the number of leaves at bolting, by Tukey's HSD. This experiment was repeated twice.

3.5 Discussion

3.5.1 JAZ6 promotes susceptibility to *B. cinerea* specifically at dusk.

Having two independent mutants in *JAZ6* for validation of *B. cinerea* resistance provides additional confidence that *JAZ6* is responsible for promoting susceptibility to *B. cinerea*. A single mutant may be displaying a phenotype due to effects of the technique which effect the expression of genes other than the target gene. For T-DNA insertion SALK and SAIL lines, there are often multiple T-DNA insertions present in a single line (O'Malley and Ecker, 2010), and so the effects attributed to a single mutant may be due to the disruption of other genes by T-DNA insertions. For CRISPR targeted gene editing, off target effects are possible with high frequency (Q. Zhang *et al.*, 2018). These off-target effects could affect other genes, obscuring the effect of modifying the target gene. However, with two independent mutant lines, the off-target effects will be different. Therefore, the infection assays with *jaz6-3* and *jaz6-31* validate the role of *JAZ6* in *B. cinerea* resistance (Figure 3.4.8) as they are independent mutants.

3.5.2 *JAZ6* promotes susceptibility to multiple necrotrophic pathogens.

Infections with *S. sclerotiorum* provide evidence for *JAZ6* controlling disease resistance for a wider range of pathogens than *B. cinerea* (Figure 3.4.9). Given the close taxonomic relationship and similar lifestyles of *B. cinerea* and *S. sclerotiorum* (Amselem *et al.*, 2011), it is common to see genes promoting susceptibility against both in the same way. One example of such a gene is *BOS1,* a MYB transcription factor, the *BOS1* mutant *bos1* is highly susceptible to both *B. cinerea* and *S. sclerotiorum* (Mengiste *et al.*, 2003). For transcriptional regulators like *BOS1* and *JAZ6* it is common to see such effects because they act upstream of plant defences. One reason for a gene having a different role between similar pathogens would be specific targeting by the pathogen via a phytotoxin like coronatine or effector protein like Jsi1 (Feys *et al.*, 1994; Darino *et al.*, 2021).

3.5.3 *JAZ6* promotes susceptibility to *B. cinerea* by repressing plant defensin gene expression.

We have shown that *JAZ6* promotes susceptibility to *B. cinerea*, with a *jaz6-3* mutant more resistant to *B. cinerea* (Figure 3.4.9), as previously described with other *jaz6* mutants (Li *et al.*, 2019). A mechanistic explanation of the resistance to *B. cinerea* in jaz6-3 mutants is

higher expression of plant defensins *PDF1.2a* and *PDF1.3* than WT, which we show both before and during *B. cinerea* infection (Figure 3.4.24). These plant defensins fatally disrupt the plasma membrane of pathogens (Thomma, Cammue and Thevissen, 2002), so higher expression of defensins can lead to *B. cinerea* resistance. Previous work shows that *JAZ6* also promotes susceptibility to *P. syringae* (Liu *et al.*, 2021), which may also be due to higher expression of plant defensins.

3.5.4 *JAZ6* represses susceptibility to *H. arabidopsidis*.

jaz6-3 and *jaz6-31* mutants are more susceptible to *H. arabidopsidis* infection (Figure 3.4.12), which is a novel phenotype for a *jaz6* mutant and contrasts to the role of *JAZ6* in infection by other pathogens. Infection by *H. arabidopsidis* may differ to other pathogens as it is an obligate biotroph with different strategies for infecting plants (Pieterse *et al.*, 2012). Similar to Arabidopsis *JAZ6*, wild grapevine *VqJAZ4* promotes susceptibility to *B. cinerea* and suppressing susceptibility to *Golovinomyces cichoracearum*, an obligate biotroph like *H. arabidopsidis* (Guofeng Zhang *et al.*, 2019). EIN3 may repress salicylic acid biosynthesis through *SID2* (Thaler, Humphrey and Whiteman, 2012), so JAZ proteins could indirectly promote SA synthesis during infection through repression of *EIN3*.

However, susceptibility to these necrotrophs can be antagonistic to susceptibility to biotrophs, due to the anatagonistic relationship between salicylic acid and jasmonic acid (Takahashi *et al.*, 2004). Genes like GLK affect susceptibility to the biotrophic pathogen *H. arabidopsidis* in the opposite way to how they affect susceptibility to necrotrophic pathogens (Murmu *et al.*, 2014).

Combining the results from the necrotrophic and biotrophic infection assays would lead us to believe that *JAZ6* promotes susceptibility to necrotrophs and represses susceptibility to biotrophs. However, it should be kept in mind that the age of the infected plants was significantly different for these two examples, and so the plant response to infection may have been different regardless of the type of pathogen. Nonetheless, both infection assays are well known standard techniques for which the results are indicative of a broad interpretation of the ecotype or mutant being studied (Denby, Kumar and Kliebenstein, 2004; Coates and Beynon, 2010).

3.5.5 *JAZ6* may have a distinct role in jasmonic acid signalling due to the proliferation of *JAZ* genes

In general, jasmonic acid signalling mutants are not fully insensitive to jasmonic acid, for example mutants of the jasmonic acid biosynthesis gene *jar-1* still exhibit induction of *JAZ10* gene expression after wounding, indicating that JA signalling still occurs (Acosta *et al.*, 2013). *JAZ6* is even more dispensable, as there are 13 *JAZ* genes in Arabidopsis which have been demonstrated to partially complement each other (Q. Guo *et al.*, 2018; Liu *et al.*, 2021).

The clear phenotypes of the JAZ6 mutant here in terms of defence and flowering may appear at first glance to be contradictory with the idea that *JAZ6* is somewhat redundant, partially complemented, and not strictly required for JA signalling. However, it has been established in multiple other works that different JAZ genes can have distinct roles (Ingle *et al.*, 2015; Liu *et al.*, 2021), so it may be inferred that there are underlying mechanisms which enable the distinct roles of the different JAZ genes.

In particular, recent work has highlighted the role of structural variation in the Jas domain to control the binding affinity a gene has for MYC2, and thus it may be inferred how well a JAZ protein can repress MYC transcription factors and dependent downstream gene expression is down to the structure of the Jas domain (Zhang *et al.*, 2015). This argument may be extended to other transcription factors repressed by JAZ proteins, but also jasmonic acid and COI1 for the targeted degradation of JAZ proteins. Synthesis of a chemical agonist similar to jasmonic acid highlights that the structure of the Jas domain controls how a JAZ protein is degraded, as a chemical specifically and uniquely targeting JAZ9 for degradation was demonstrated (Takaoka *et al.*, 2018).

As the above established structural variation in JAZ proteins belies their distinct functions, it can be understood that JAZ6 has a distinct role due to its specific protein structure. It may be hypothesised that the proliferation of *JAZ* genes has enabled such functional diversity, which is not possible for solitary genes involved in jasmonic acid signalling like COI1, as *JAZ* genes can evolve independently of each other to distinct roles.

3.5.6 *PFP* promotes resistance to *B. cinerea*, but not through plant defensins.

PFP has not previously been linked to defence in Arabidopsis, and here we show it represses susceptibility to *B. cinerea* (Figure 3.4.10). However, *PFP* does not affect the key defensin defence genes which *JAZ6* represses (Figure 3.4.24). A mechanistic explanation for the higher susceptibility of *pfp-1* than WT to *B. cinerea* infection may be the expression of *KMD1* and *MEA* (Figure 3.4.26). *KMD1* is upregulated in *pfp-1* mutants compared to WT plants prior to infection only. It forms SCF^{KMD} complexes which negatively regulate cytokinin signalling and degrade phenylalanine ammonia-lyase (PAL) (Kim *et al.*, 2013; Zhang, Gou and Liu, 2013). This degradation of PAL can lead to impaired lignin production (Zhang, Gou and Liu, 2013), which would make plants more susceptible to *B. cinerea* infection. In contrast, *MEA* is upregulated in *pfp-1* mutants compared to WT plants during infection only. *MEA* is a polycomb group protein which has previously been linked to *P. syringae* disease susceptibility (Roy *et al.*, 2018). *mea* mutants less susceptible to *P. syringae* infection than WT, while over-expressors are more susceptible with lower expression of *PDF1.2a*. It is plausible *MEA* could act in a similar manner during *B. cinerea* infection.

Both JAZ6 and PFP may be acting on defence genes through a DLNxxP EAR domain which they both possess (Kagale, Links and Rozwadowski, 2010). This EAR domain may be key for transcriptional repression or protein binding, resulting in disease resistance or susceptibility for *JAZ6* and *PFP* mutants. Future work could test this by attempting to complement the jaz6-3 mutant line with JAZ6mEARs. If disease susceptibility is rescued, the JAZ6 EAR domains are not essential for JAZ6 to regulate disease resistance or susceptibility.

3.5.7 *PFP* promotes susceptibility to a range of biotrophic pathogens.

The role of *PFP* in *H. arabidopsidis* infection may be compared to the role of its homologue *NbUBR7* in TMV infection (Yongliang Zhang *et al.*, 2019). Nb*ubr7* mutants are more resistant to TMV infection, which contrasts to Arabidopsis *pfp-1* mutants which are more susceptible to *B. cinerea* and more resistant to *H. arabidopsidis* (Figure 3.4.12). These contrasts may be explained by the different host plants and pathogens involved in these pathosystems. Indeed, *N. benthamiana* has a significantly different set of JAZ proteins to *A. thaliana* (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019), as there are many recent

duplications of JAZ proteins in plants. As such, it is indeed possible that *PFP* responds differently to TMV and *B. cinerea* and may be involved in different plant defence mechanisms in different plant species.

3.5.8 *JAZ6* promotes flowering in short day conditions.

This is in contrast to *PFP* repressing flowering in short days (Figures 3.4.36 & 37), which has also been reported for long day and half-short day flowering assays (Yokoyama, Kobayashi and Kidou, 2019), all showing *pfp-1* mutants flowering earlier than WT. While the early flowering phenotype for *jaz6* mutants was not evident in previous flowering time experiments (Liu *et al.*, 2021), the aforementioned flowering assay was performed in flowering-inductive long day 16 hour photoperiods, as opposed to the short day 8 hour photoperiods in this work (Figure 3.4.37). The different photoperiod lengths will change which flowering pathways are active and how fast WT plants flower (Song *et al.*, 2015). A flowering phenotype only evident in short days is also seen for *NO FLOWERING IN SHORT DAY* (*NFL)* (Sharma *et al.*, 2016), and is indicative of involvement with the autonomous and/or vernalisation flowering pathways. A late flowering phenotype is also apparent for the JAZ4 mutant *jaz4-1* (Oblessuc *et al.*, 2020). However, the *jaz4-1* mutant phenotype may be the result of JAZ4 repressing TOE1/2 (Zhai *et al.*, 2015b), and as JAZ6 does not bind to TOE1/2 a different mechanism may be important here. Interaction with MYC2/3/4 or SPL9 could be ways JAZ6 accelerates flowering independently of PFP (Mao *et al.*, 2017; H. Wang *et al.*, 2017) (Figure 3.4.27).

3.5.9 The jasmonic acid marker gene *VSP2* is not differentially expressed by *JAZ6* and *PFP*

A classic Arabidopsis marker gene for jasmonic acid responses is *VSP2*, classically associated with wounding responses on the MYC2 branch of the jasmonic acid pathway (Verhage *et al.*, 2011). However, *VSP2* induction in response to jasmonic acid is inhibited by ethylene and prior work has demonstrated that *VSP2* may not be significantly differentially expressed in response to *B. cinerea* infection (Zander *et al.*, 2010; Vos *et al.*, 2015).

With *jaz6* mutants exhibiting a *B. cinerea* infection phenotype and increases in ethylene-dependent plant defensin gene expression (Figures NN, NN), it is not surprising that changes

in *VSP2* expression were not seen. Indeed, the lack of significant change in *VSP2* expression may be indicative of a degree of specialisation of *JAZ* genes to repress different target transcription factors, as has been demonstrated for *JAZ9* (Takaoka *et al.*, 2018). *PFP* has no previously described role for mediating the *MYC2* branch of the jasmonic acid pathway, and no evidence is seen here for such a role.

While the results described above are consistent with a lack of involvement of *JAZ6* and *PFP* in the *MYC2* branch of the jasmonic acid pathway, and wounding responses thereon, this could be tested experimentally with wounding or herbivorous *P. rapae* caterpillars to more comprehensively understand or refute the effects of *JAZ6* and *PFP* on crosstalk with the *MYC2* branch of the jasmonic acid pathway (Vos *et al.*, 2015).

3.5.10 There are effects of *JAZ6* and *PFP* on gene regulation by ethylene

Ethylene has a major role in regulating responses to *B. cinerea*, and so it is unsurprising that *jaz6-3* mutants exhibit differential expression of genes related to ethylene responses. However, from the data presented here it is not evident if there is any regulation of the responses commonly associated with other hormones, such as ABA and GA, by hormone crosstalk. As previously stated regarding the jasmonic acid response marker gene *VSP2*, this may be due to specialisation of the *JAZ* genes into distinct regulatory roles, where *JAZ6* is specifically regulating JA and ethylene specific responses. *PFP* has no previously described role for mediating ABA and GA hormone responses, and no evidence is seen here for such a role.

However, given the disease phenotypes of *jaz6-3* and *pfp-1* mutants in *Hyaloperonospra arabidopsidis* infection it may be possible for *JAZ6* and *PFP* to regulate salicylic acid responses, which may not be apparent from the transcriptional response to *B. cinerea* as it is a necrotroph and genes may be subject to control of expression on a different timescale in *Hyaloperonospra arabidopsidis* infection (Figure 3.4.12). While the plant defensin genes differentially expressed in *jaz6-3* mutants are also known to be regulated by salicylic acid such crosstalk may be occurring upstream of *JAZ6* with *TGA* transcription factors (Zander *et al.*, 2010). As such, to further elucidate the possible roles of *JAZ6* and *PFP* in regulating salicylic acid responses, whether by direct control or antagonistic crosstalk between he

jasmonic acid and salicylic acid pathways, RNAseq analysis of gene expression of *jaz6-3* and *pfp-1* mutants in response to *Hyaloperonospra arabidopsidis* infection may assist in discovering any such possible crosstalk or regulatory mechanisms.

3.5.11 There is insufficient evidence for epigenetic regulation of genes by *JAZ6* and *PFP*

The interaction of *JAZ6* and *PFP* controls the expression of the epigenetic regulator MEDEA (Figure 3.4.26). As MEDEA is an epigenetic regulator with a demonstrated role in mediating plant defence it is tempting to speculate that this is representative of an epigenetic mechanism for controlling defence which is dependent on JAZ6, particularly in light of the demonstrated role of ethylene in controlling epigenetic marks of histone H3 like H3K14Ac and H3K23Ac, and how BABA treatment priming against *Phytophthora infestans* infection can result in an increase in histone 3 H3K4me2 marks in SA-dependent genes (W. Zhang *et al.*, 2017; Meller *et al.*, 2018; Roy *et al.*, 2018).

However, there is not further support for *JAZ6* and *PFP* controlling wider epigenetic marks associated with defence from the data presented here. There is no clear evidence for genes other than *MEDEA* such as the DNA methyltransferases or DNA demethylases being controlled by *JAZ6* and *PFP,* GO analysis did not indicate any contrast where genes controlled by JAZ6 or PFP were related to epigenetic regulation, expect for *MEDEA* itself (Figures 3.4.20, 21, 22, 23). For example, while *MEDEA* is known to be subject to epigenetic control of gene expression through DNA demethylation by DEMETER, this is understood to happen during seed development and endosperm proliferation, so there is no clear link to wider epigenetic control through DEMETER itself (Lucibelli, Valoroso and Aceto, 2022).

The lack of experimental support for a role of *JAZ6* and *PFP* controlling epigentic marks is perhaps unsurprising considering that no experiments were performed to interrogate the epigenetic marks and how they may change in the *jaz6-3, pfp-1*, and *jaz6-3 pfp-1* double mutant. In view of at least furthering the understanding of how MEDEA may impact defence through such epigenetic marks, it may be worth performing whole genome bisulphite sequencing in the *jaz6-3, pfp-1*, and *jaz6-3 pfp-1* double mutant to look for possible changes in epigenetic marks. It may also be worth performing such bisulphite sequencing both

before and during *B. cinerea* infection, to identify genes where epigenetic marks dynamically respond to *B. cinerea* infection, and if such dynamic responses are maintained in the *jaz6-3, pfp-1*, and *jaz6-3 pfp-1* double mutants. Such bisulfite sequencing results could be cross-referenced with the RNAseq results in this study to understand how they impact, or reflect, gene expression.

3.5.10 The genetic interaction of *JAZ6* and *PFP* appears to be additive

While JAZ6 binds PFP, the genetic interaction of *JAZ6* and *PFP* appears to be additive, by the defence and flowering assay intermediate phenotypes of the double *jaz6-3 pfp-1* mutant (Figures 3.4.10, 12 & 37). The additive effect suggests that neither *JAZ6* nor *PFP* is necessary for the other to act, though there is some overlap in what genes they regulate individually and what is regulated synergistically or antagonistically (Figure 3.4.18).

Given the regulation of *MEDEA* by *PFP* in a *JAZ6*-dependent manner (Figure 3.4.26), we may hypothesise that JAZ6 and PFP could act together in a protein complex to control gene expression. Considering previous work on the activity of *JAZ6* and *PFP*, this complex may be a polycomb repressive complex with the ability to alter gene expression (Figure 3.5.1). PFP is known to ubiquitinate H2A histones (Yokoyama, Kobayashi and Kidou, 2019), which is essential for writer group proteins of polycomb repressive complex 1 (Kim and Sung, 2014). REDUCED VERNALIZATION RESPONSE 1 (VRN1) is a known component of polycomb repressive complex 1 in Arabidopsis essential for *FLC* repression (Greb *et al.*, 2007), which *PFP* also represses (Yokoyama, Kobayashi and Kidou, 2019). JAZ6 is known to bind to LIKE-HETEROCHROMATIN PROTEIN (LHP1) (Li *et al.*, 2021), which is also a part of polycomb repressive complex 1 in Arabidopsis.

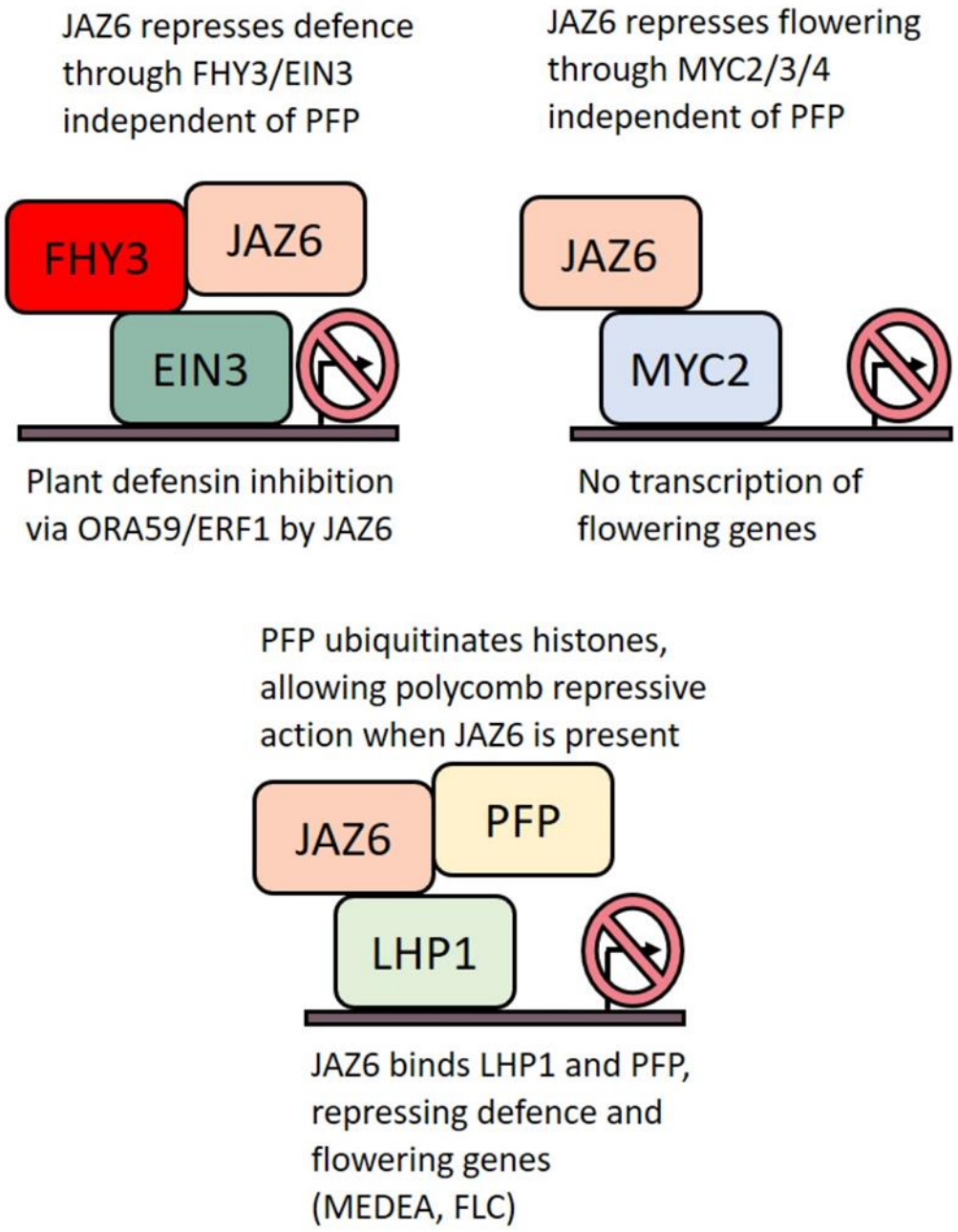


Figure 3.5.1: A working model of *JAZ6* and *PFP* dependent gene regulation.
JAZ6 represses the transcription of defensin defence genes indirectly, through binding to a multimeric complex with FHY3 and EIN3 to repress transcriptional activation of ORA59/ERF1. JAZ6 represses MYC transcription factors through binding to them. This prevents MYC transcription factors from inhibiting FT transcription and delaying flowering.
JAZ6 and PFP bind on a polycomb repressive complex with LHP1, repressing gene transcription through epigenetic regulation.

The genetic interaction may otherwise appear to be additive because the mutants are being partially complemented by similar related proteins. This is possible for JAZ6, with 13 similar JAZ proteins which have overlapping roles it is likely that JAZ6 could be partially complemented by other JAZ proteins (Q. Guo *et al.*, 2018). The exact differences between similar JAZ proteins include their binding affinity for transcription factors like MYC2 (Zhang *et al.*, 2015), though further work could help us better understand the exact molecular differences between JAZ proteins and how so many copies evolved.

It is also possible that a similar protein is acting in place of PFP in the *pfp-1* mutant. A candidate for this is *BIG*, the most similar gene to *PFP* and a member of the UBR family (Garzón *et al.*, 2007). The disease phenotype of *big* mutants is consistent with this (Ruo-Xi Zhang *et al.*, 2019), as they are more susceptible to *B. cinerea* infection. Further work investigating the extent to which *BIG* and *PFP* affect each other could improve our understanding of how the circadian clock and jasmonic acid responses are interconnected.

Taken together, these data suggest that JAZ6 acts to modify defence related gene expression independently of PFP. As JAZ6 has other protein-protein binding partners which it can repress to affect gene expression, these other protein-protein binding partners may be how JAZ6 controls defence, as follows. Among JAZ6 protein-protein binding partners is FHY3 (FAR-RED ELONGATED HYPOCOTYL 3) (Y. Liu *et al.*, 2019), a light-responsive transcription factor with a homologous partner *FAR1 (FAR-RED IMPAIRED RESPONSE 1)*. From mutant analysis of *fhy3 far1* mutant plants (Wang *et al.*, 2016), it is evident that several defence related genes regulated by *JAZ6* independently of *PFP* are regulated by *FHY3*. This includes *PDF1.3, MYB43,* and *MYB47*. But also, genes regulated by the interaction of *JAZ6* and *PFP* are regulated by *FHY3*, including *RAV1, WRKY53, PUB23, ERF5,* and *TIR-NBS-LRRs.* FHY3 may regulate defence related genes by binding in a protein complex with EIN3 and JAZ proteins (Y. Liu *et al.*, 2019), as EIN3 also has an established role as a transcription factor regulating defence genes including plant defensins (Zhu *et al.*, 2011). As FHY3 gates inputs to the circadian clock (Allen *et al.*, 2006), and directly regulates clock genes *ELF4* and *CCA1* (Li *et al.*, 2011; Liu *et al.*, 2020), this represents a likely aspect for the control of

circadian pathogen susceptibility (Ingle *et al.*, 2015). Future work could look at roles of FHY3 in circadian susceptibility and how a *fhy3* mutation interacts with *jaz6-3*.

In conclusion, JAZ6 and PFP both have roles in regulating defence against necrotrophic and biotrophic pathogens. They also regulate flowering time. However, the genetic interaction of JAZ6 and PFP appears to be additive, so they likely act independently of each other. A putative model might suggest JAZ6 controls defence by repressing EIN3 via FHY3 (Figure 3.5.1), while it represses MYC transcription factors to control flowering time. Both JAZ6 and PFP could act together in multimeric polycomb complexes to control gene expression through epigenetic modification.

3.5.11 *B. cinerea* gene expression reflects the presence of *JAZ6*

The control of gene expression in *B. cinerea* has been established to reflect gene expression in its plant host, through co-transcriptome network analysis. Here, it was clearly shown that *B. cinerea* gene expression also responds to the presence and absence of JAZ6 and resultant gene expression. However, despite not responding much to the absence of PFP, *B. cinerea* responds with more differential expression in response to the double *jaz6-3 pfp-1* mutant compared to just *jaz6-3* mutants (Figure 3.4.32). This intriguing difference in gene expression may reflect a change in the dynamics of infection on these mutants, and a possible response to the different levels of plant defensins present (Figure 3.4.24).

The particular genes which appear to respond to interaction effects of *JAZ6* and *PFP* include the *B. cinerea* laccase 2 enzyme Bcin14g (Figure 3.4.35). This gene has previously been identified to have potential roles in pathogenicity (Schouten *et al.*, 2002), and may reflect a response by *B. cinerea* to the heightened defences of the jaz6-3 mutant, which for whatever reason are not consistently applied to the *jaz6-3 pfp-1* double mutant. Therefore, this presents a future gene of interest for looking at how *B. cinerea* responds to different host plants.

More speculatively, it has been established that pathogens can induce changes in host gene expression, notably by export of siRNAs such as Bc-siR37 to host cells (M. Wang *et al.*, 2017). No difference in the expression of Bc-siR37 due to *JAZ6* or *PFP* was evident, though it is

possible that the RNA extraction method did not adequately capture enough small RNAs for quantitative comparisons. While WRKY transcription factors including WRKY57 were notably differentially expressed in the Arabidopsis mutants, without evidence of a difference in expression of Bc-siR37 it is difficult to distinguish genes subject to pathogen manipulation of host genes. However, as Arabidopsis gene expression was measured prior to infection, it can at least be established that some differences in Arabidopsis gene expression can be independent of pathogen manipulation, such as repression of plant defensin expression by *JAZ6*.

4: *JAZ* orthologues in lettuce are differentially expressed by biotic and abiotic stress

4.1 Introduction

JASMONATE ZIM DOMAIN (JAZ) proteins have been well characterised in the model plant Arabidopsis (Liu *et al.*, 2021). The overall aim of this Section is to translate this knowledge to the crop plant *Lactuca sativa* (lettuce). The reason for investigating JAZ proteins in lettuce is to understand their involvement in defence against pathogens. Since this is a crop plant, improving lettuce resistance to diseases caused by necrotrophic pathogens like *B. cinerea* is important to food security.

JAZ proteins are found across the Viridaeplantae (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). Comparative genomics approaches have identified JAZ proteins in many plant species, and just as in Arabidopsis these JAZ proteins exhibit conserved function in regulating disease resistance; abiotic stress resistance; and ethylene responses (Lv *et al.*, 2017; Zhu and Napier, 2017; Guofeng Zhang *et al.*, 2019; S. Liu *et al.*, 2019; Hu *et al.*, 2022). This extends even to the bryophytes where a single JAZ protein has been reported in the liverwort *Marchantia polymorpha* (Monte *et al.*, 2019). The *M. polymorpha* MpJAZ is typical of JAZ proteins in that it contains both TIFY and Jas domains. It is also degraded when jasmonic acid is present, illustrating conserved function with Arabidopsis AtJAZ proteins. It also shows conserved function by binding to MpMYC transcription factors (Peñuelas *et al.*, 2019). This conservation of function may also be true for lettuce LsJAZ proteins.

JAZ proteins from various species have been implicated in the regulation of plant defence. Wild grapevine VqJAZ4 promotes resistance to biotrophic powdery mildew golovinomyces and susceptibility to necrotrophic *Botrytis cinerea* (Guofeng Zhang *et al.*, 2019). This is comparable to the roles of Arabidopsis JAZ proteins (Liu *et al.*, 2021), particularly AtJAZ6 in Chapter 4 of this work. Effector proteins HopX1 and HopZ1a from the bacterial pathogen *P. syringae* have been demonstrated to bind Arabidopsis JAZ proteins and target them for degradation (Jiang *et al.*, 2013; Gimenez-Ibanez *et al.*, 2014), in order to impair plant defence responses. *P. syringae* is a generalist plant pathogen with many host species (Xin,

Kvitko and He, 2018), and reflecting this broad range of host species HopZ1a also binds to soybean GmJAZ1 to target it for degradation (Jiang *et al.*, 2013). However, Poplar PtJAZ6 is instead protected from degradation by the effector MiSSP7 produced by the mutualistic fungus *Laccaria bicolor* (Daguerre *et al.*, 2020).

In *N. benthamiana* NbJAZ3 is necessary for jasmonic acid to control trichome development, by binding to and repressing the transcription factor NbWo (Yan *et al.*, 2022). Tomato SlJAZ4 controls trichome development by binding to HOMEODOMAIN 8 (SlHD8) (Hua *et al.*, 2021). In *Artemisia annua* the similar AaHD1 is repressed by AaJAZ8 to regulate trichome development (Yan *et al.*, 2017). This is similar to the role of Arabidopsis JAZ proteins in regulating the WD-repeat/bHLH/MYB transcriptional complex including GL3 to regulate trichome development (Qi *et al.*, 2011).

Apple JAZ controls fruit ripening by gating ethylene responses (Hu *et al.*, 2022). This reflects the Arabidopsis function of JAZ proteins at the intersection of jasmonic acid and ethylene, which is exemplified by the repression of EIN3 by AtJAZ1/3/9 (Zhu *et al.*, 2011).

In several species there is conserved function of JAZ proteins in regulating abiotic stress resistance. Rice OsJAZ9 suppresses OsMYB30 to promote cold tolerance (Lv *et al.*, 2017). This reflects how Arabidopsis JAZ proteins repress ICE1 and ICE2 in order to control cold tolerance (Hu *et al.*, 2013). JAZ proteins also mediate stress tolerance in plants. Grapevine *VrJAZ* genes are induced by salinity stress and are thought to mediate resistance to salinity stress (Ismail, Riemann and Nick, 2012). This is also seen in Bryophytes with moss PnJAZ1 promoting resistance to salinity stress (S. Liu *et al.*, 2019).

From phylogenies of JAZ proteins across different species, it is evident that JAZ proteins have diverged significantly during the evolution of the Angiosperms (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). One example of JAZ protein divergence is the monocot-exclusive clade of JAZ proteins (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019), which may prove worth investigation for novel unique functions of JAZ proteins. Another example of relatively recent JAZ divergence is the divergence of Arabidopsis JAZ5

and JAZ6, which is only present in Brassicales (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). As such, there are no 1:1 orthologues for most JAZ proteins between two species, as the JAZ proteins would have diverged after the ancestral species diverged. Additionally, even comparing JAZ genes in the closely related Brassica species Arabidopsis and *Brassica rapa* (turnip), 1:1 orthologues are not readily apparent due to the recent genome triplication of *Brassica rapa* (Jia *et al.*, 2021). Therefore, approaches used here have focussed on many-to-many orthologues.

The variation in genome sizes across Angiosperms as exemplified by the closely related Arabidopsis and *Brassica rapa* also presents a challenge for accurate analysis of synteny, because the sizes of plant genomes vary so greatly (Zhao and Schranz, 2019). Therefore, a microsynteny approach was used to find syntenic orthologues of *JAZ* genes across species. Microsynteny has previously been used to evaluate the evolution of APETALLA 2 type transcription factors in Angiosperms (Kerstens, Schranz and Bouwmeester, 2020), illustrating groups of genes limited to specific clades of plants like brassicales and monocots.

Lettuce is a dicot angiosperm. As a commercial crop it is worth £200M per year in the UK (Defra Horticulture Statistics, 2021). However, it is susceptible to infections by both *B. cinerea* and *S. sclerotiorum* (Young *et al.*, 2004; Amselem *et al.*, 2011), especially from lettuce drop caused by *S. sclerotiorum* (Wu and Subbarao, 2006). Furthermore, the effectiveness of fungicides is decreasing as the organisms have evolved resistance to the fungicides (Rupp *et al.*, 2017; Hou *et al.*, 2018). Therefore, developing new, alternative means of reducing plant disease is timely. As JAZ proteins regulate defence responses against both these pathogens in Arabidopsis, as detailed in chapter 3 of this thesis, the complement of JAZ proteins and expression of these lettuce JAZ proteins was investigated.

Through transcriptome-wide searches for JAZ proteins, nine JAZ proteins were identified in lettuce. Evidence of conserved function in these lettuce JAZ proteins consists of orthology to JAZ proteins in other plants, and similar expression patterns to these orthologues in response to necrotrophic pathogen attack and abiotic cold stress. Further work could establish exactly which transcription factors JAZ proteins are regulating, and how those

could affect pathogen defence, providing potential targets for lettuce breeding and defence improvements. The potential of editing JAZ genes to develop disease resistant plants has previously been demonstrated with targeted deletion of the Jas domain in tomato SlJAZ2 (Ortigosa *et al.*, 2019). Further work could also look at conservation of TF binding to *JAZ* promoters, and so the regulation of *JAZ* gene expression across species.

4.2 Aims and objectives

The main aim of this chapter is to identify and characterise *JAZ* genes in the lettuce genome of the cultivar Salinas (Reyes-Chin-Wo *et al.*, 2017). Several hypotheses were tested to compare the lettuce *JAZ* genes with previously known Arabidopsis *JAZ* genes. Firstly, to see if their expression changes after infection as in Arabidopsis. Secondly, that lettuce *JAZ* genes are involved in the response to cold abiotic stress with similar changes in expression to *JAZ* genes in Arabidopsis. Thirdly, that similar cis regulatory elements in lettuce and Arabidopsis can be found to explain their change in expression in response to biotic and abiotic stresses.

This work presents a basis for future investigation of the roles of lettuce *JAZ* genes in pathogen resistance, with an aim to translating work performed in the model plant *Arabidopsis thaliana* to crop plants grown commercially.

4.3 Methods

4.3.1 JAZ protein and gene sequence data

Using the online tool HMMER (Finn, Clements and Eddy, 2011), the lettuce reference proteome for *Lactuca sativa* hosted by the Ensembl Genomes Plants reference database was scanned for putative lettuce orthologues for the Arabidopsis JAZ proteins with sequences obtained manually from Uniprot, results were discovered based on their inclusion of sequences significantly similar to ($p<0.05$) the Jas domain characteristic of TIFY family proteins. The discovered putative lettuce JAZ proteins were then verified for inclusion of both Jas and ZIM domains characteristic of JAZ proteins by InterProScan (Jones *et al.*, 2014), which identifies known domains in input sequences. For the remaining 9 discovered putative lettuce JAZ proteins, the most up to date protein, cDNA, and (1kb upstream) promoter sequences were obtained from the *Lactuca sativa* V8 assembly hosted on

Phytozome (Goodstein *et al.*, 2012; Reyes-Chin-Wo *et al.*, 2017). JAZ protein sequences from other species (liverwort, moss, lycophyte, gymnosperms, monocots and dicots) were recovered from previous publications (Chao *et al.*, 2019; Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019; Tian *et al.*, 2019; Jia *et al.*, 2021).

4.3.2 Mapping lettuce JAZ genes

Phytozome (Goodstein *et al.*, 2012) provided the chromosome locations for lettuce JAZ genes identified as above, which were imaged using MapGene2Chrom (Jiangtao *et al.*, 2015).

4.3.3 Phylogenetic analysis

Phylogenetic trees were computed and visualised with MEGA11 (Tamura, Stecher and Kumar, 2021). Expectation maximisation algorithms were used, with 100 bootstraps for smaller trees, and 1000 bootstraps for the larger tree with 369 nodes. Trees were visualised in iTOL (Letunic and Bork, 2021).

4.3.4 Protein motif analysis

JAZ protein sequences from Arabidopsis, lettuce, and *M. polymorpha* were analysed for consensus TIFY, Jas, EAR, and CMID motifs with leitmotif (Biđin *et al.*, 2020). Consensus motifs were constructed by leitmotif with the input of all Arabidopsis motifs in JAZ proteins. Leitmotif searches a protein sequence for the best match to a consensus motif in several different proteins simultaneously. As TIFY is always present towards the N-terminus and Jas towards the C-terminus, the order of TIFY then Jas was also input as a constraint on the search. EAR motifs can be present multiple times in multiple locations relative to the TIFY and Jas domains, so no constraint was placed on this search. The CMID has only more recently been experimentally validated, and it is not yet established if it is present in JAZ proteins of other species towards the N-terminal end of JAZ proteins, so no constraint was placed on this search. De novo motif analysis was performed on the protein sequences using XSTREME from the MEME suite, which was not targeted towards any particular known motifs (Bailey *et al.*, 2015; Grant and Bailey, 2021).

4.3.5 Promoter analysis

Promoter sequences of 1000bp upstream of the transcriptional start site for Arabidopsis JAZ genes were retrieved from the Arabidopsis information resource, TAIR: (https://www.arabidopsis.org/tools/bulk/sequences/index.jsp), and 1000bp lettuce JAZ gene promoters from Phytozome (Goodstein *et al.*, 2012). Promoter DNA sequences were analysed for motifs using SEA in the MEME suite (Bailey *et al.*, 2015; Bailey and Grant, 2021), for known JASPAR motifs, DAP-seq motifs, and novel motifs respectively (Bartlett *et al.*, 2017; Castro-Mondragon *et al.*, 2022). Promoters from Arabidopsis and lettuce syntenic orthologue JAZ genes were further analysed using EARS (Picot *et al.*, 2010), for which regions of significant similarity were again analysed by MEME and SEA in the MEME suite (Bailey *et al.*, 2015; Bailey and Grant, 2021), to identify common regulators.

4.3.6 Time series *JAZ* expression data

Time series expression data for Arabidopsis *B. cinerea* infection was extracted from a formerly published dataset (Windram *et al.*, 2012). Cold responsive expression data for lettuce was extracted from (Park, Shi and Mou, 2020), and for Arabidopsis from (Kilian *et al.*, 2007).

Time series expression data for lettuce *B. cinerea* and *S. sclerotiorum* infection was extracted from unpublished datasets provided by Harry Pink (Harry Pink, Personal Communications). The infection assays were performed on Saladin cultivar lettuce as previously described (Pink *et al.*, 2022). Samples were taken with a 1.75 cm diameter cork borer around the inoculation site, every 3 hours from 9 to 42 hours post inoculation for *S. sclerotiorum* and 9 to 48 hours post inoculation for *B. cinerea*. Differential expression analysis over time series was conducted as previously described (Windram *et al.*, 2012; Ransom, 2018).

4.4 Results

4.4.1 Lettuce JAZ proteins contain Jas, TIFY, and EAR domains

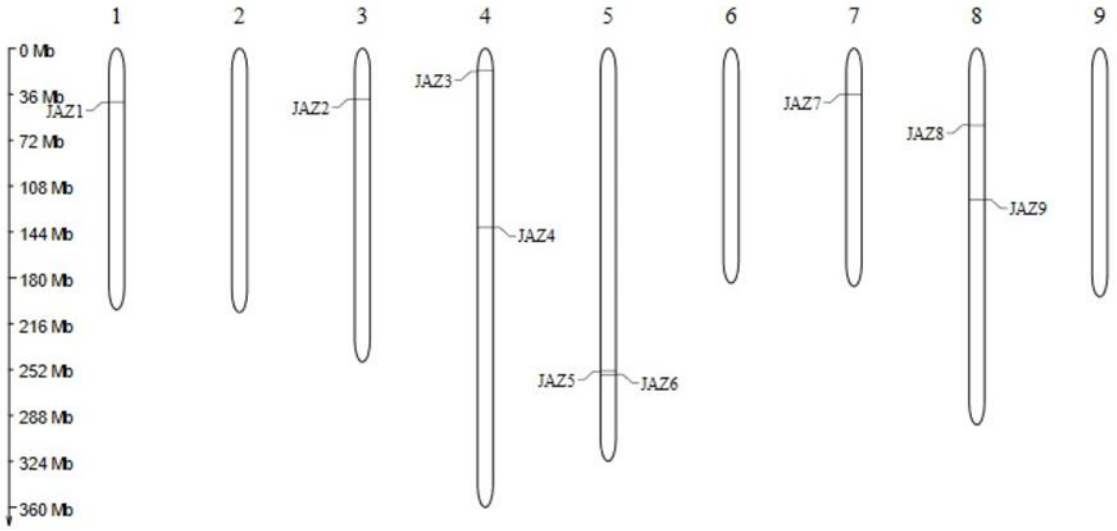
In order to identify lettuce JAZ proteins, the protein sequences of all 13 Arabidopsis JAZ proteins were used to search for similar proteins in lettuce Salinas v8 (Reyes-Chin-Wo *et al.*, 2017) using HMMER. While this identified proteins with similar sequences to Arabidopsis

JAZ proteins, to verify that these candidates were actual JAZ genes instead of other TIFY family members InterProScan was used to test for the presence of both JAZ and TIFY domains. Nine lettuce proteins were found which contained both TIFY and Jas domains and named LsJAZ proteins. These were found across six of the nine lettuce chromosomes, detailed in Figure 4.4.1. There is one *LsJAZ* gene each on chromosomes 1, 3, and 7, while there are two *LsJAZ* genes each on chromosomes 4, 5, and 8. These *LsJAZ* genes were named according to their location in the genome, specifically the order in which they appear along the chromosomes from 1 to 9.

The function of JAZ proteins is dependent on the sequences of key domains. For example, AtJAZ8 does not possess a canonical degron in its Jas domain (Shyu *et al.*, 2012), but does possesses an EAR domain which facilitates direct binding to the transcriptional co-repressor TOPLESS (Shyu *et al.*, 2012). This leads it to behave differently to other JAZ proteins, as this divergent protein domain sequence enables AtJAZ8 to repress transcription of genes during infection and wounding when jasmonic acid is present.

In order to characterise any such divergent functions as seen with AtJAZ8 for lettuce JAZ proteins, an analysis was performed using Leitmotif to identify divergent domain sequences in lettuce JAZ proteins. As TIFY and Jas domains are the functional determinants of JAZ protein properties, Leitmotif analysed lettuce JAZ proteins for sequential TIFY and Jas motifs appearing one after the other with the TIFY motif at the N-terminal end of the protein, as detailed in Figure 4.4.2. This was performed in comparison to sequential TIFY and Jas motifs in Arabidopsis and *Marchantia polymorpha*. This comparative analysis assessed the sequential order, and amino acid sequence of TIFY and Jas domains in lettuce JAZ proteins. Arabidopsis and *M. polymorpha* JAZ proteins were also included because they serve as references for the impacts of sequence divergence of TIFY and Jas domains from consensus.

A



B

Lettuce JAZ name	Corresponding v5 gene ID	Corresponding v8 gene ID	UniProt protein accession	Lettuce Chromosome	Chromosome Start bp	Chromosome End bp	Strand
JAZ1	Lsat_1_v5_gn_1_37841	LOC111882352	A0A2J6MCF9	1	43,943,746	43,944,766	+
JAZ2	Lsat_1_v5_gn_3_31461	LOC111912330	A0A2J6LDT0	3	41,907,236	41,908,662	+
JAZ3	Lsat_1_v5_gn_4_12800.2	LOC111915834	A0A2J6L6D2	4	18,403,483	18,405,244	+
JAZ4	Lsat_1_v5_gn_4_91521	LOC111883555	A0A2J6KBH6	4	147,305,597	147,306,733	+
JAZ5	Lsat_1_v5_gn_5_137041	LOC111910354	A0A2J6LID2	5	265,385,547	265,387,013	+
JAZ6	Lsat_1_v5_gn_5_139561	LOC111892588	A0A2J6JSF0	5	268,433,330	268,434,667	-
JAZ7	Lsat_1_v5_gn_7_28101	LOC111880744	A0A2J6KHK9	7	38,124,786	38,126,324	-
JAZ8	Lsat_1_v5_gn_8_45621	LOC111886704	A0A2J6K4H8	8	63,401,322	63,404,527	+
JAZ9	Lsat_1_v5_gn_8_85820	LOC111894246	A0A2J6JNX1	8	124,353,568	124,354,743	-

Figure 4.4.1: Locations of the nine *LsJAZ* genes in lettuce.

(A) Ideogram indicating the positions of the nine L*sJAZ* genes on lettuce chromosomes. Chromosomes are numbered at the top of the figure.

(B) Table detailing the lettuce *JAZ* gene accession codes and corresponding proteins, and their precise locations on the genome

Arabidopsis JAZ proteins are the best characterised JAZ proteins for the impacts of domain amino acid sequence divergence, as detailed for AtJAZ8 above. The sequential order was checked as TIFY and Jas motifs are sequential in all known JAZ proteins, with TIFY domains located towards the N-terminus and Jas domains located towards the C-terminus (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). To represent a phylogenetic variety of well characterised Angiosperm JAZ proteins, the input consensus motif alignments were comprised of *Oryza sativa* and Arabidopsis TIFY and Jas motifs.

Leitmotif analysis showed that lettuce JAZ proteins all contain sequential TIFY and Jas motifs (Figure 4.4.2), as was suggested by HMMER and InterProScan, with a good fit to the consensus sequences. Figure 4.4.2 shows that some proteins are a better match for consensus TIFY and Jas motifs than others. For example, the divergent AtJAZ8 and 13 proteins are the worst matches for TIFY and Jas motifs (Figure 4.4.2), which matches their lack of canonical Jas and TIFY domains respectively (Shyu *et al.*, 2012; Thireault *et al.*, 2015). Among lettuce JAZ proteins the worst match is LsJAZ7, which contains a divergent Jas domain sequence like AtJAZ8. The spacing between the TIFY and Jas motifs varies around an average of approximately 50aa, from 31aa in AtJAZ13 to 108aa in AtJAZ3. Lettuce JAZ proteins have a more consistent inter-domain spacing around the average of 50aa.

Since EAR domains are also important functional determinants of JAZ protein co-repressor activity, LxLxL and DLNxPT EAR motifs were searched for with consensus sequences of *Oryza sativa* and Arabidopsis JAZ protein EAR motifs (Table 4.4.2). Here LsJAZ4, 5, and 7 possess LxLxL EAR domains (Table 4.4.2) which are also found in AtJAZ1, 2, 5, 6, 7, and 8. No DLNxPT EAR domains were found in lettuce JAZ proteins as are found in AtJAZ5 and 6 (Table 4.4.3).

Table 4.4.1: Lettuce JAZ proteins contain TIFY and Jas domains.

Jas and TIFY protein domain structure for JAZ proteins in Arabidopsis, lettuce, and *Marchantia polymorpha*. Leitmotif was used to evaluate matches to TIFY and Jas domains (A), or EAR motifs (B) in JAZ proteins across the three species. Closer matches to consensus motifs constructed from characterised *Arabidopsis thaliana* and *Oryza sativa* JAZ proteins receive a higher score. The colour of amino acids indicates their polarity, while red highlight indicates missing amino acids.

RANK	SCORE	SEQUENCE ID						
1	54.257	AtJAZ2	118	PLTIFYGGRVMVFDD	69	LPIARRASLHRFLEKRKDRITSKAPY	21
2	51.418	LsJAZ9 | Lsat_1_v5_gn_8_85820.1	86	PMTIFYNGQVIVFND	47	LPIARKASLARFLEKRKDRITARSPY	29
3	50.426	AtJAZ1	125	PLTIFYAGQVIVFND	60	LPIARRASLHRFLEKRKDRVTSKAPY	27
4	49.599	LsJAZ6 | Lsat_1_v5_gn_5_139561.1	90	QMTIFYAGQVIVLDD	58	LPIARKASLARFLEKRKERITARSPY	34
5	49.531	AtJAZ10	103	PMTIFYNGSVSVFQV	48	LPIARRKSLQRFLEKRKERLVSTSPY	5
6	48.913	AtJAZ3	177	QLTIFYAGSVCVYDD	108	LPLARKASLARFLEKRKERVTSVSPY	26
7	48.797	LsJAZ1 | Lsat_1_v5_gn_1_37841.1	118	QMTIFYNGKVIVFDD	56	LPIARRASLHKFLAKRKERAAVRSPY	24
8	48.008	LsJAZ8 | Lsat_1_v5_gn_8_45621.1	131	QLTIFYGGMVNVYDD	98	VPQARKASLARFLEKRKERVMASSPY	24
9	46.958	AtJAZ12	56	QLTIFFGGSVTVFDG	66	LPIARRHSLQRFLEKRRDRLVNKNPY	24
10	46.410	AtJAZ9	117	QLTIFYGGTISVFND	85	VPQARKASLARFLEKRKERLMSAMPY	23
11	45.523	LsJAZ4 | Lsat_1_v5_gn_4_91521.1	106	QMTIFYGGNILVLDD	56	LPIARRASLHKFLAKRKDRATVRAPY	18
12	44.610	LsJAZ5 | Lsat_1_v5_gn_5_137041.1	112	QMTIFYKGQVLVFDC	52	LPIARRVSLHKFLEKRKDRATGRAPY	23
13	44.178	AtJAZ6	105	QLTIFFGGKVMVFNE	63	ERIARRASLHRFFAKRKDRAVARAPY	60
14	43.504	LsJAZ2 | Lsat_1_v5_gn_3_31461.1	88	PLTIFYNGTVSVFDV	47	LPLSRKKSLRRFLEKRKERQVSASPY	10
15	43.394	AtJAZ4	146	QLTIFYAGSVLVYQD	96	LPQTRKASLARFLEKRKERVINVSPY	27
16	42.368	MpJAZ | Mp6g06230.1	189	QLTIFYSGTVNVYDD	98	LPQARKASLARFLEKRKDRVRGKAPY	131
17	42.102	AtJAZ5	97	QLTIFFGGKVLVYNE	67	ERIARRASLHRFFAKRKDRAVARAPY	69
18	41.551	LsJAZ3 | Lsat_1_v5_gn_4_12800.2	37	QLTIFYKGTVNVYDD	61	VPQMRGASLARFLEKRKERVMASAPY	20
19	39.973	AtJAZ11	130	QLTIIFGGSFSVFDG	50	VPIARRRSLQRFFEKRRHRFVHTKPY	17
20	37.218	LsJAZ7 | Lsat_1_v5_gn_7_28101.1	58	KLTIFYDGKVSVCDV	44	GGLSMKRSLQRFLQKRKHRIQATSPY	1
21	31.872	AtJAZ7	59	ILTIFYNGHMCVSSD	44	QKASMKRSLHSFLQKRSLRIQATSPY	4
22	27.570	AtJAZ8	44	RITIFYNGKMCFSSD	42	PKASMKKSLQSFLQKRKIRIQATSPY	4
23	20.517	AtJAZ13	36	EINAFYSGRLSEYDL	31	LGCSVKRSVKRFLEKRKKRSKSFTLT	17

Table 4.4.2: Some lettuce JAZ proteins contain LxLxL EAR motifs.

EAR protein domain structure for JAZ proteins in Arabidopsis, lettuce, and *Marchantia polymorpha*. Leitmotif was used to evaluate matches to EAR LxLxL motifs in JAZ proteins across the three species. Closer matches to consensus motifs constructed from characterised *Arabidopsis thaliana* and *Oryza sativa* JAZ proteins receive a higher score. The colour of amino acids indicates their polarity, while red highlight indicates missing amino acids.

RANK	SCORE	SEQUENCE ID			
1	-1.225	AtJAZ8	8	LELRL	118
2	-1.356	LsJAZ7 | Lsat_1_v5_gn_7_28101.1	6	LELRL	133
3	-1.396	AtJAZ7	25	LELRL	118
4	-1.556	AtJAZ13	5	LDLHL	115
5	-2.740	AtJAZ5	269	LDLRL	0
6	-3.612	LsJAZ4 | Lsat_1_v5_gn_4_91521.1	25	LSLFL	191
7	-3.682	LsJAZ5 | Lsat_1_v5_gn_5_137041.1	27	LSLFL	196
8	-3.752	AtJAZ6	264	LELKL	0
9	-5.092	LsJAZ6 | Lsat_1_v5_gn_5_139561.1	43	LSLGM	175
10	-5.354	AtJAZ2	37	LSLGM	207
11	-5.394	AtJAZ1	42	LSLGM	206
12	-6.308	LsJAZ1 | Lsat_1_v5_gn_1_37841.1	29	LSVFL	205
13	-7.741	AtJAZ10	7	LDFLGL	184
14	-8.057	LsJAZ9 | Lsat_1_v5_gn_8_85820.1	188	LGLGA	10
15	-8.133	LsJAZ3 | Lsat_1_v5_gn_4_12800.2	121	LARFL	33
16	-8.781	AtJAZ11	23	LDL-L	211
17	-9.208	AtJAZ9	225	LARFL	36
18	-9.476	AtJAZ12	145	LQRFL	37
19	-9.486	AtJAZ4	7	LGSKL	298
20	-9.490	LsJAZ8 | Lsat_1_v5_gn_8_45621.1	252	LARFL	37
21	-9.939	AtJAZ3	5	LGLGS	342
22	-10.502	LsJAZ2 | Lsat_1_v5_gn_3_31461.1	89	LTIFY	92
23	-11.148	MpJAZ | Mp6g06230.1	310	LARFL	144

Table 4.4.3: No lettuce JAZ proteins contain DLNxPT EAR motifs.

EAR protein domain structure for JAZ proteins in Arabidopsis, lettuce, and *Marchantia polymorpha*. Leitmotif was used to evaluate matches to EAR DLNxPT motifs in JAZ proteins across the three species. Closer matches to consensus motifs constructed from characterised *Arabidopsis thaliana* and *Oryza sativa* JAZ proteins receive a higher score. The colour of amino acids indicates their polarity, while red highlight indicates missing amino acids.

RANK	SCORE	SEQUENCE ID			
1	3.953	AtJAZ6	160	DLNEPT	103
2	3.902	AtJAZ5	154	DLNEPT	114
3	-7.745	AtJAZ11	228	DKNETS	4
4	-7.908	LsJAZ7 | Lsat_1_v5_gn_7_28101.1	101	SL-EPS	38
5	-8.133	AtJAZ12	46	QTAEPT	135
6	-8.817	LsJAZ5 | Lsat_1_v5_gn_5_137041.1	205	QLHNPS	17
7	-8.947	AtJAZ4	101	SMSEPN	203
8	-8.963	AtJAZ7	89	DVEEKS	53
9	-9.136	AtJAZ1	82	DV-KPT	166
10	-9.671	LsJAZ4 | Lsat_1_v5_gn_4_91521.1	48	DASETT	167
11	-9.724	LsJAZ2 | Lsat_1_v5_gn_3_31461.1	128	TLVKPT	52
12	-10.528	LsJAZ3 | Lsat_1_v5_gn_4_12800.2	51	DMS-PE	103
13	-10.533	LsJAZ9 | Lsat_1_v5_gn_8_85820.1	14	GQNMPS	183
14	-10.570	LsJAZ6 | Lsat_1_v5_gn_5_139561.1	133	DWIPKPT	83
15	-11.045	AtJAZ13	6	DLHLSPM	112
16	-11.301	AtJAZ3	280	NINCPV	66
17	-11.389	LsJAZ1 | Lsat_1_v5_gn_1_37841.1	215	QLHNPP	18
18	-11.411	AtJAZ8	37	QPNEES	88
19	-11.625	AtJAZ2	143	DLANKGS	99
20	-12.369	AtJAZ9	131	DIS-PD	130
21	-12.640	LsJAZ8 | Lsat_1_v5_gn_8_45621.1	145	DIS-PE	144
22	-12.791	MpJAZ | Mp6g06230.1	226	FMNPPA	227
23	-13.557	AtJAZ10	17	TNNAPK	174

However, these Leitmotif analyses were targeted to find and examine only TIFY, Jas, and EAR domains. *De novo* motif analysis was thus performed with MEME which identified TIFY Jas domains, which correspond to red consensus motifs (DLPIARRASLARFLEKRKERVTARAPYQLDN) and TIFY domains, which correspond to blue consensus motifs (QLTIFYGGSVSVFBDIPAEKAQ), EAR domains were not found (Figure 4.4.2). MEME additionally identified the duplication of the Jas and TIFY motifs in AtJAZ11, a recently duplicated JAZ protein (Yan *et al.*, 2007), which is not apparent for any of the lettuce JAZ proteins.

MEME also identifies another motif in the JAZ proteins (Figure 4.4.2), which consistently appears towards the N-terminal end of the protein. A motif which does not clearly correspond to a known JAZ domain in green (PEKSSFSQTCNRLSRYLKEKGS). This motif appears in AtJAZ1, 2, 5, and 6 as well as LsJAZ1, 4, 5, 6, and 9. This motif may correspond to the cryptic MYC interacting domain (CMID) experimentally suggested for JAZ1 (Withers *et al.*, 2012), but not explicitly defined. Subsequent analysis of JAZ proteins suggested that the CMID was conserved in AtJAZ1, 2, 5, 6, and 10 (F. Zhang *et al.*, 2017), though AtJAZ10 does not appear to possess a good match to the green domain in this analysis. Subsequent work identified the CMID domain in MED25 and showed it was essential for MED25 binding to MYC3 (Takaoka *et al.*, 2021), with a CMID consensus sequence corresponding to the latter half of the green domain found in this analysis.

To verify the CMID domain was present in lettuce JAZ proteins, Leitmotif was used to identify matches for a consensus CMID motif to Arabidopsis, lettuce, and *M. polymorpha* JAZ proteins, as shown in Table 4.4.4. The consensus CMID motif was taken from a recent publication investigating the CMID in Arabidopsis (Takaoka *et al.*, 2021). Here we see that the CMID motif appears in AtJAZ1, 2, 5, 6, and 10 as well as LsJAZ1, 4, 5, 6, and 9. Other proteins possess partial matches, but these are located far from the N-terminal end of the protein and correspond to the Jas domain as previously identified (Table 4.4.1). This analysis correctly identified the CMID motif in AtJAZ10 as previously identified (F. Zhang *et al.*, 2017), and concurs with MEME analysis that LsJAZ1, 4, 5, 6, and 9 possess CMID motifs. It might be hypothesised that lettuce JAZ proteins with this domain are able to bind MYC proteins via

this motif in addition to the Jas motif, just like Arabidopsis JAZ proteins do (Takaoka *et al.*, 2021).

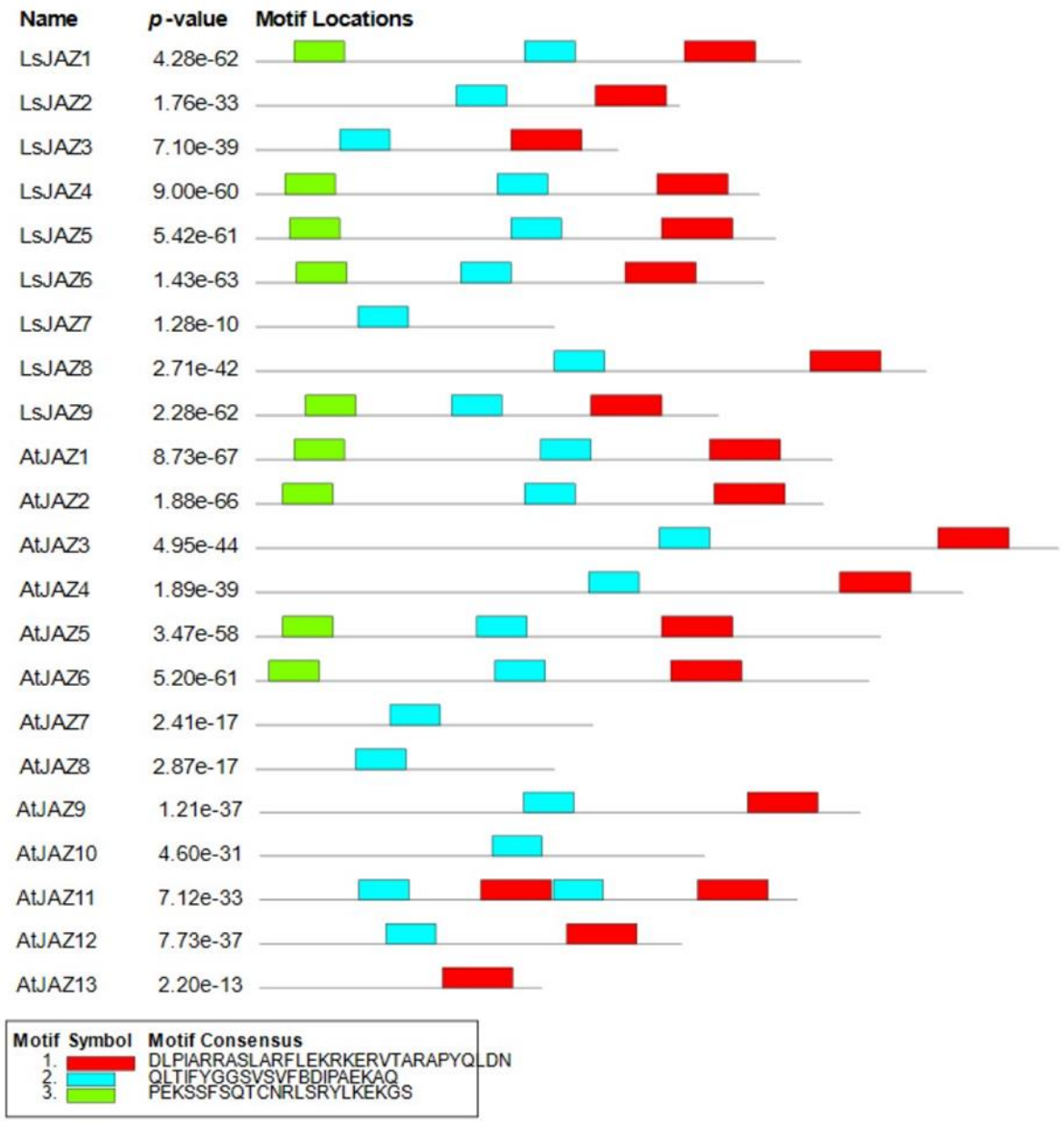


Figure 4.4.2: Protein *de novo* domain discovery for JAZ proteins in Arabidopsis and lettuce. Positional representation of the 3 top motifs in the MEME output for *de novo* motif discovery, enriched in JAZ protein amino acid sequences.

Table 4.4.4: Some lettuce JAZ proteins contain a cryptic MYC interacting domain.
CMID protein domain structure for JAZ proteins in Arabidopsis, lettuce, and *Marchantia polymorpha*. Leitmotif was used to evaluate matches to CMID motifs in JAZ proteins across the three species. Closer matches to consensus motifs constructed from characterised *Arabidopsis thaliana* JAZ proteins receive a higher score. The colour of amino acids indicates their polarity, while red highlight indicates missing amino acids.

RANK	SCORE	SEQUENCE ID			
1	43.930	AtJAZ2	20	TCTRLSRYLKEKGSFGDLSLGMTCKPD	202
2	43.652	AtJAZ1	25	TCSRLSQYLKENGSFGDLSLGMACKPD	201
3	41.927	AtJAZ5	20	RCSLLSRYLKEKGSFGNIDLGLYRKPD	227
4	40.249	AtJAZ6	14	RCSLLSRYLKEKGSFGNINMGLARKSD	228
5	37.021	AtJAZ10	21	PKPKFQKFLDRRRSFRDIQ-GAISKID	150
6	14.601	LsJAZ6 | Lsat_1_v5_gn_5_139561.1	26	TFNLLSQYLKENGTFGDLSLGMATGTM	170
7	12.939	LsJAZ4 | Lsat_1_v5_gn_4_91521.1	21	TCNRLSLFLKENGNQFRDLSFGINAKFD	172
8	8.009	LsJAZ1 | Lsat_1_v5_gn_1_37841.1	25	TCNQLSVFLKERGSLKDLHRGINPKFD	187
9	7.022	LsJAZ9 | Lsat_1_v5_gn_8_85820.1	30	TCNLFSQYMKENATFPDLTLGRRTTPS	146
10	-1.733	LsJAZ5 | Lsat_1_v5_gn_5_137041.1	23	TCNRLSLFLKEKGNLTDL--GINGNFD	180
11	-10.213	AtJAZ12	141	RRHSLQRFLEKRRD-RLVNKNPYPTSD	20
12	-12.795	AtJAZ3	304	RKASLARFL-EKRKERVTSVSPYC-LD	23
13	-13.019	LsJAZ2 | Lsat_1_v5_gn_3_31461.1	154	RKKSLRRFL-EKRKERQVSASPYAYSQ	6
14	-13.088	AtJAZ4	261	RKASLARFL-EKRKERVINVSPY-YVD	24
15	-14.744	AtJAZ11	104	RRS-LQRFLEKRRDRSTKPDGSMILPSQLT	105
16	-14.748	AtJAZ9	221	RKASLARFL-EKRKERLMSAMPYKKML	19
17	-15.823	LsJAZ7 | Lsat_1_v5_gn_7_28101.1	109	KRS-LQRFL-QKRKHR-IQ--ATS-PY	1
18	-16.550	LsJAZ8 | Lsat_1_v5_gn_8_45621.1	248	RKASLARFL-EKRKER-V---MASSPY	24
19	-17.186	AtJAZ7	123	KRSLHS-FL-QKRSLR-IQ--ATS-PY	4
20	-17.302	AtJAZ8	106	KKS-LQSFL-QKRKIR-IQ--ATS-PY	4
21	-19.114	MpJAZ | Mp6g06230.1	306	RKASLARFL-EKRKDR-VR-GKAPYVA	129
22	-20.575	LsJAZ3 | Lsat_1_v5_gn_4_12800.2	117	RGASLARFL-EKRKER-V---MASAPY	20
23	-21.710	AtJAZ13	90	VKRFLEKRKKRSKSF-TLTPNYTSSTS	9

4.4.2 *Lactuca sativa* JAZ proteins cluster into distinct groups of homologous genes

To evaluate the evolution of the *JAZ* genes in lettuce, orthologous genes in Arabidopsis and tomato were identified, as these two species have well-characterised JAZ genes and can represent the super-rosids and super-asterids respectively. Arabidopsis JAZ genes are the best characterised, while tomato has perhaps a closer evolutionary relationship to lettuce with a slightly more recent ancestral divergence date of 102-112 mya compared to 114-125 mya (Wikström, Savolainen and Chase, 2001). Recent JAZ gene diversification often results in many-to-many or many-to-one orthology when comparing JAZ genes in different species (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019), so analyses focused on groups of orthologous genes instead of one-to-one reciprocal orthologues.

In an initial analysis to identify putative orthologs, Arabidopsis, lettuce, and tomato JAZ protein orthology was evaluated by OrthoFinder (Table 4.4.5). This suggested there may be six orthologous groups of *JAZ* genes based on protein similarity. A plurality of JAZ genes from each species are found in group 1, including AtJAZ6. More divergent JAZ proteins are found in other groups, such as AtJAZ7 and 8. A few Arabidopsis and tomato JAZ proteins have no orthologues in the other two species, such as the highly divergent AtJAZ13. All the lettuce JAZ proteins identified in this study possess orthologues in Arabidopsis, lettuce, or both.

Orthologues should be syntenic, in which case they are present in ancestrally related blocks of DNA with some level of conserved gene order (Stein, 2013). For this analysis, syntenic orthologues were extracted for Arabidopsis, lettuce, and tomato JAZ genes from previously published microsynteny analysis of 107 Angiosperm species (Zhao and Schranz, 2019), detailed in Table 4.4.6. As with orthologues identified by protein similarity alone, a plurality of JAZ genes is found in a single orthologous group. Compared with the putative orthologues found by OrthoFinder (Table 4.4.5), the syntenic orthologue groups for Arabidopsis, lettuce and tomato JAZ proteins are similar (Table 4.4.7), but the distribution of JAZ proteins between orthogroups changes. Perhaps most notable is the loss of tomato SlJAZ9, 10, and 11 from the orthogroup with LsJAZ7, which implies that despite high protein similarity, the genomic regions they were located in are not comparable. The use of syntenic

relationships to refine orthology also changes the relationships of many JAZ proteins, including the consolidation of LsJAZ3 and LsJAZ8 in a single orthogroup, and isolation of AtJAZ13 and SlJAZ7 in their own groups.

Membership of proteins in orthogroups broadly matches the protein domain structure they possess. For example, the CMID motif as identified by Leitmotif is only present in JAZ orthogroup 1 and 4 (Table 4.4.6), highlighting the similarity of orthologous genes across species. The isolation of AtJAZ13 also makes sense, as it has a divergent TIFY domain.

Looking at broader patterns in JAZ gene synteny across the angiosperms, it is evident that a plurality of JAZ genes is in the same syntenic orthologous group for most species. However, there are exceptions such as the basal Angiosperm *Amborella trichopodia* which possesses single copy syntenic orthologues for each of the five groups of JAZ genes lettuce has. Some groups of related species share a unique group of syntenic orthologues, such as the one containing AtJAZ13 which is mostly contained in the brassicales. A paraphyletic subset of species including *N. benthamiana* appear to possess no syntenic JAZ genes, which is unlikely given their possession of JAZ genes (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019; Yan *et al.*, 2022), and is more likely to reflect incomplete or erroneous genome drafts used in the analysis (Zhao and Schranz, 2019).

To elucidate possible evolutionary relationships and divergences between the JAZ proteins, amino acid sequences of these lettuce JAZ proteins were aligned to JAZ proteins from Arabidopsis and *M. polymorpha* as an ancestral outgroup (Figure 4.4.3). The lettuce JAZ proteins formed clades in accordance with their orthologous genes, as in the orthogroups (Table 4.4.7).

The lettuce JAZ proteins were then combined with characterised JAZ proteins from across the Viridaeplantae to give a more thorough picture of the variation in JAZ proteins (Figure 4.4.4), protein sequences from (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019; Tian *et al.*, 2019; Zheng *et al.*, 2020; Jia *et al.*, 2021). The 369 characterised JAZ proteins included in this analysis could be divided into nine clades, similar to previous publications (Garrido-

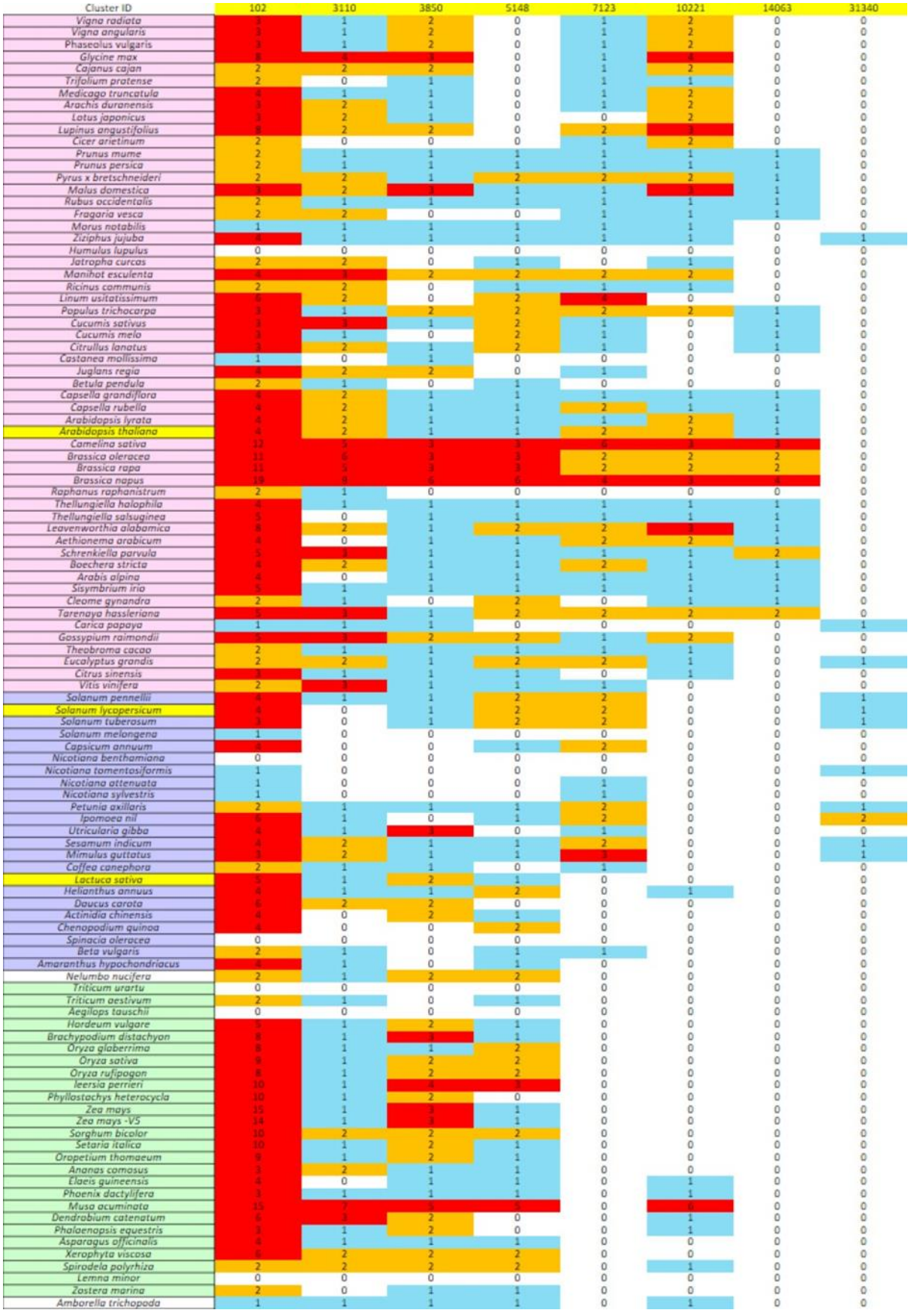
Bigotes, Valenzuela-Riffo and Figueroa, 2019). Some clades were exclusive to particular plant lineages here as well as in the literature – such as Clade II which is exclusively found in Monocots (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). The analysis shows that JAZ proteins in lettuce fall into typical Dicot clades of JAZ proteins, no LsJAZ proteins are found in Monocot exclusive clades. Arabidopsis and lettuce JAZ orthologues are always found in the same clades, as suggested by their orthogroups (Table 4.4.2).

Table 4.4.5: *JAZ* genes can be grouped by protein similarity in Arabidopsis, lettuce, and tomato. OrthoFinder was used to identify groups of orthologous *JAZ* genes using the amino acid sequences of the 13 AtJAZ, 9 LsJAZ, and 12 SlJAZ proteins. AtJAZ13 was not assigned to an orthogroup.

Putative JAZ orthogroup	Arabidopsis JAZ	Lettuce JAZ	Tomato JAZ
1	1, 2, 5, 6	1, 4, 5, 6, 9	1, 2, 3, 4, 12
2	7, 8	7	9, 10, 11
3		8	5, 6, 7, 8
4	3, 4, 9	3	
5	11, 12		
6	10	2	

Table 4.4.6 (overleaf): Syntenic orthologues can be found across the Angiosperms for lettuce JAZ genes.

Syntenic clusters which contain Arabidopsis, lettuce, or tomato JAZ genes, across 107 angiosperm plant species. Rosid species are coloured in pink, asterids in blue, and monocots in green. Arabidopsis (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum*), and lettuce (*Lactuca sativa*) are highlighted in yellow. The number of JAZ genes in a particular species in a syntenic orthologous group is colour-coded. None found – white, one – blue, two – yellow, three or more – red. Data from Zhou et al. 2019.



Cluster ID	102	3110	3850	5148	7123	10221	14063	31340
Vigna radiata	1	1	2	0	1	2	0	0
Vigna angularis	1	1	2	0	1	2	0	0
Phaseolus vulgaris	1	1	2	0	1	2	0	0
Glycine max	8	3	3	0	1	4	0	0
Cajanus cajan	2	2	2	0	1	2	0	0
Trifolium pratense	2	0	1	0	1	1	0	0
Medicago truncatula	4	1	1	0	1	2	0	0
Arachis duranensis	3	2	1	0	1	2	0	0
Lotus japonicus	3	2	1	0	0	2	0	0
Lupinus angustifolius	8	2	2	0	2	3	0	0
Cicer arietinum	2	0	0	0	1	2	0	0
Prunus mume	2	1	1	1	1	1	1	0
Prunus persica	2	1	1	1	1	1	1	0
Pyrus x bretschneideri	2	2	1	2	2	2	1	0
Malus domestica	3	2	3	1	1	3	1	0
Rubus occidentalis	2	1	1	1	1	1	1	0
Fragaria vesca	2	2	0	0	1	1	1	0
Morus notabilis	1	1	1	1	1	1	0	0
Ziziphus jujuba	4	1	1	1	1	1	0	1
Humulus lupulus	0	0	0	0	0	0	0	0
Jatropha curcas	2	2	0	1	0	1	0	0
Manihot esculenta	4	3	2	2	2	2	0	0
Ricinus communis	2	2	0	1	1	1	0	0
Linum usitatissimum	6	2	0	2	3	0	0	0
Populus trichocarpa	3	1	2	2	2	2	1	0
Cucumis sativus	3	3	1	2	1	0	1	0
Cucumis melo	3	1	0	2	1	0	1	0
Citrullus lanatus	3	2	1	2	1	0	1	0
Castanea mollissima	1	0	1	0	0	0	0	0
Juglans regia	4	2	2	0	1	0	0	0
Betula pendula	2	1	0	1	0	0	0	0
Capsella grandiflora	4	2	1	1	1	1	1	0
Capsella rubella	4	2	1	1	2	1	1	0
Arabidopsis lyrata	4	2	1	1	1	2	1	0
Arabidopsis thaliana	4	2	1	1	2	2	1	0
Camelina sativa	12	5	4	4	6	4	4	0
Brassica oleracea	11	6	4	4	2	2	2	0
Brassica rapa	11	5	4	4	2	2	2	0
Brassica napus	19	9	6	6	4	4	4	0
Raphanus raphanistrum	2	1	0	0	0	0	0	0
Thellungiella halophila	4	1	1	1	1	1	1	0
Thellungiella salsuginea	5	0	1	1	1	1	1	0
Leavenworthia alabamica	6	2	1	2	2	3	1	0
Aethionema arabicum	4	0	1	1	2	2	1	0
Schrenkiella parvula	5	3	1	1	1	1	2	0
Boechera stricta	4	2	1	1	2	1	1	0
Arabis alpina	4	0	1	1	1	1	1	0
Sisymbrium irio	5	1	1	1	1	1	1	0
Cleome gynandra	2	1	0	2	0	1	1	0
Tarenaya hassleriana	3	3	1	2	2	2	2	0
Carica papaya	1	1	1	0	0	0	0	1
Gossypium raimondii	5	3	2	2	1	2	0	0
Theobroma cacao	2	1	1	1	1	1	0	0
Eucalyptus grandis	2	2	1	2	2	1	0	1
Citrus sinensis	3	1	1	1	0	1	0	0
Vitis vinifera	2	3	1	1	1	0	0	0
Solanum pennellii	4	1	1	2	2	0	0	1
Solanum lycopersicum	4	0	1	2	2	0	0	1
Solanum tuberosum	3	0	1	2	2	0	0	1
Solanum melongena	1	0	0	0	0	0	0	0
Capsicum annuum	4	0	0	1	2	0	0	0
Nicotiana benthamiana	0	0	0	0	0	0	0	0
Nicotiana tomentosiformis	1	0	0	0	0	0	0	1
Nicotiana attenuata	1	0	0	0	1	0	0	0
Nicotiana sylvestris	1	0	0	0	1	0	0	0
Petunia axillaris	2	1	1	1	2	0	0	1
Ipomoea nil	6	1	0	1	2	0	0	2
Utricularia gibba	4	1	3	0	1	0	0	0
Sesamum indicum	4	2	1	1	2	0	0	1
Mimulus guttatus	3	2	1	1	3	0	0	1
Coffea canephora	2	1	1	0	1	0	0	0
Lactuca sativa	5	1	2	1	0	0	0	0
Helianthus annuus	4	1	1	2	0	1	0	0
Daucus carota	6	2	2	0	0	0	0	0
Actinidia chinensis	4	0	2	1	0	0	0	0
Chenopodium quinoa	4	0	0	2	0	0	0	0
Spinacia oleracea	0	0	0	0	0	0	0	0
Beta vulgaris	2	1	0	1	1	0	0	0
Amaranthus hypochondriacus	4	1	0	1	0	0	0	0
Nelumbo nucifera	2	1	2	2	0	0	0	0
Triticum urartu	0	0	0	0	0	0	0	0
Triticum aestivum	2	1	0	1	0	0	0	0
Aegilops tauschii	0	0	0	0	0	0	0	0
Hordeum vulgare	5	1	2	1	0	0	0	0
Brachypodium distachyon	8	1	3	1	0	0	0	0
Oryza glaberrima	8	1	1	2	0	0	0	0
Oryza sativa	9	1	2	2	0	0	0	0
Oryza rufipogon	8	1	2	2	0	0	0	0
Leersia perrieri	10	1	4	3	0	0	0	0
Phyllostachys heterocycla	10	1	2	0	0	0	0	0
Zea mays	15	1	3	1	0	0	0	0
Zea mays -V5	14	1	3	1	0	0	0	0
Sorghum bicolor	10	2	2	2	0	0	0	0
Setaria italica	10	1	2	1	0	0	0	0
Oropetium thomaeum	9	1	2	1	0	0	0	0
Ananas comosus	3	2	1	1	0	0	0	0
Elaeis guineensis	4	0	1	1	0	1	0	0
Phoenix dactylifera	3	1	1	1	0	1	0	0
Musa acuminata	15	7	5	5	0	6	0	0
Dendrobium catenatum	6	3	2	0	0	1	0	0
Phalaenopsis equestris	3	1	2	0	0	1	0	0
Asparagus officinalis	4	1	1	1	0	0	0	0
Xerophyta viscosa	6	2	2	2	0	0	0	0
Spirodela polyrhiza	2	2	2	2	0	1	0	0
Lemna minor	0	0	0	0	0	0	0	0
Zostera marina	2	0	1	1	0	0	0	0
Amborella trichopoda	1	1	1	1	0	1	0	0

Table 4.4.7: Groups of syntenic orthologue *JAZ* genes in Arabidopsis, lettuce, and tomato. Previously constructed synteny data was used to identify clusters including lettuce JAZ genes. SlJAZ9, 10, and 11 were not assigned to syntenic clusters. Data from Zhou et al. 2019.

JAZ orthogroup	Synteny cluster ID	Arabidopsis JAZ	Lettuce JAZ	Tomato JAZ
1	102	1, 2, 5, 6	1, 4, 5, 6, 9	1, 2, 3, 4
2	3110	7, 8	7	
3	3850	9	3, 8	6
4	5148	10	2	12
5	7123	3, 4		5, 8
6	10221	11, 12		
7	14063	13		
8	31340			7

To elucidate possible evolutionary relationships and divergences between the JAZ proteins, amino acid sequences of these lettuce JAZ proteins were aligned to JAZ proteins from Arabidopsis and *M. polymorpha* as an ancestral outgroup (Figure 5.3.3). The lettuce JAZ proteins formed clades in accordance with their orthologous genes, as in the orthogroups (Table 5.3.7).

The lettuce JAZ proteins were then combined with characterised JAZ proteins from across the Viridaeplantae to give a more thorough picture of the variation in JAZ proteins (Figure 5.3.4), protein sequences from (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019; Tian *et al.*, 2019; Zheng *et al.*, 2020; Jia *et al.*, 2021). The 369 characterised JAZ proteins included in this analysis could be divided into nine clades, similar to previous publications (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). Some clades were exclusive to particular plant lineages here as well as in the literature – such as Clade II which is exclusively found in Monocots (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019). The analysis shows that JAZ proteins in lettuce fall into typical Dicot clades of JAZ proteins, no LsJAZ proteins are found in Monocot exclusive clades. Arabidopsis and lettuce JAZ orthologues are always found in the same clades, as suggested by their orthogroups (Table 4.4.2).

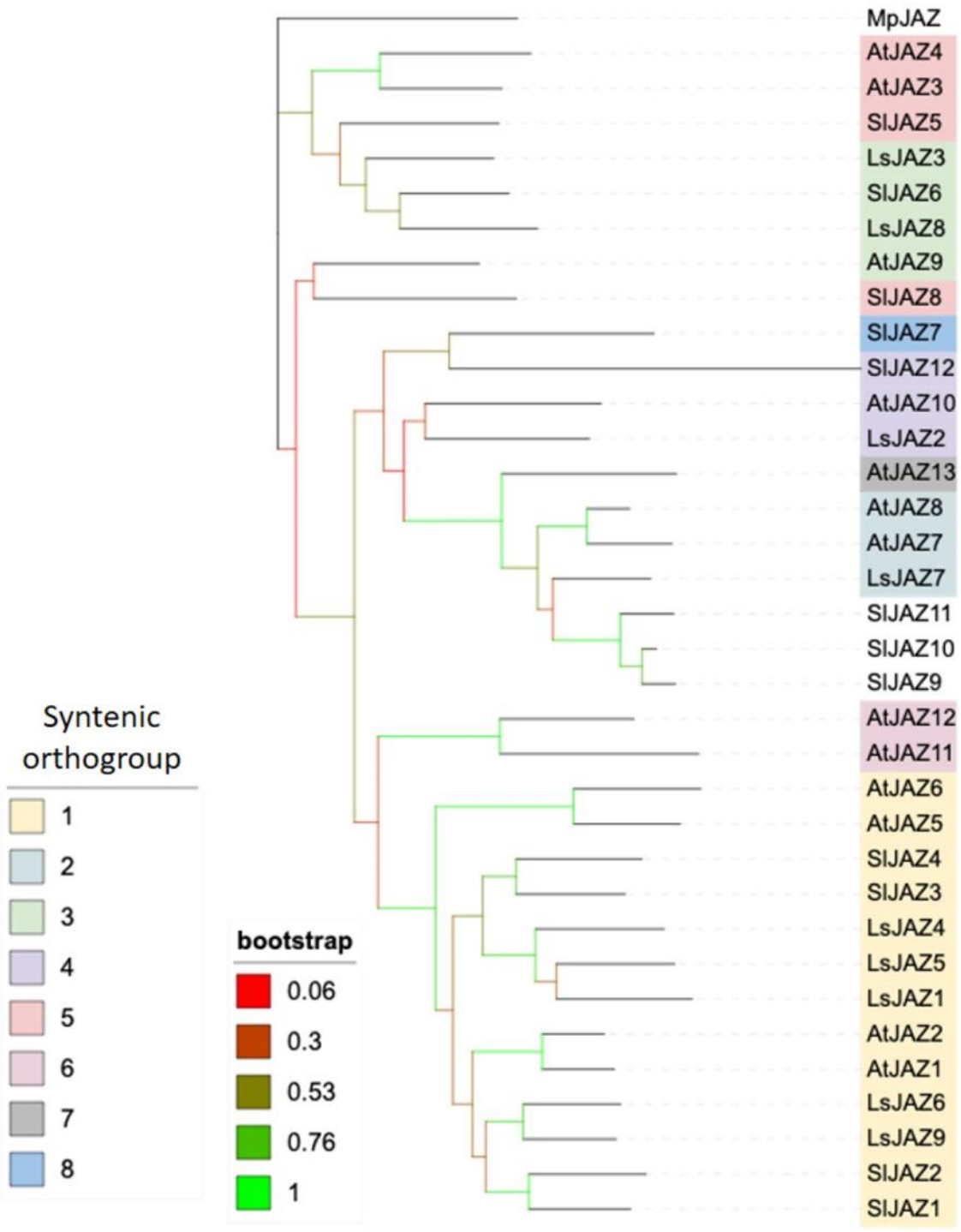


Figure 4.4.3: Orthologous JAZ proteins cluster together in phylogenetic analysis.
A maximum-likelihood phylogenetic tree was constructed in MEGA11 using full-length animo acid sequences for the thirteen AtJAZ, nine LsJAZ, twelve SlJAZ, and singular MpJAZ. The MpJAZ was assigned as the outgroup. Bootstrap values for branches are displayed. JAZ proteins are coloured according to their syntenic orthogroups.

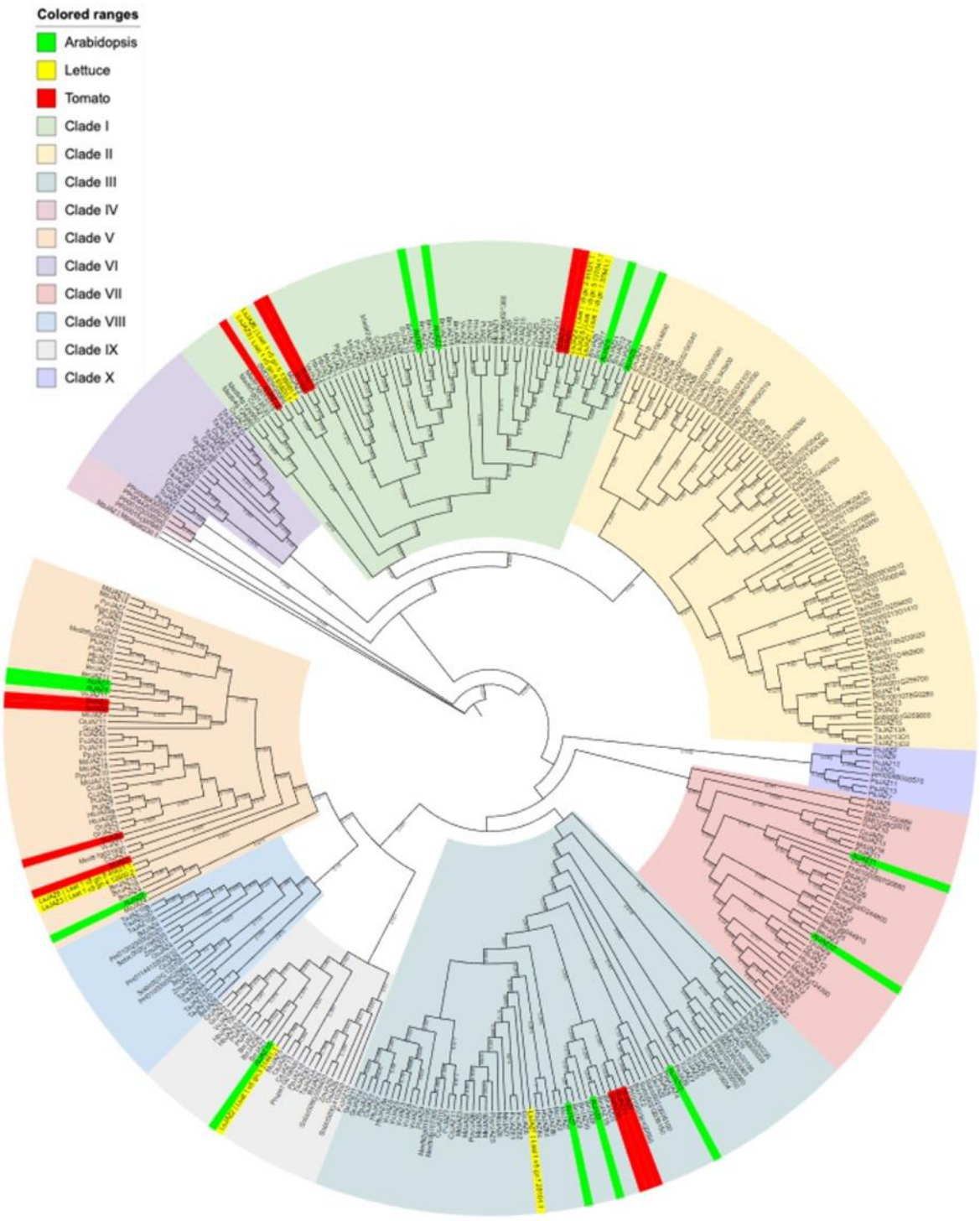


Figure 4.4.4: Phylogenetic relationships of characterised JAZ proteins across the *Viridaeplantae.* A maximum-likelihood phylogenetic tree was constructed in MEGA11 using full-length animo acid sequences for 369 JAZ proteins across the *viridaeplantae.* The MpJAZ was assigned as the outgroup. JAZ proteins from Arabidopsis (green), lettuce (yellow), and tomato (red) are highlighted. Ten clades of JAZ proteins are also highlighted, largely following Garrado-Brigotes et al., 2019.

4.4.3 Lettuce *JAZ* gene expression is regulated by necrotrophic pathogen infection and cold stress

Analysis of protein sequence and motifs indicates conserved structure of the Arabidopsis and lettuce JAZ proteins, encoded by syntenic orthologue *JAZ* genes. To investigate if the function of the lettuce and Arabidopsis *JAZ* genes is conserved, gene expression was used as a proxy for function. Because Arabidopsis *JAZ* genes are known to be involved in necrotrophic pathogen resistance, expression dynamics were investigated for lettuce *JAZ* genes after necrotrophic pathogen infection, which induces JA accumulation, *JAZ* gene upregulation and JAZ protein degradation (Windram *et al.*, 2012; Widemann *et al.*, 2016).

A time series of lettuce gene expression after inoculation with the necrotrophic pathogens *B. cinerea* and *H. arabidopsidis* has recently been analysed by Harry Pink. In this time series, the expression of lettuce genes from 9-48 hours after inoculation by either pathogen or mock treatment was captured. The dataset was analysed for differential expression of lettuce genes in pathogen inoculated samples, from which the LsJAZ genes have been subsetted here to investigate lettuce JAZ gene function (Figure 4.4.5).

All but one of the lettuce *JAZ* genes were upregulated in response to both *B. cinerea* and *S. sclerotiorum* infection (Figure 4.4.5), the exception being LsJAZ8. Upregulation after *S. sclerotiorum* inoculation is slower than upregulation after *B. cinerea* inoculation in all cases, likely due to the slower speed of infection by *S. sclerotiorum* as previously described (Pink *et al.*, 2022). The initial level of expression is notably lower for *LsJAZ7*, which is not expressed prior to infection, unlike other *LsJAZ* genes which are expressed prior to infection. While *LsJAZ8* may be slightly upregulated, the degree of upregulation is less than a two-fold change, which is considerably less than other *LsJAZ* genes.

The expression of lettuce JAZ genes after *B. cinerea* inoculation contrasts to the expression of Arabidopsis *JAZ* genes after *B. cinerea* inoculation (Windram *et al.*, 2012), detailed in Figure 4.4.6. Comparatively fewer *AtJAZ* genes are upregulated during the infection. Specifically, *AtJAZ1, 5, 6, 7, 8, 9, 10,* and *13* are upregulated after *B. cinerea* infection, *AtJAZ8* exhibits the largest magnitude change, and *AtJAZ5* the smallest. The dynamics of the changes in gene expression may also be contrasted, with *AtJAZ5* and *6* exhibiting more

transient changes while *AtJAZ8* and *10* maintain a higher level of expression for longer. The response of *AtJAZ* genes to *B. cinerea* divides orthogroups (Table 4.4.7), *AtJAZ2* is the only member of orthogroup 1 which is not upregulated in response to *B. cinerea*.

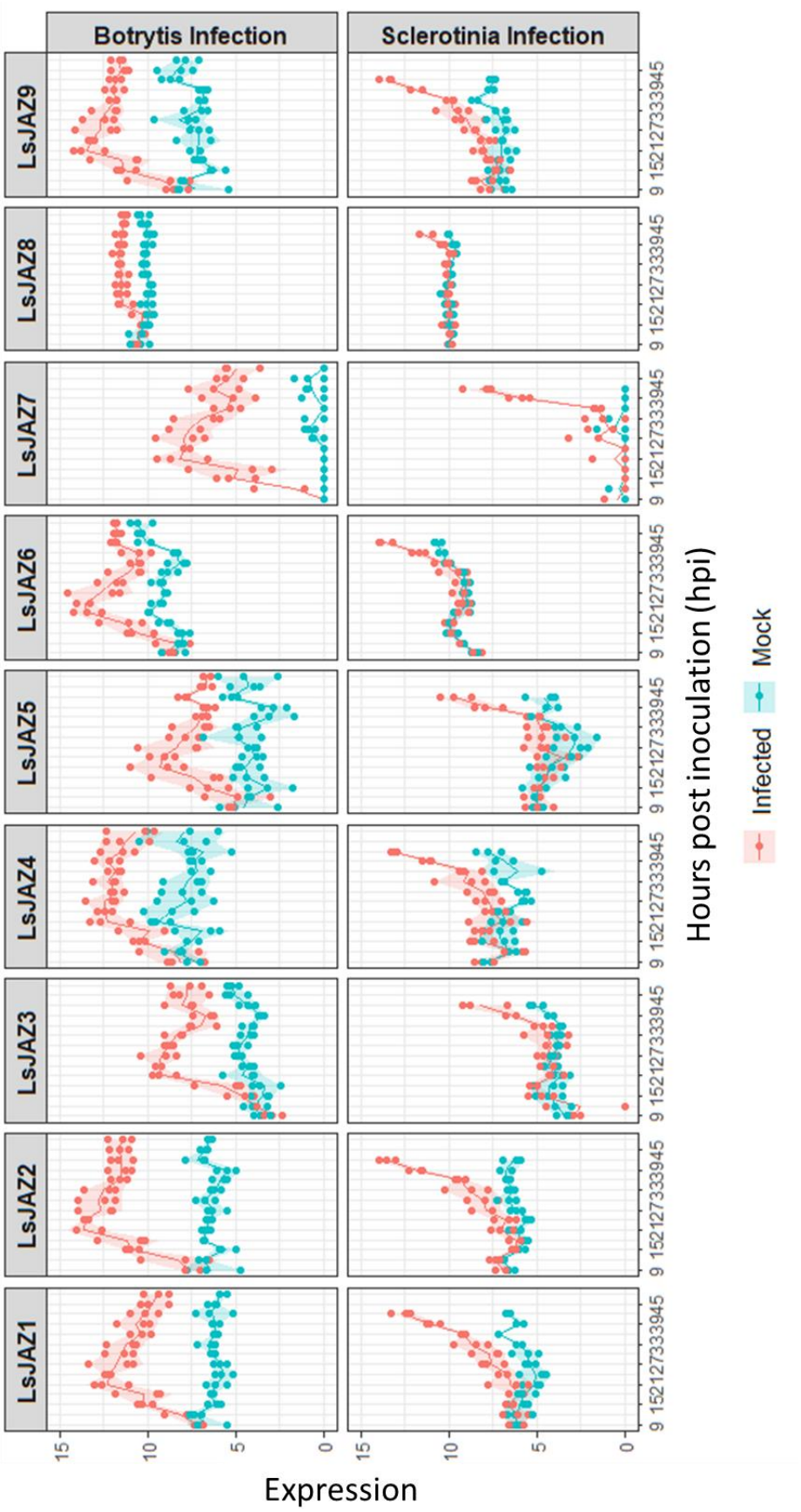


Figure 4.4.5: The expression of most lettuce *JAZ* genes is upregulated by necrotrophic pathogen infection. Time series of lettuce *JAZ* gene expression 9-48 hours post inoculation with *B. cinerea* (top) and *S. sclerotiorum* (bottom). Data and analysis courtesy of Harry Pink, from RNAseq analysis of necrotrophic infections of lettuce over the first few days of infection.

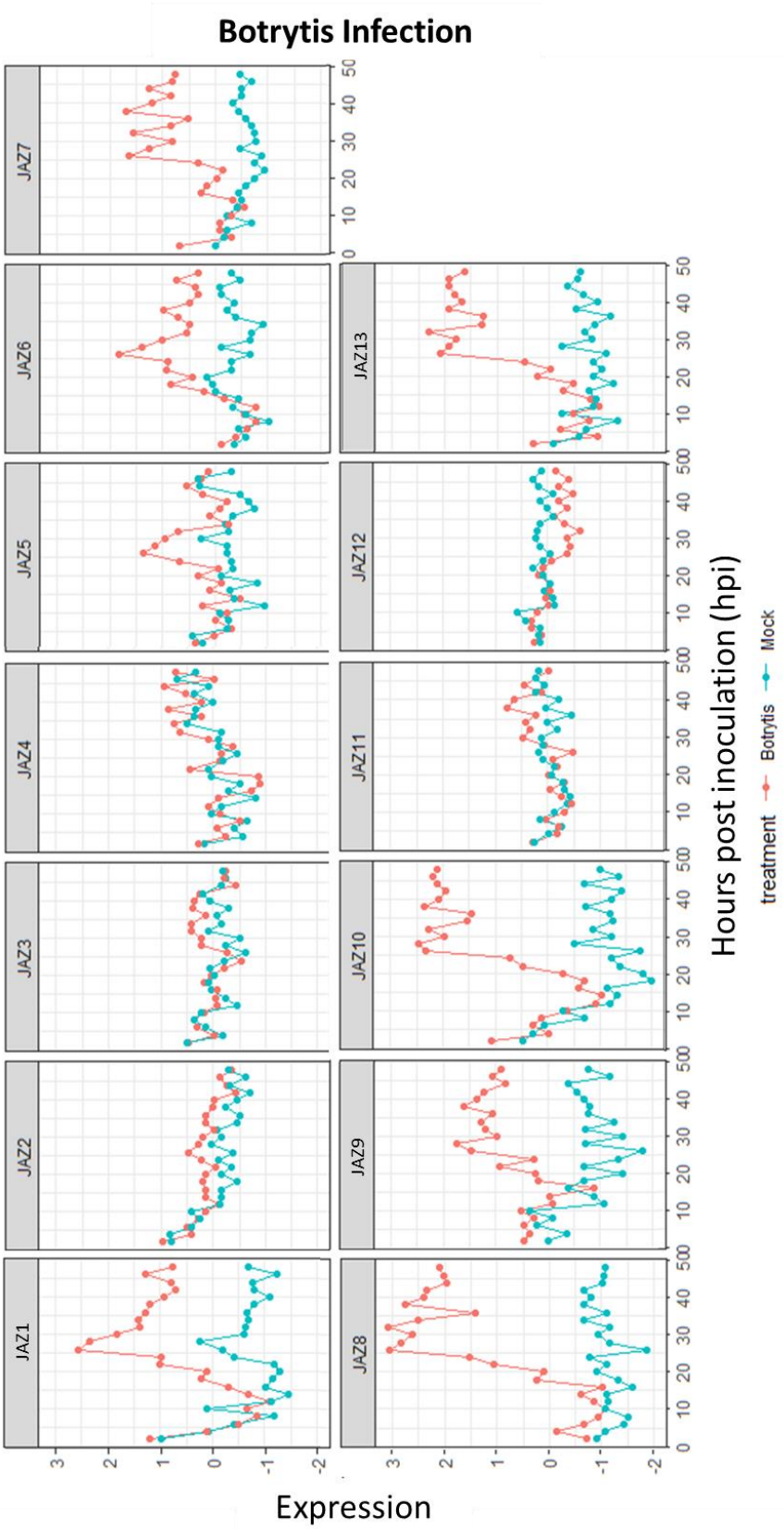


Figure 4.4.6: The expression of most Arabidopsis *JAZ* genes is upregulated by necrotrophic pathogen infection.

Time series analysis of Arabidopsis *JAZ* gene expression up to 48 hours post inoculation with *B. cinerea*. Data and differential expression analysis from Windram et al. (2012).

As Arabidopsis JAZ proteins are involved with the response to cold stress, particularly through binding ICE proteins (Hu *et al.*, 2013), I investigated if lettuce and Arabidopsis *JAZ* genes are responsive to cold stress in order to infer the function of lettuce *JAZ* genes in abiotic stress (Figure 4.4.7). In lettuce six of the nine *JAZ* genes are differentially expressed by cold stress (Figure 4.4.7A). *LsJAZ1* was downregulated whereas *LsJAZ2, 4, 5, 6,* and *9* were all initially slightly upregulated before being downregulated (Park, Shi and Mou, 2020). All *LsJAZ* genes differentially expressed by cold are members of orthogroup 1 or 6 (Table 4.4.7), and conversely all members of those orthogroups are differentially expressed. *LsJAZ7* and *8* were not identified as cold-regulated, *LsJAZ3* may not have been identified as a single gene in this dataset. Also differentially expressed in the cold response are their targets *LsMYC4* and *LsEIN3* (Park, Shi and Mou, 2020), as well as many components of ethylene responses.

This may be compared to how *AtJAZ* genes are responsive to cold treatment (Figure 4.4.7B), only differentially expressed genes in response to cold are shown, data and statistical analysis from (Kilian *et al.*, 2007). Here five of the thirteen *AtJAZ* genes were differentially expressed by cold treatment: *AtJAZ1, 3, 5, 6, 9*. All these genes are also upregulated by *B. cinerea* infection (Figure 4.4.6).

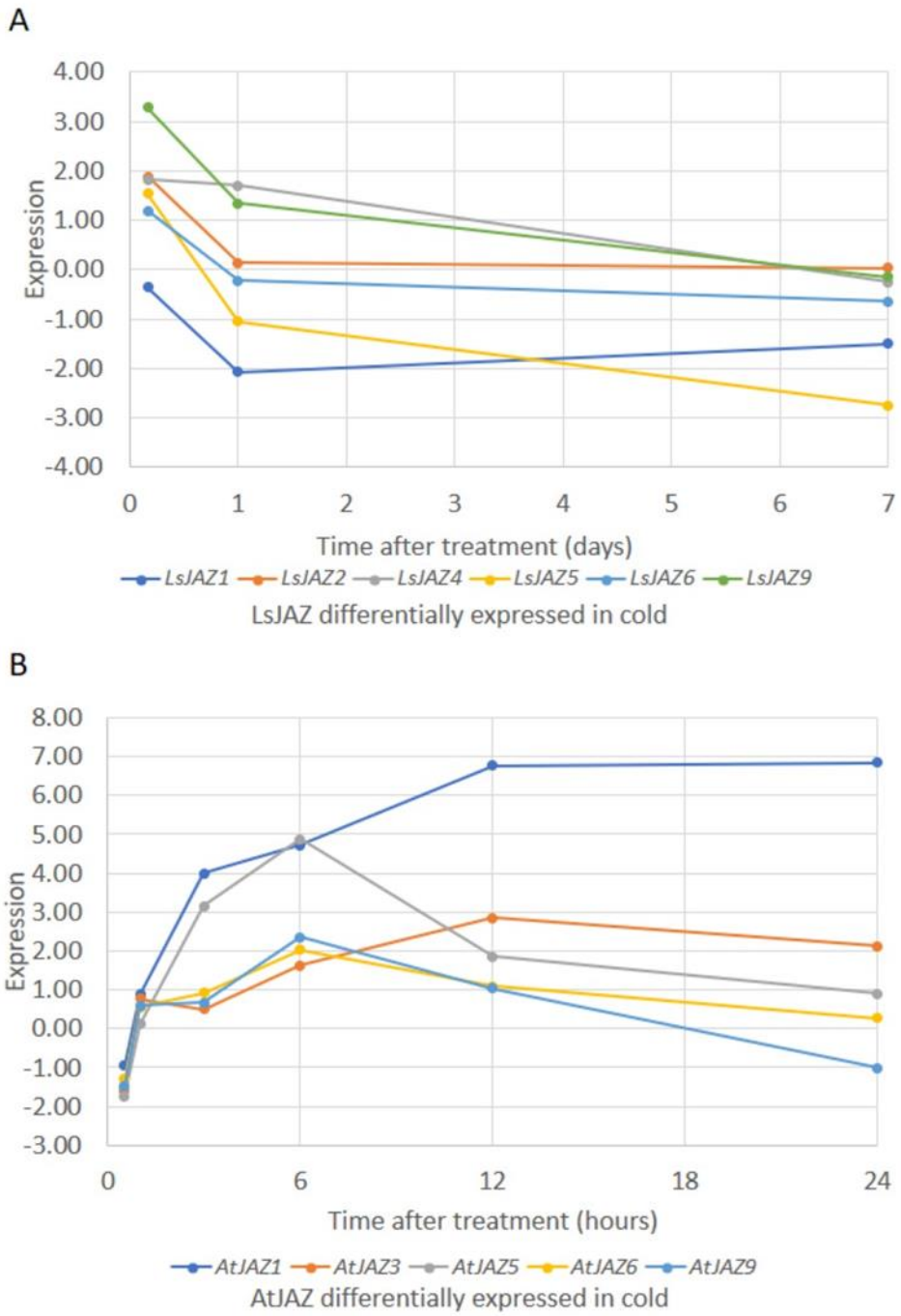


Figure 4.4.7: Several Arabidopsis and lettuce *JAZ* genes are differentially expressed in response to cold stress.

(A) Time series of lettuce *JAZ* gene expression up to 7 days after cold treatment, for lettuce JAZ genes differentially expressed in response to cold. Data and differential expression analysis from Park et al., 2020.

(B) Time series of Arabidopsis *JAZ* gene expression up to 1 day after cold treatment, for lettuce JAZ genes differentially expressed in response to cold. Data and differential expression analysis from Kilian et al., 2007.

4.4.4 Lettuce and Arabidopsis *JAZ* gene promoters contain similar cis-regulatory elements

Cis-acting regulatory sequences can determine gene regulation under external stress, and external stress such as wounding induces Arabidopsis *JAZ* gene expression (Chung *et al.*, 2008). This regulation reflects the role of *JAZ* genes in mediating responses to biotic and abiotic stress across the Viridaeplantae (Lv *et al.*, 2017; Zhu and Napier, 2017; Guofeng Zhang *et al.*, 2019; S. Liu *et al.*, 2019; Hu *et al.*, 2022).

Hypothesising that lettuce *JAZ* genes are also involved in stress responses; all Arabidopsis and lettuce *JAZ* gene promoters 1000bp upstream of the transcriptional start were analysed for cis-acting regulatory sequences. This analysis aimed to find common conserved stress-related aspects of *JAZ* gene regulation within each species, with all promoters from each species grouped together for straightforward comparison of the results. Through the MEME suite, SEA was used to test the enrichment of known plant transcription factor DNA binding motifs. These motifs were sourced from the JASPAR plant collection (Castro-Mondragon *et al.*, 2022), as well as motifs from DAPseq high-throughput screening of transcription factor binding sites (Bartlett *et al.*, 2017) and protein binding microarray (PBM) analysis of 63 transcription factors (Franco-Zorrilla *et al.*, 2014). The sources of motifs and the range of motifs these three data sources cover varies significantly, and so including all three in the analysis covers the widest possible range of transcription factors, as some motifs are only present in one of the three databases. Some motifs are present in all three sources, and extremely similar motifs can be repeated in a single source, resulting in considerable overlap. While the DAP-seq and PBM datasets each provide high confidence experimentally derived data, using both covers a wider range of transcription factors. Similarly, while the JASPAR plant collection motifs may not necessarily be as high confidence as the experimentally derived motifs, using the JASPAR plant collection in addition permits analysis of a broader range of transcription factor binding sites.

This analysis shows a variety of cis regulatory elements are enriched in both lettuce and Arabidopsis *JAZ* gene promoters, of which some key regulators of jasmonate responses are shown in Table 4.4.8. Here we see enrichment of G-box (CACGTG) and ethylene responsive (CACCGWC) regulatory elements, which could reflect conserved gene regulation between

species. Top hits for JASPAR and PBM motif enrichment in JAZ promoters in both Arabidopsis and lettuce include MYC binding motif G-boxes (Table 4.4.8). As transcription of *AtJAZ* genes is promoted by MYC transcription factors (Figueroa and Browse, 2012; Wang *et al.*, 2019), it is notable that MYC regulatory elements are enriched in *LsJAZ* promoters as well as *AtJAZ* promoters. ERF binding motifs are also enriched in both lettuce and Arabidopsis *JAZ* gene promoters. As ERF and MYC transcription factors are involved in regulating stress responses (Xie *et al.*, 2019; Song *et al.*, 2022), this raised the possibility that the lettuce and Arabidopsis *JAZ* genes would behave similarly in response to biotic and abiotic stress.

Most lettuce and Arabidopsis *JAZ* genes are unregulated in response to necrotroph infection (Figures 4.4.5 & 6). Considering that the induction of gene expression is driven by cis regulatory elements, the promoter regions of *JAZ* genes from both species significantly upregulated by necrotroph infection were compared to the promoters of non-responsive *JAZ* genes from both species (Figure 4.4.9). While no DAP-seq motifs were significantly enriched for the responsive promoters, MYB3 and MYB4 JASPAR motifs were significantly enriched (Figure 4.4.9). MYB4 is directly activated by kinase cascades through MPK3 in pathogen infection responses (Lin *et al.*, 2022), regulating lignin biosynthesis. This is indicative of conserved regulatory mechanisms underlying the response of defence regulators in pathogen attack.

Many lettuce and Arabidopsis JAZ genes are differentially expressed in response to cold stress (Figure 4.4.7). Again, considering that the induction of gene expression is driven by cis regulatory elements, the promoters for *JAZ* genes from both species differentially expressed in response to cold stress were compared to the promoters of non-responsive *JAZ* genes from both species. This shows a variety of both JASPAR and DAP-seq motifs are enriched in the cold-responsive promoters (Table 4.4.10). These analyses show motifs from cold responsive transcription factors called CBFs (Novillo *et al.*, 2004; Fowler, Cook and Thomashow, 2005), which are conserved in lettuce (Park, Shi and Mou, 2020). This is indicative of conserved regulatory mechanisms underlying responses to abiotic stress. They

may also be part of feedback loops involving JAZ proteins, as Arabidopsis JAZ proteins repress ICE1 and ICE2 transcription factors which promote CBF expression (Hu *et al.*, 2013).

Table 4.4.8: Jasmonic acid response cis-regulatory elements are present in both Arabidopsis and lettuce *JAZ* gene promoters.
Significantly enriched cis-regulatory element enrichment in Ls*JAZ* or AtJAZ gene promoters. Genomic sequences 1,000bp upstream of *JAZ* genes were analysed by SEA in the MEME suite for JASPAR plant 2022, DAP-seq, and PBM cis-regulatory element enrichment, relative to shuffled input sequences.

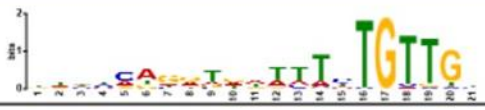
Motif	Enrichment ratio (lettuce)	Enrichment ratio (Arabidopsis)	Motif logo
TOE2	6	3.5	
MYC2	8	4.5	
MYC3	4	4.5	
MYC4	8	4.5	
DREB2D	4.5	8	
ERF14	5	5	
ERF54	5	7	

Table 4.4.9: MYB3 and 4 motifs are present Arabidopsis and lettuce *JAZ* promoters upregulated by necrotroph infection.

JASPAR curated cis-regulatory element enrichment in pathogen-responsive *JAZ* gene promoters. Genomic sequences 1,000bp upstream of differentially expressed *JAZ* genes in Arabidopsis and lettuce were analysed by SEA in the MEME suite for JASPAR plant 2022 cis-regulatory element enrichment, relative to non-differentially expressed *JAZ* gene promoters.

Motif	Enrichment ratio	Motif logo
MYB4	6.59	
MYB3	6.18	

Table 4.4.10: Motifs from transcription factors regulating the response to cold are present in Arabidopsis and lettuce *JAZ* promoters responsive to cold.
JASPAR curated and DAP-seq cis-regulatory element enrichment in cold-responsive *JAZ* gene promoters. Genomic sequences 1,000bp upstream of differentially expressed *JAZ* genes in Arabidopsis and lettuce were analysed by SEA in the MEME suite for JASPAR plant 2022 cis-regulatory element enrichment, relative to non-responsive JAZ promoters.

Motif	Enrichment ratio	Motif logo
RAV1	8.27	
CBF3	4.73	
CBF4	4.73	
CBF1	7.09	
ARF1	9.45	
DREB1A	5.32	

4.4.5 Lettuce and Arabidopsis *JAZ* gene promoters contain conserved non-coding regions

Given syntenic orthologous *JAZ* genes in lettuce and Arabidopsis, their promoters can be compared to identify conserved regulatory regions. The approach used in this work to identify conserved elements of lettuce and Arabidopsis JAZ gene promoters is EARS (Evolutionary analysis of regulatory sequences) analysis (Picot *et al.*, 2010). EARS finds conserved non-coding regions of DNA in gene promoters of orthologues from different species by comparing 60bp windows of the two promoters, as originally illustrated for CCA1 (Picot *et al.*, 2010). The conserved non-coding sequences which EARS finds are enriched for transcription factor binding sites (Baxter *et al.*, 2012), so analysing them is likely to identify putative transcription factors regulating lettuce *JAZ* genes. While EARS was originally used with 2000bp regions upstream of the transcriptional start site (Picot *et al.*, 2010), this results in large unknown gaps in lettuce so 1000bp sequences were instead used.

Assuming that conserved non-coding regions contain regulatory sequences, they indicate conserved function of orthologues. In order to find conservation of non-coding sequences over evolution EARS analysis was conducted for all individual lettuce gene promoters against their Arabidopsis syntenic orthologues (Figure 4.4.8-12), as identified by microsynteny (Table 4.4.7). Firstly, considering similarity between individual *LsJAZ* gene promoters and their *AtJAZ* gene orthologues (Figure 4.4.8-12), islands of similarity above a 10^-4 p-value threshold are evident for all lettuce JAZ gene promoters. The position of these islands of similarity is not consistent between different orthogroups of *LsJAZ* promoters, only the group 1 LsJAZ1 and LsJAZ5 exhibit shared peak locations around -175 to -110 bp from the transcriptional start site (Figure 4.4.8).

Extraction of the coding sequences corresponding to the EARS analysis peaks allowed for motif searching in the MEME suite for *de novo* motifs. The top six consensus motifs can be categorised as MYB, zinc-finger, TCP/ARF, bHLH/bZIP, ERF, and NAC transcription factors (Table 4.4.11). The MYB transcription factors include DREB2F, an abiotic stress responsive transcription factor, and similarly the zinc finger transcription factors include MYB93. TCP/ARFs contained ARF10, while bHLH/bZIP contained bZIP16. The ERF transcription factors were previously identified as important for the response to cold, and generally

widely found in *LsJAZ* promoters. The NAC transcription factors include NAC18, and 25. The conservation of motifs related to stress responses in JAZ promoters reflects their function in regulating stress responses, and how they are upregulated after necrotrophic pathogen attack and cold stress.

These data provide evidence for conservation of lettuce and Arabidopsis *JAZ* gene upstream non-coding sequences which may determine transcriptional regulation through cis regulatory elements bound by transcription factors.

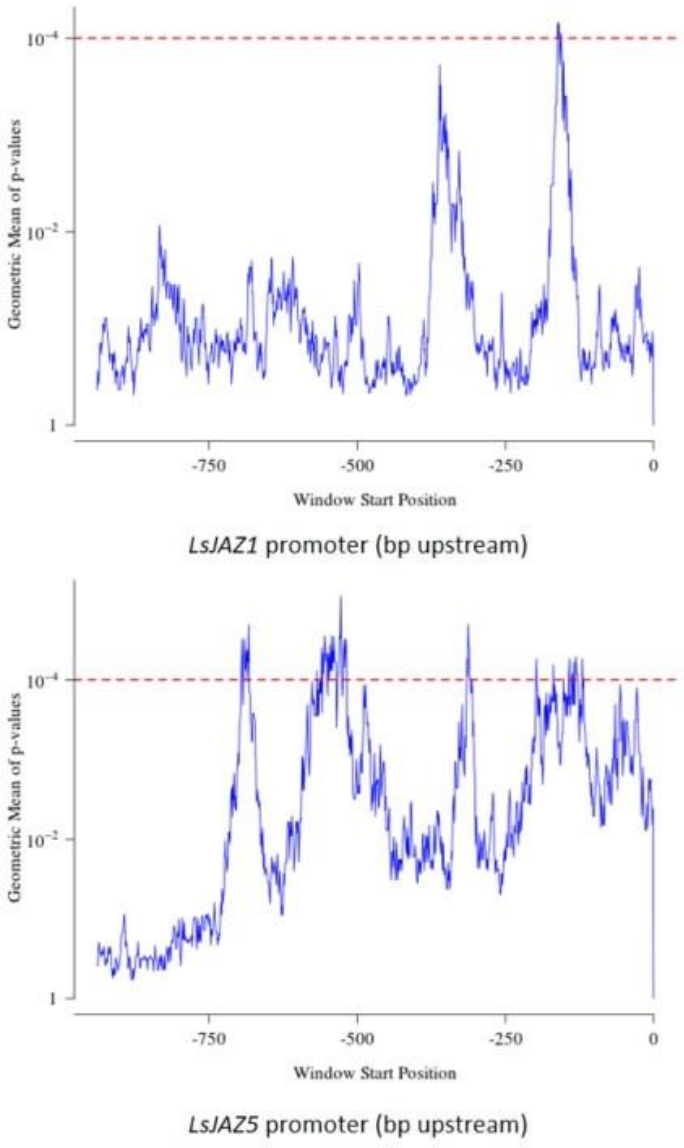


Figure 4.4.8: Some Arabidopsis and lettuce *JAZ* group 1 syntenic orthologues have conserved upstream non-coding regions.

Evolutionary analysis of *JAZ* gene promoters, comparing similarity from group 1 syntenic orthologues in lettuce and Arabidopsis. Genomic sequences 1,000bp upstream of orthologous *JAZ* genes in lettuce and Arabidopsis were compared by EARS. 60bp windows of the LsJAZpromoter sequences were compared to 60bp windows of AtJAZ1, 2, 5, 6 promoters.

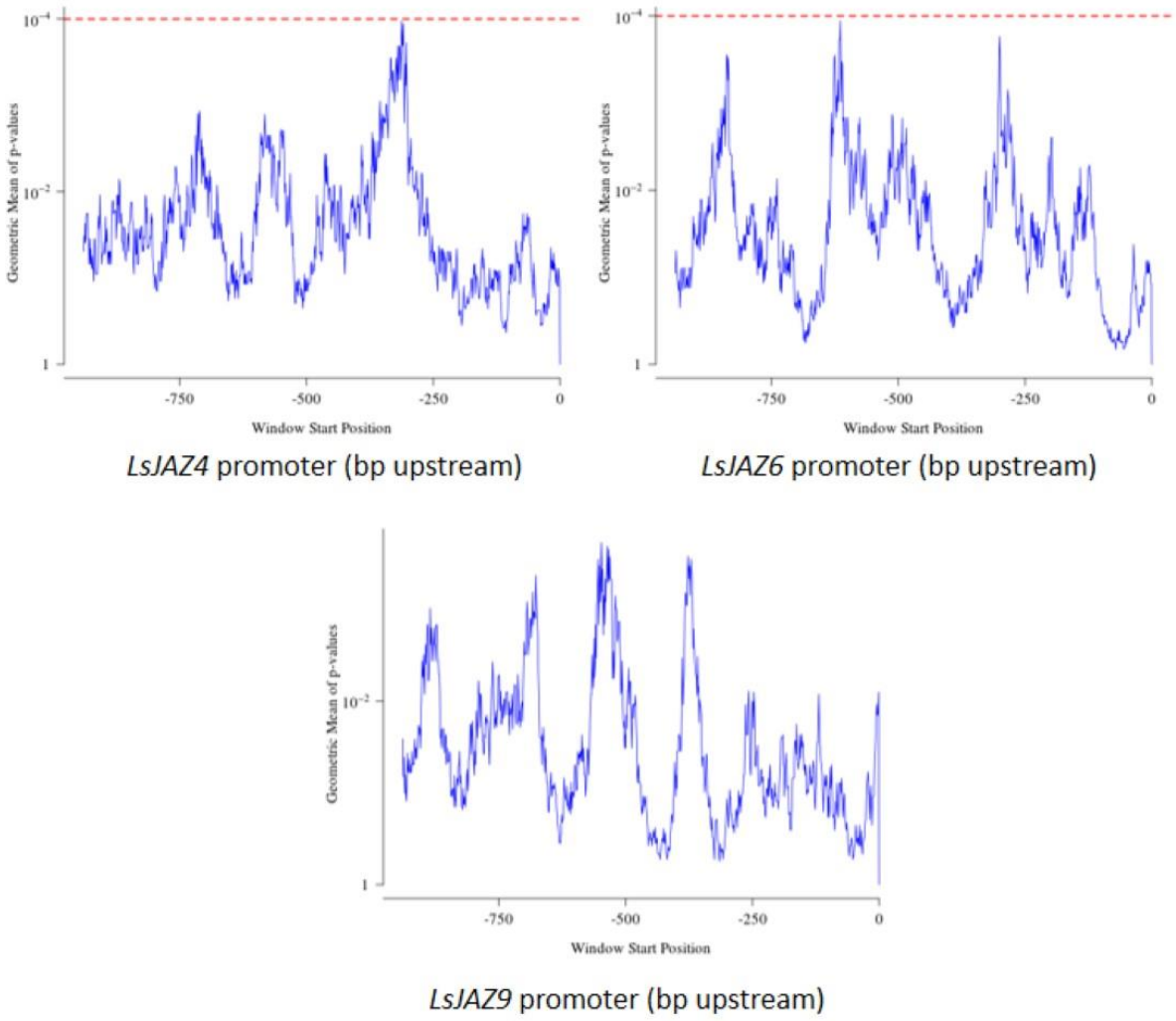


Figure 4.4.9: Some Arabidopsis and lettuce *JAZ* group 1 syntenic orthologues do not have conserved upstream non-coding regions.

Evolutionary analysis of *JAZ* gene promoters, comparing similarity from group 1 syntenic orthologues in lettuce and Arabidopsis. Genomic sequences 1,000bp upstream of orthologous *JAZ* genes in lettuce and Arabidopsis were compared by EARS. 60bp windows of the LsJAZ4, 6, or 9 promoter sequences were compared to 60bp windows of AtJAZ1, 2, 5, 6 promoters.

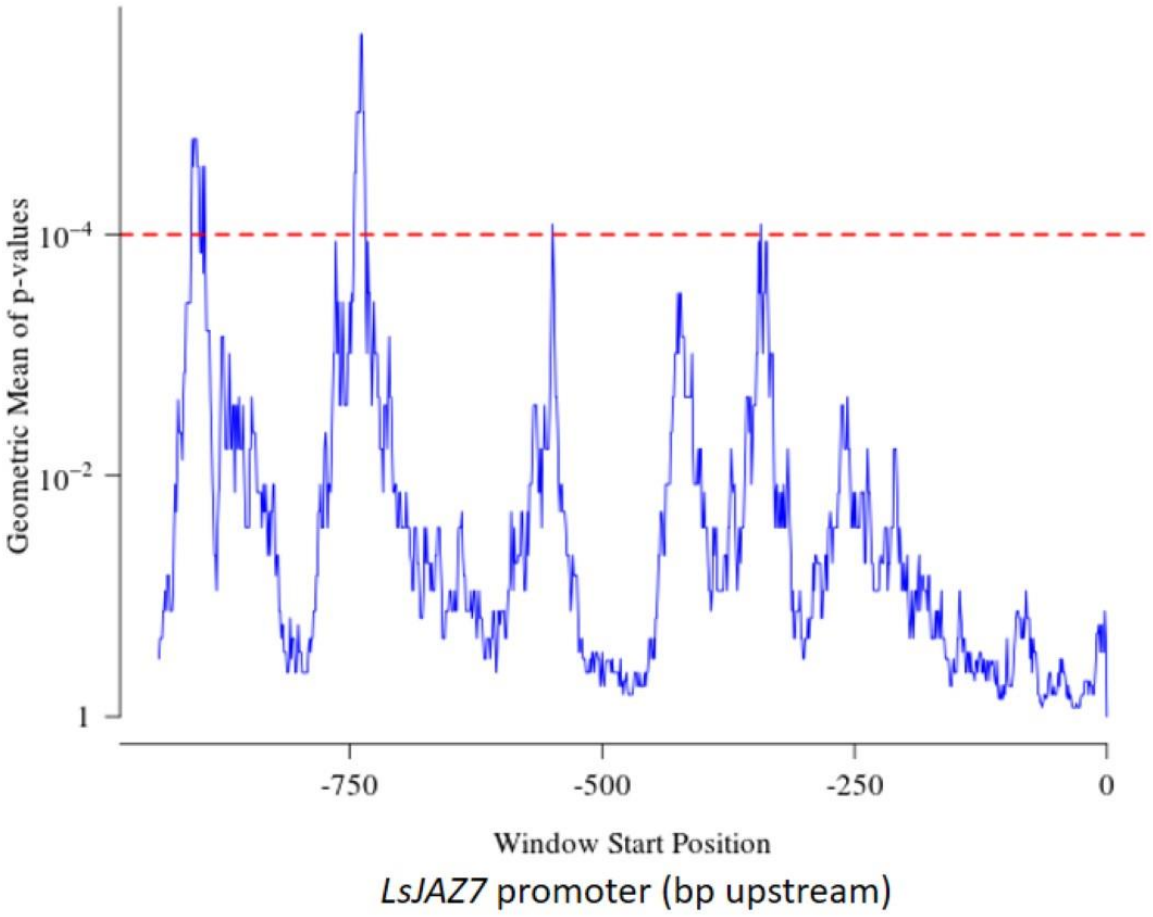


Figure 4.4.10: Arabidopsis and lettuce *JAZ* group 2 syntenic orthologues have conserved upstream non-coding regions.

Evolutionary analysis of *JAZ* gene promoters, comparing similarity from group 2 syntenic orthologues in lettuce and Arabidopsis. Genomic sequences 1,000bp upstream of orthologous *JAZ* genes in lettuce and Arabidopsis were compared by EARS. 60bp windows of the LsJAZ7 promoter sequence were compared to 60bp windows of the AtJAZ7 and AtJAZ8 promoters.

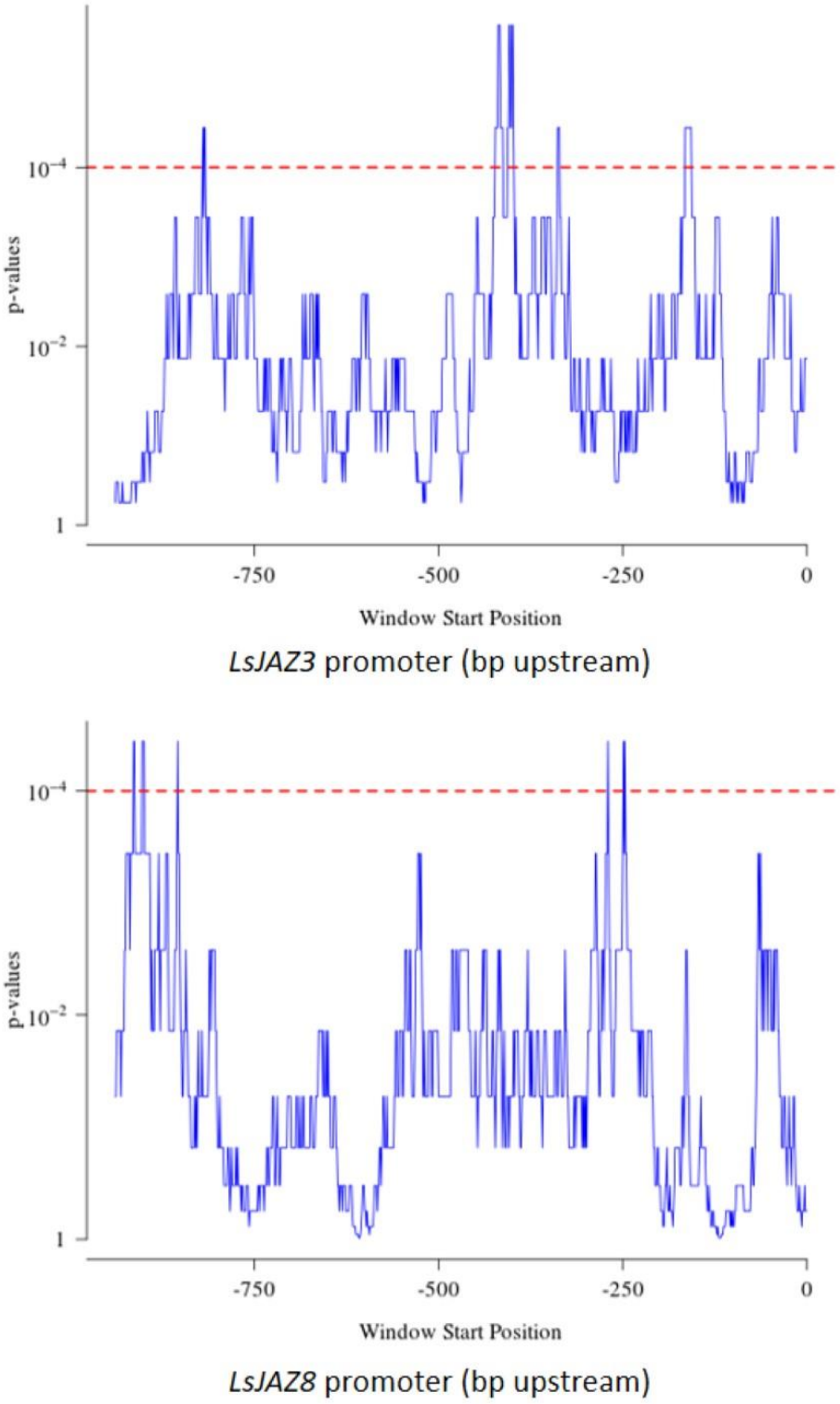


Figure 4.4.11: Arabidopsis and lettuce *JAZ* group 3 syntenic orthologues have conserved upstream non-coding regions.

Evolutionary analysis of *JAZ* gene promoters, comparing similarity from group 3 syntenic orthologues in lettuce and Arabidopsis. Genomic sequences 1,000bp upstream of orthologous *JAZ* genes in lettuce and Arabidopsis were compared by EARS. 60bp windows of the LsJAZ3 (upper) or LsJAZ8 (lower) promoter sequence were compared to 60bp windows of the AtJAZ9 promoter.

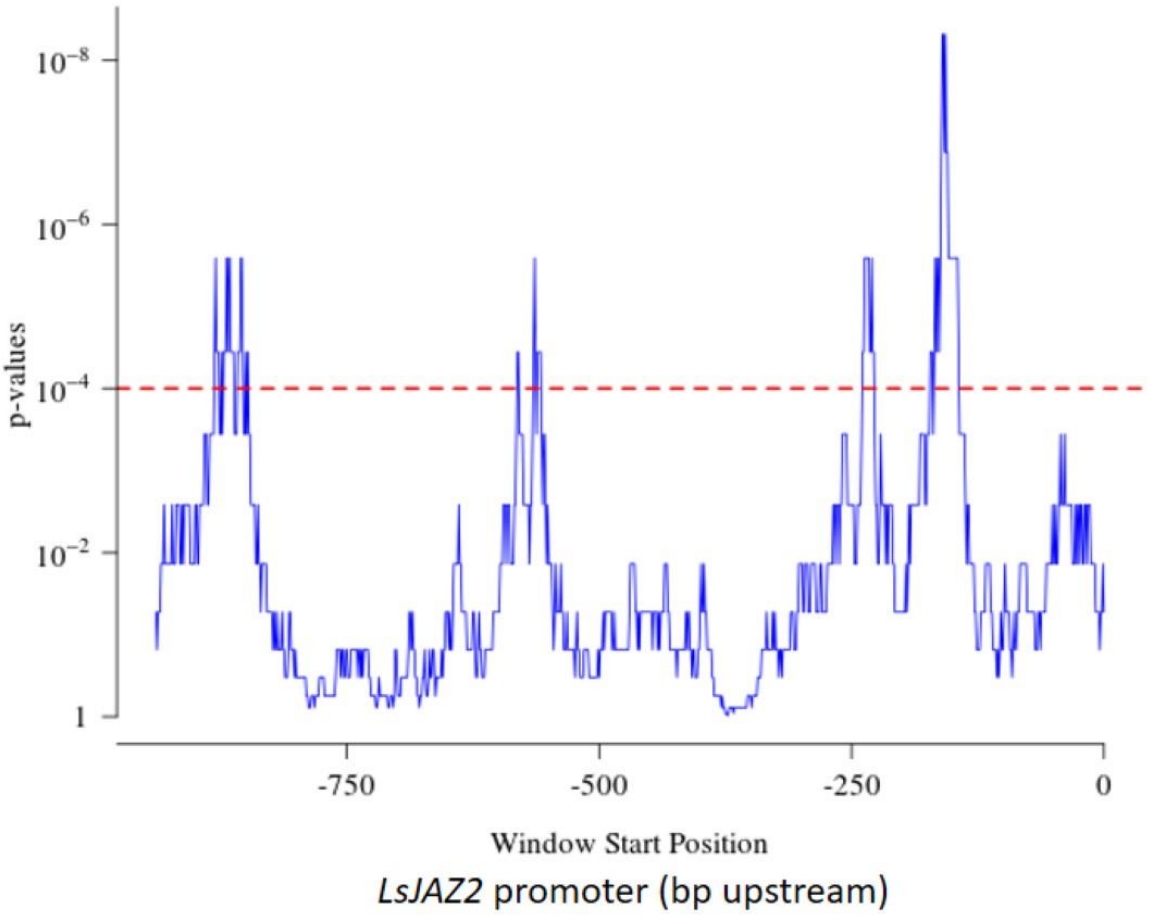


Figure 4.4.12: Arabidopsis and lettuce *JAZ* group 4 syntenic orthologues have conserved upstream non-coding regions.

Evolutionary analysis of *JAZ* gene promoters, comparing similarity from group 4 syntenic orthologues in lettuce and Arabidopsis. Genomic sequences 1,000bp upstream of orthologous *JAZ* genes in lettuce and Arabidopsis were compared by EARS. 60bp windows of the LsJAZ2 promoter sequence were compared to 60bp windows of the AtJAZ10 promoter.

Table 4.4.11: Transcription factor binding sites are conserved in syntenic Arabidopsis and lettuce *JAZ* promoters.

Windows of high similarity in LsJAZ genes to their syntenic orthologues in EARS were scanned for potential shared motifs using MEME. Matching transcription factors for these were found using TOMTOM.

Motif type	Example transcription factors	Consensus motif logo
MYB	ABI4, MYB46, DREB2F	
Zinc-finger	TRP1, IDD6, MYB93	
TCP/ARF	ARF10, ARF14, TCP4	
bHLH/bZIP	bHLH130, bZIP16, bZIP68	
ERF	ERF14, DREB2A, LBD13	
NAC	NAC18, NAC25, NAC29	

4.5 Discussion

The key results of this analysis are that nine JAZ proteins were identified in lettuce (Figure 4.4.1), containing TIFY and Jas domains (Figure 4.4.2). All lettuce JAZ proteins are syntenic orthologues of Arabidopsis and tomato JAZ proteins. *JAZ* genes are upregulated in response to necrotrophic pathogens and cold stress in both lettuce and Arabidopsis (Figures 4.4.5-7). The same cis elements are found in the promoters of lettuce and Arabidopsis *JAZ* genes (Table 4.4.8-10, 11), especially similar regions in the promoters of syntenic orthologues (Figures 4.4.8-12). Similarities between Arabidopsis and lettuce JAZ protein content and expression regulation suggest that they have conserved functions.

4.5.1 Nine lettuce *JAZ* genes are within expected limits

The number of JAZ proteins in Arabidopsis (13) is higher than the number of JAZ proteins in lettuce (9) (Figure 4.4.1). Across plant species there is considerable variation in the number of JAZ proteins (Garrido-Bigotes, Valenzuela-Riffo and Figueroa, 2019), from five to thirty five in the 66 species in the phylogenetic analysis in this work (Figure 4.4.4), and so the number of lettuce JAZ proteins is within the expected range. Species with high numbers of JAZ proteins at the upper end of the range, like *Brassica napus* (Jia *et al.*, 2021), are likely due to whole genome duplication. It is plausible that newer versions of the lettuce genome or transcriptome could reveal further *JAZ* genes, just as *LsJAZ4* was not originally annotated as a gene in previous versions of the lettuce genome (v5). It is possible that JAZ proteins with highly divergent TIFY and Jas domains like AtJAZ13 are yet to be found in lettuce. There may be an orthologue to the orthogroup 6 AtJAZ11 and AtJAZ12 in lettuce yet undiscovered, as sunflower (*Helianthus annu*s), which is closely related to lettuce, possesses an orthologue in orthogroup 6 (Table 4.4.5).

4.5.2 JAZ protein structure is suggestive of divergent protein function

Lettuce JAZ proteins possess TIFY and Jas domains (Table 4.4.1). This implies several aspects of their activity and function are conserved with Arabidopsis JAZ proteins. Specifically, a conserved Jas domain implies conserved binding to transcription factors and degradation in the presence of jasmonic acid (Thines *et al.*, 2007), while a conserved TIFY domain implies conserved homo- and heterodimerisation between JAZ proteins (Chini *et al.*, 2009). Further

work could examine the extent of their functional similarity. All lettuce JAZ proteins are likely to be localised to the nucleus, due to the nuclear localising signal in the Jas domain (Withers *et al.*, 2012), as it binds to MYC2. The new *de novo* motif found by MEME (Figure 4.4.2) could be tested for its function as a MYC2 interacting domain, as it appears to coincide with the cryptic MYC interacting domain in AtJAZ1 (Withers *et al.*, 2012).

Divergence from the conserved domains of JAZ proteins implies divergent protein function. For example LsJAZ7 is the only lettuce JAZ protein to possess a non-canonical Jas domain degron amino acid sequence (Table 4.4.1), which is similar to AtJAZ8 (Shyu *et al.*, 2012). As AtJAZ8 is impaired in binding to COI1 for degradation in the presence of jasmonic acid, LsJAZ7 may also be unable to bind to COI1 for degradation.

LsJAZ4, 5, and 7 possess canonical LxLxL EAR domains (Table 4.4.2), and so may be capable of binding to proteins like the lettuce TOPLESS orthologue. Due to these structural deviations, it is likely LsJAZ4, 5, and 7 have a distinct function to other LsJAZ proteins, a point for further investigation.

4.5.3 Lettuce *JAZ* genes are orthologous to Arabidopsis and tomato *JAZ* genes

All lettuce JAZ proteins are found in groups of syntenic orthologoue proteins covering 107 Angiosperm species (Zhao and Schranz, 2019). The most surprising syntenic relationship is that most JAZ genes in Angiosperms are clustered in a single orthologous group (Table 4.4.6). As *Amborella trichopodia* JAZ proteins do not exhibit this bias towards JAZ orthogroup 1, it may be inferred that the duplication and diversification of the JAZ proteins in this group has largely occurred in the past 150 million years since the radiation of the Angiosperms (Wikström, Savolainen and Chase, 2001). The lack of any Arabidopsis or lettuce JAZ proteins in Monocot exclusive clades of the phylogenetic assessment of JAZ proteins across the Viridaeplantae (Figure 4.4.4), supports duplication and divergence of JAZ proteins after the divergence of Monocots and Dicots. It is tempting to hypothesise that the recent divergence of JAZ genes may be a cyclical process, repeating to replace former *JAZ* duplications which have accumulated too many mutations to produce functional JAZ proteins. Alternatively, *JAZ* gene duplications may represent a recent plant innovation in

response to herbivore and pathogen selective pressure, which would put evolutionary pressure on the growth-defence tradeoff. Such analysis is beyond the scope of this thesis.

The colocalization of Arabidopsis and lettuce orthologues to the same clades of JAZ proteins in phylogenetic analysis (Figure 4.4.2 & 3), may indicate conserved functions for the diverse range of JAZ proteins. This is supported by evidence *JAZ* genes undergoing purifying in petunia (Tian *et al.*, 2019). It is in orthogroup 1 that AtJAZ6 is found (Table 4.4.5), so a lettuce orthologue with the same functions might be predicted to be part of orthogroup 1 as well. Functional similarity between Arabidopsis and lettuce orthogroup 1 proteins is likely considering the similarity of their domain structures (Table 4.4.1), including the cryptic MYC interacting domain.

4.5.4 There are similarities in the regulation of *JAZ* gene expression in Arabidopsis and lettuce

Arabidopsis and lettuce *JAZ* gene promoters contain conserved sections of DNA and share many cis-regulatory elements (Figures 4.4.8-12). These suggest that *JAZ* genes in both species are controlled by similar transcription factors and respond similarly to the same environmental stresses. Together with the presence of conserved Jas motifs in lettuce *JAZ* genes (Figure 4.4.2), the presence of G-box MYC motifs in their promoters is indicative of lettuce MYC orthologues involved in *LsJAZ* regulation. In Arabidopsis, JAZ proteins repress MYC2/3/4 by binding to them through the Jas domain (Zhang *et al.*, 2015). However, when JAZ proteins are degraded, MYC2/3/4 activate JAZ gene expression by binding to G-boxes (Wang *et al.*, 2019), in a negative feedback loop. Testing LsJAZ binding to lettuce MYC2/3/4 orthologues could further support the hypothesis that this feedback loop regulation is also active in lettuce. The conservation of cis regulatory elements between different species has been noted for the promoters of cytokinin response factors across Angiosperms (Powell *et al.*, 2019), so it is possible that the cis regulatory elements of JAZ genes are similarly conserved.

Upregulation of both lettuce and Arabidopsis *JAZ* genes is apparent after necrotrophic pathogen attack (Figure 4.4.5 & 6). The different speeds and degrees of lettuce *JAZ* gene upregulation after attack by necrotrophic pathogens are indicative of different roles and

functions. For example, *LsJAZ8* may be less relevant for pathogen responses as it is not upregulated to the same degree as other lettuce JAZ genes. In contrast to *LsJAZ8*, *LsJAZ7* is not expressed prior to infection and so is upregulated to the highest fold change seen for any lettuce *JAZ* gene. Together with the unique motif sequences found in the LsJAZ7 protein (Table 4.4.1-3), this further suggests a unique role for LsJAZ7 in regulating jasmonic acid responses in lettuce.

A smaller proportion of the Arabidopsis *JAZ* genes than lettuce *JAZ* genes are upregulated after necrotrophic pathogen infection compared to lettuce *JAZ* genes (Figures 4.4.5, 6). However, the same total number of *JAZ* genes (8) are differentially expressed in both species. It may be hypothesised that *JAZ* genes beyond these eight genes face less pressure to respond to necrotrophic pathogens and undergo neo-subfunctionalisation into different roles.

Only a subset of Arabidopsis and lettuce *JAZ* genes are responsive to cold stress (Figure 4.4.7). As with the response to necrotropic pathogens, this may be a result of the duplication of *JAZ* genes allowing for diversification in the roles they assume and pathways they act in. This is evident with the cold response, as members of the same orthogroup have different responses to cold. While a smaller ratio of *JAZ* genes is responsive to cold in Arabidopsis than in lettuce, the different timescales used for sampling may lead to different results.

The conserved MYB and CBF motifs in both lettuce and Arabidopsis which are enriched for responsive *JAZ* gene promoters may indicate conserved mechanisms underlying responses to biotic and abiotic stress in plants (Tables 4.4.8 & 9). This matches the conservation of cis regulatory elements for the promoters of cytokinin response factors across Angiosperms (Powell *et al.*, 2019). The specificity of the motifs for some *JAZ* genes may reflect regulatory divergence between *JAZ* genes, which may be concurrent with functional differentiation (Arsovski *et al.*, 2015). While the CBF genes have previously been linked to JAZ genes in cold responses (Hu *et al.*, 2013), this regulatory relationship puts CBF genes as indirect targets of JAZ repression. It is possible that CBFs then regulate JAZ gene expression in a feedback loop

in the event of cold stress. MYB4 has previously been identified as a target of the MAPK kinase cascade which occurs in early stages of plant infection (Lin *et al.*, 2022), but not as a regulator of JAZ genes. However, as a target of the MAPK kinase cascade during infection, it is possible MYB4 is also regulating JAZ genes during infection.

4.5.5 Further work can establish the functional roles and mechanisms of action of lettuce *JAZ* genes

While this work demonstrates that lettuce *JAZ* genes are responsive to biotic and abiotic stress, it does not show how they affect downstream gene expression as transcriptional co-repressors. One approach to identifying targets of JAZ co-repression in lettuce would be *in silico* network prediction techniques such as OutPredict (Cirrone *et al.*, 2020). While the fact that JAZ proteins act post-transcriptionally as transcriptional-corepressors makes this a non-ideal method, the negative feedback loop which promotes JAZ gene transcription as JAZ proteins are degraded allows for computational simplification of the double-negative JAZ gene - JAZ protein - JAZ target gene to a simple positive JAZ gene -> JAZ target gene (Frank, pers. communications). However, due to the computational difficulty of handling large numbers of genes, these are usually run with and optimised for transcription factors (Cirrone *et al.*, 2020), which excludes *JAZ* genes as they are transcriptional co-repressors which act post-transcriptionally.

Reverse genetic approaches involving mutant and overexpression lines would be especially useful for determining direct and indirect targets of JAZ co-repression. There is currently no T-DNA mutant library for lettuce genes as there is for Arabidopsis genes (O'Malley, Barragan and Ecker, 2015), so generating and verifying lettuce mutants is significantly different to Arabidopsis. It is possible to generate lettuce mutants with RNA interference as seen for *LsFT* (Chen *et al.*, 2018), and CRISPR as seen for *LsNCED4* (Bertier *et al.*, 2018), though these approaches can be time consuming due to the long generation time of lettuce and technically challenging (Ransom, 2018). Generating overexpression lines in lettuce presents similar difficulties, though it is possible as demonstrated with *LsFT* and *LsGRF5* (Chen *et al.*, 2018; B. Zhang *et al.*, 2021). With respect to JAZ genes, stable overexpression has been used to demonstrate the role of chrysanthemum *CmJAZ1-like* in promoting flowering (Guan *et al.*, 2021). Stable ectopic expression lines of lettuce genes can be made in Arabidopsis, as has

been done for *LsERF1* and *LsFT* (Chen *et al.*, 2018; Ransom, 2018), which is a faster way to gain functional information due to the shorter generation time of Arabidopsis. Functional conservation of JAZ genes from a variety of plant species has previously been demonstrated by ectopic expression in Arabidopsis, including wild grapevine *VqJAZ4*, *Erigeron breviscapus EbJAZ1,* and all known rice *OsJAZ* genes (Guofeng Zhang *et al.*, 2019; B. Sun *et al.*, 2022; Chen *et al.*, 2022). Ectopic expression of lettuce genes in Arabidopsis can also illustrate functional conservation through expression of lettuce genes in the mutant of their Arabidopsis orthologue and showing recovery of the mutant phenotype, which has been demonstrated with *LsFT* in an *ft-2* mutant background (Chen *et al.*, 2018). However, due to the different cellular environments and proteins present in Arabidopsis and lettuce, as well as divergence of functional roles of orthologues, expression of lettuce genes in Arabidopsis may not present an accurate picture of their behaviour.

Transient overexpression of lettuce genes is feasible in lettuce and *N. benthamiana,* allowing for functional assessment of their roles in defence through infection assays considerably faster than making stable lines of Arabidopsis or lettuce. Transient overexpression in *N. benthamiana* is a well-established technique and easy to perform (Bally *et al.*, 2018), as demonstrated for *JAZ* genes in Chapter 3 of this work. However, as with Arabidopsis, *N. benthamiana* presents a non-native cellular environment so there are caveats for interpreting the results. Transient overexpression in lettuce was initially developed before CRISPR (Wroblewski, Tomczak and Michelmore, 2005), and has recently shown considerable promise for functional analysis of genes (Yamamoto *et al.*, 2018). This has been demonstrated with transient expression of the effector protein AvrRps4 showing it is recognised by a broad range of plant species (Su *et al.*, 2021). Unfortunately, transient expression in lettuce can be technically challenging to perform successfully (Harry Pink, pers. communication). Additionally, due to the short duration of transient expression, it would not be feasible for investigating the roles of *JAZ* genes in flowering and other long-term developmental processes. While transient expression would be considerably easier in *N. benthamiana*, overexpression in lettuce would be particularly valuable as it allows for assessment of lettuce *JAZ* function in a close to native context.

Protein-protein binding testing with lettuce JAZ proteins and lettuce orthologues of known JAZ binding proteins would validate their involvement in JA responsive pathways and establish potential targets of JAZ co-repression. Protein-protein binding testing was used to characterise rubber tree HbJAZ and *Mentha canadensis* McJAZ proteins through Y2H experiments showing they can bind the orthologues of CORONATINE INSENSITIVE 1 (COI1) and other JAZ proteins (Chao *et al.*, 2019; Xu *et al.*, 2021). While testing known interactors of JAZ in Arabidopsis would validate the action of lettuce JAZ proteins as transcriptional repressors binding to the orthologues of MYC transcription factors and COI1, it would not find new lettuce-specific targets of JAZ. New lettuce-specific targets of JAZ could be found with Y2H library screening as previously performed for AtJAZ6 (Pruneda-Paz *et al.*, 2014; Stoker, 2016), though this would require the construction of a Y2H library for lettuce. Such library screening would provide a basis for high-throughput analysis of the specificity of JAZ binding partners, just as AtJAZ6 was compared to AtJAZ5 previously (Stoker, 2016). However, such results may not wholly reflect *in planta* binding due to the different cellular environment in yeast, as demonstrated in Chapter 2 of this work. As in Chapter 2, BiFC and co-immunoprecipitation of protein-protein binding partners transiently expressed in *N. benthamiana* would be feasible techniques to assess protein-protein binding of lettuce JAZ proteins with potential target transcription factors.

Transient or stable overexpression of tagged lettuce *JAZ* genes in lettuce would allow for affinity purification mass spectroscopy to identify binding partners of lettuce JAZ proteins (Youjun Zhang *et al.*, 2019), and protein complexes they are involved with. This would thoroughly assess the role of lettuce JAZ proteins as transcriptional co-repressors, finding targets which may be unique to specific lettuce JAZ proteins. Binding affinity assays can reveal more about the dynamics of protein-protein interaction, which is particularly important for protein complex stability. The binding affinity of lettuce JAZ proteins to these targets could be assessed by biochemical *in vitro* methods, such as COI1 lettuce orthologue binding at varying concentrations of jasmonic acid, or competitive binding assays with multiple target transcription factors. Binding affinity assays have recently been used to elucidate the dynamics of Arabidopsis MYC3 repression by JAZ proteins (Takaoka *et al.*,

2021), illustrating the importance of splice variants like JAZ10.4 which possess divergent Jas domains.

In conclusion, this work has identified 9 JAZ proteins in lettuce. These are all syntenic orthologues of Arabidopsis and tomato JAZ proteins. Many lettuce JAZ genes are differentially expressed in response to biotic and abiotic stress like Arabidopsis JAZ genes, which may be related to similar cis-regulatory elements in their promoter regions. This work could be expanded upon by investigating lettuce *JAZ* gene responses to other biotic and abiotic stress conditions, finding proteins which natively interact with lettuce JAZ proteins, and investigating what genes the lettuce JAZ proteins regulate. CRISPR gene editing of lettuce *JAZ* genes, or ectopic expression in Arabidopsis, will give further evidence in credence of conserved function in the response to necrotrophic pathogens, and other stress responses.

5: Discussion

We live in a World with an ever-increasing population size, and this directly leads to increasing pressure on agricultural productivity. Although in recent decades agricultural practices have improved crop yield, there are still significant losses due to plant diseases (Savary *et al.*, 2019). Such losses could be reduced by improving crop resistance to disease. However, in order to achieve this, we first need to more fully understanding of how plants currently protect themselves from infection. The research described in this thesis is timely, not just due to the increasing World population, but also because future climate change will increase the threat of fungal and oomycete plant pathogens to global food security (Chaloner, Gurr and Bebber, 2021).

Early in plant evolution, defence mechanisms developed to protect plants from microbial pathogens. One key process involves the hormone jasmonic acid which plants produce in response to wounding and the detection of necrotrophic pathogens (Widemann *et al.*, 2016). The induced jasmonic acid, in the bioactive form iso-jasmonyl-L-isoleucine, is then detected by various JASMONATE ZIM DOMAIN (JAZ) proteins (Fonseca *et al.*, 2009; Wasternack and Hause, 2013). In the model plant Arabidopsis there are 13 JAZ proteins, which complement each other and share protein-protein binding partners like MYC2 (Niu, Figueroa and Browse, 2011; Major *et al.*, 2020). JAZ proteins act by repressing the transcription factors they are bound to, and this repression is relieved when bioactive jasmonic acid allows JAZ proteins to be targeted for degradation by COI1 (Wasternack and Hause, 2013). As many of these protein-protein binding partners are transcription factors, the outcome of jasmonic acid detection includes changes in gene expression. There is some degree of functional specificity of different JAZ proteins to different physiological roles suggested by mutant analysis (Liu *et al.*, 2021), which reflects how different JAZ proteins possess different key domains for binding other proteins. Specific roles in disease resistance have been established for JAZ6, including regulating diurnal variation in susceptibility to *B. cinerea* (Ingle *et al.*, 2015), and repressing resistance to *B. cinerea* and *P. syringae* (Li *et al.*, 2019; Liu *et al.*, 2021). My thesis sought to investigate the regulation of *JAZ6*, verify and characterise potential protein-protein interactors of JAZ6, and to extend our knowledge of JAZ proteins from Arabidopsis to the crop plant lettuce.

As JAZ proteins are transcriptional co-repressors, to understand their actions it is vital to understand the proteins which they bind to. JAZ proteins alone do not directly regulate gene transcription. Instead, they bind to other transcription factors and epigenetic regulators in protein complexes which in turn interact with DNA and chromatin (Zander, 2021). However, their differing protein sequence and motif content leads to different activities because they will preferentially bind to different transcription factors. As Arabidopsis contains 13 JAZ proteins, the multiple possibilities for regulatory logic gives a great complexity to the system. At the same time, similarities between JAZ proteins allow them to complement each other (Major *et al.*, 2020), leading to challenges in understanding the actions of a single JAZ protein such as JAZ6.

5.1 JAZ6 is under transcriptional and post-transcriptional control by the circadian clock

In chapter 2 it is predicted that JAZ6 is under direct transcriptional control of the circadian clock and could be under indirect post-translational control by the circadian clock. The control of JAZ6 by the circadian clock reflects the extensive system of control the circadian clock exerts on jasmonic acid related processes (Y. Zhang *et al.*, 2018). Jasmonic acid synthesis is under control of the circadian clock, with TOC1 repressing transcription of the jasmonic acid synthesis genes *LOX2, LOX3,* and *LOX4* (Huang *et al.*, 2012). The clock also controls jasmonic acid responses downstream of JAZ6, an example of this is TIC which controls *MYC2* transcription (Shin *et al.*, 2012).

The control of JAZ6 by the circadian clock may be one part of a feedback mechanism in which the clock and jasmonic acid control each other. Jasmonic acid is known to affect the circadian clock and dampen oscillations of clock genes like CCA1 (Chong Zhang *et al.*, 2019). Although the precise mechanism by which this happens remains unclear, COI1 is necessary so it is likely that some JAZ proteins are necessary for this feedback as well. Although JAZ6 itself does not appear to regulate circadian clock genes (as none were differentially expressed in *jaz6-3* mutant plants as detailed in chapter 3), it may be the case that other JAZ

proteins are involved in the regulation of circadian clock genes. This could occur via the transcriptional co-repressor TPL (Plant, Larrieu and Causier, 2021), which binds JAZ proteins and regulates CCA1/LHY. JAZ proteins may also regulate the circadian clock through binding FHY3, which is able to gate light inputs into the circadian clock (Allen *et al.*, 2006). While JAZ6 may be involved in the regulation of clock genes, it may be compensated for in the *jaz6-3* mutant by other JAZ proteins such as the very similar JAZ5. It could be hypothesised that jasmonic acid represses salicylic acid production which leads to indirect effects on the circadian clock. This could be further linked into salicylic acid-jasmonic acid antagonism as the clock is responsive to salicylic acid levels. Salicylic acid feeds into the circadian clock through NPR1 (Jingjing Zhang *et al.*, 2019), which directly transcriptionally regulates three circadian clock genes.

The reason for this feedback mechanism between jasmonic acid and the circadian clock may be to increase fitness by providing responsiveness to plants which enables them to predict attack by pathogens and herbivores (Goodspeed *et al.*, 2012). Future work could further elaborate on the interconnectedness of jasmonic acid responses and the circadian clock, in particular obtaining biochemical data for JAZ protein production and degradation rates would permit more accurate models of such control of the JAZ proteins through the clock, in addition to any putative feedback mechanisms which might be present.

The wider implications of a clock-JAZ feedback loop are difficult to fully appreciate, as unlike the clock-NPR1 feedback system, there are 13 JAZ proteins which may be involved to varying degrees.

5.2 JAZ6 acts in plant defence against multiple pathogens

Several recent works have shown that *JAZ6* acts to repress resistance to the necrotroph *B. cinerea* (Li *et al.*, 2019; Liu *et al.*, 2021). It has also been shown that JAZ6 controls circadian susceptibility to *B. cinerea* (Ingle *et al.*, 2015). In this thesis (chapter 2) JAZ6 is shown to repress resistance to *B. cinerea* at dusk. Therefore, we now have further details on the dynamics of how JAZ6 acts in susceptibility to *B. cinerea*.

In chapter 3, JAZ6 is shown to promote Arabidopsis susceptibility to *S. sclerotiorum*. This suggests that JAZ6 is functioning to promote susceptibility to a wider range of pathogens than *B. cinerea* alone. This is supported by how JAZ6 also promotes susceptibility to the hemibiotrophic pathogen *P. syringae* (Liu *et al.*, 2021). This may be related to the *P. syringae* virulence factor coronatine which acts as a jasmonic acid mimic by binding to and degrading JAZ proteins (Geng *et al.*, 2014). However, coronatine binds to many JAZ proteins and only a few JAZ proteins promote susceptibility to *P. syringae* (Zhao and Schranz, 2019). The mechanism for JAZ6 repressing resistance to *P. syringae* would be particularly interesting to investigate, especially to see how much of the mechanism is conserved with the repression of resistance to *B. cinerea*.

A novel role of JAZ6 was found by infecting Arabidopsis with *H. arabidopsidis* oomycete pathogens. In chapter 3 it was shown that *jaz6* mutants are more susceptible to *H. arabidopsidis* infection than WT plants. This concurs with a previous report showing that wild grapevine VqJAZ4 promotes resistance to the biotrophic pathogen *Golovinomyces cichoracearum* (Guofeng Zhang *et al.*, 2019). This could be further explored in other biotrophs to see if the role of JAZ6 is specific to the Arabidposis-*H. arabidopsidis* pathosystem, possibly with *G. cichoracearum* or viruses like CMV.

The dual role of JAZ6 in controlling disease resistance in contrasting ways to biotrophs and necrotrophs is not necessarily surprising, especially given the similar demonstrated role of wild grapevine VqJAZ4, it is interesting that this particular JAZ protein has these two divergent roles.

While the contrasting roles of JAZ6 in mediating both biotrophic and necrotrophic plant pathogen resistance could be construed as an extension of how the JAZ proteins can act to translate the hormone signals of abscisic acid, jasmonic acid, and ethylene into control of plant defences optimised for the particular herbivore or pathogen threat identified, it has not yet been fully explained why JAZ6 in particular is so indispensable for balancing the responses to these different pathogens.

While prior work highlights the specificity of JAZ proteins though their individual binding affinities to proteins like MYC2, and there is experimental evidence for a variety of different roles for different JAZ proteins, the current body of work is insufficient to fully explain the difference in function between JAZ5 and JAZ6. Specifically, multiple different experiments have demonstrated that JAZ5 is dispensable for plant defence, while mutants which lack JAZ6 have altered susceptibility to pathogens. JAZ5 and JAZ6 comprise highly similar protein domain structures, their Jas and ZIM domains are identical. As shown in Chapter 2, both JAZ5 and JAZ6 bind PFP, while there is no evidence to suggest JAZ1 and JAZ8 can do such. It may be hypothesised that the difference in function of JAZ5 and JAZ6 is found at the transcriptional level, or possibly with contrasting protein levels or localisation. These would all be points for future investigation to better elucidate the difference between JAZ5 and JAZ6.

On another level, it would be interesting to see if the divergence in roles between JAZ5 and JAZ6 is seen in their closest lettuce orthologues, for which genetic engineering of lettuce could highlight possible roles in plant defence for these orthologues.

5.3 The role of JAZ6 in promoting the transition to flowering

The novel role of JAZ6 in promoting the transition to flowering was a surprise since previous reports stated that *jaz6-5* mutants did not display an altered flowering time phenotype in long day growth conditions compared to WT plants (Liu *et al.*, 2021).

Roles for JAZs in flowering is not entirely unexpected, however there is significant variation in the roles of different JAZ genes in flowering. JAZ4 and JAZ8 bind to TOE1 and TOE2 to control flowering (Zhai *et al.*, 2015a), but this does not obviously involve JAZ6 as JAZ6 was not demonstrated to bind TOE1 or TOE2.

PFP is known to target FLC for repression and binds to histones. The known mechanisms regulating FLC via histone modification involve either polycomb group repressor complexes, or a protein complex of FLD-HDA6-FVE or MSI5 (He, 2012). Proteins within these complexes, HDA6 and LHP1 respectively, are known to bind JAZ proteins (Zhu *et al.*, 2011; Li *et al.*,

2021), JAZ6 for LHP1 and its paralog JAZ1 for HDA6. The PRC1-like complex targeting FLC is a particularly interesting prospect for JAZ6 and PFP regulation, as LHP1 in this complex is known to bind JAZ6, and the complex is targeted to FLC by histone monoubiquitination (Cao *et al.*, 2008), which the orthologues of PFP are capable of (Adhikary *et al.*, 2019).

Control of flowering by JAZ6 though FLC also makes sense from the demonstrated late flowering phenotype of *jaz6-3* mutants exclusively in short day growth conditions. This is because a flowering time phenotype only seen in short day growth conditions is consistent with control of flowering through the autonomous or vernalisation pathways, which regulate FLC expression (Sharma *et al.*, 2016). Therefore, the demonstrated phenotype of *jaz6-3* mutants late flowering in short day conditions may lead us to hypothesise that JAZ6 is controlling flowering through FLC, possibly through a PRC-1 like complex including LHP1 which is targeted to FLC by histone monoubiquitination by PFP.

While the flowering time phenotype of the double jaz6-3 pfp-1 mutant is intermediate between the two parental lines, there are a few possible ways of interpreting this. Firstly, as this work has demonstrated that JAZ5 can also bind to PFP, it is possible that even without JAZ6, another JAZ protein can bind to PFP albeit possibly with weaker binding affinity, partially complementing the lack of JAZ6. The potential of JAZ proteins to complement for each other is well established and has been tested by stacking multiple JAZ gene mutations (Major *et al.*, 2020; Liu *et al.*, 2021). It can be argued that *jazD*, a mutant line which lacks 10 of the 13 Arabidopsis JAZ genes, displays phenotypes of reduced seed production and the like comparable to COI1 the jasmonic acid receptor itself (Major *et al.*, 2020). As such, beyond such extreme levels of JAZ mutations, it may be expected for at least partial complementarity of the JAZ genes in the face of a single mutant.

5.4 JAZ6 is dissimilar to master regulators in plants

JAZ6 may be compared to master regulators in plants such as FERRONIA. FERONIA is a master regulator in plants, which is regulated by CCA1 of the circadian clock and can influence flowering time (Wang *et al.*, 2020; L. Zhang *et al.*, 2021). The above features of FERONIA can be compared to JAZ6, which may be regulated by LUX of the circadian complex

and influence flowering time in short day conditions. It also regulates defence against pathogens as a component of immune complexes containing the NB-LRR receptor proteins such as FLS2 and BAK1 for pathogen perception , as well as repressing jasmonic acid associated responses (H. Guo *et al.*, 2018; Duan *et al.*, 2022).

The regulation of flowering time by *JAZ6* and *FERONIA* is comparable, as they both have late flowering mutants, so act as promoters of flowering (Wang *et al.*, 2020). They may further be similar in that both target *FLC*, though there is no evidence that JAZ6 mediates alternative splicing of *FLC* like FERONIA does.

However, FERONIA may be contrasted with JAZ6 localisation as FERONIA is a membrane protein and JAZ6 acts in the nucleus. It is this nuclear localisation which enables JAZ6 to influence gene expression downstream of pathogen perception, while FERONIA has a role upstream. *JAZ6* and *FERONIA* are also regulated by different clock genes and have distinct patterns of expression across the day.

5.5 PFP in plant disease resistance

The role of PFP in *B. cinerea* disease resistance is a novel phenotype of great interest. PFP is a highly conserved gene across eukaryotes, with single-copy orthologues present in most species (Adhikary *et al.*, 2019; Zhao and Schranz, 2019). The demonstrated role of PFP in Arabidopsis is to bind histones (Yokoyama, Kobayashi and Kidou, 2019), which is proposed as the mechanism by which it regulates transcription of the flowering gene regulator FLC. The human and rice orthologues of PFP (HsUBR7, OsUBR7) are able to monoubiquitinate histones to alter gene transcription (Adhikary *et al.*, 2019; Zheng *et al.*, 2022). Given the high protein similarity of these orthologues PFP is likely to show conserved function in monoibiquitinating histones as well.

The role of PFP in controlling plant gene transcription is small in uninfected plants, with only 12 genes differentially expressed. However, during infection by *B. cinerea* more genes are differentially expressed. These genes include several genes previously linked to both defence and flowering time, such as PCC1 (Mir *et al.*, 2013). Thus PFP may serve as a regulator of both defence and flowering time.

The tobacco orthologue of PFP (NbUBR7) exhibits another mechanism by which it contributes to defence. NbUBR7 binds the N R-gene which confers resistance to viruses (Yongliang Zhang *et al.*, 2019), but this is disrupted by the TMV effector p50. It may be worth investigating if PFP acts to stabilise or destabilise R-genes in Arabidopsis, given the function of UBR7. In particular, the potential role of PFP in mediating virus infection through control of R genes would be interesting to evaluate. While PFP has been shown to have a role in regulating defence against the biotrophic pathogen *H. arabidopsidis* in chapter 3, it would be interesting to see if this role extends to viral pathogens. PFP does not appear to stabilise or destabilise JAZ6. This contrasts with the stabilising effect KEG has on JAZ12 (Pauwels *et al.*, 2015). JAZ12 is bound and ubiquitinated by KEG, destabilising JAZ12 when there are high concentrations of abscisic acid.

In another sense, the control of disease resistance by PFP may hint to links between some level of functional conservation between PFP and the other closely related UBR proteins such as PRT6. As the N-end rule proteins such as PRT6 are known to have established roles in disease resistance, there may be some potential mechanism for PFP to affect the N-end rule pathway, despite no confirm affinity for an N-terminal moiety itself. Conversely, the role of PFP in controlling disease resistance may reflect one possible way in which the other N-end rule pathway proteins are able to affect disease resistance, by mediating jasmonic acid responses, potentially through binding to other JAZ proteins.

5.6 Potential agricultural impact

The presence of JAZ proteins in species other than Arabidopsis raises the possibility of editing the growth-defence balance in commercial crop species. This has already begun with the targeting of a tomato *JAZ* gene to improve resistance to bacterial speck disease (Ortigosa *et al.*, 2019), and further characterisation of JAZ genes in crop species will permit the improvement of a greater diversity of crop plants.

Mutating both a *JAZ* gene and the orthologue of *PFP* in crop plants could lead to control of both flowering time and disease resistance, which could help complement current work into

CRISPR gene editing in lettuce to modify flowering, which has demonstrated a conserved role for the flowering regulator FT (Chen *et al.*, 2018). This would be particularly useful in crop improvement as flowering time is crucial in the development of many crops and can contribute to crop yield (Blümel, Dally and Jung, 2015). This is especially true for any kind of horticultural or agricultural production where flowers, fruits, and seeds are harvested.

An initial barrier to translating knowledge of *JAZ6* to a crop plant would be finding a *JAZ* gene with a corresponding role, as shown in Chapter 4 *JAZ* genes often display many-to-many orthology, though clear syntenic groupings can be identified. As such, it may be useful to ectopically express *JAZ* genes from the target crop plant in Arabidopsis *jaz6* mutants, and evaluating their function based on to what degree wild type disease resistance and gene expression are rescued.

In light of the current restrictions on gene editing in Europe, even variants of CRISPR methods which do not add material to the genome, it is a long road ahead for getting approval for any CRISPR edited plants regulatory approval in view of perceived controversies regarding the safety of the genetically modified plants (Smyth *et al.*, 2021). However, as evidenced with *Camelina sativa* varieties with engineered secondary metabolism to produce long chain omega-3 oils, in the UK at least new rules have enabled field trials to proceed (Clarke, 2022; Han, Silvestre, *et al.*, 2022). As such, there is reason to be optimistic about the implementation of disease resistant crops harbouring mutations in *JAZ6* in crop plants in the field.

However, starting such field trials is not the end of the challenges facing the deployment of a new genetically modified crop variety. One reason to undertake such field trials is that the conditions plants grow under are not the same as controlled laboratory conditions. This can result in significant differences in the results intended, and the results obtained, from making such modifications in a crop plant. One such example was wheat designed to release E-beta-farnesene to repel aphids, which did not occur under field conditions (Bruce *et al.*, 2015). Laboratory results with *B. cinerea* may similarly not translate to field conditions, one potential explanation would be that the 'pepper' isolate used in this work does not fully

reflect the isolates currently infecting crop plants, particularly in light of recent evolutionary pressure on *B. cinerea* to adapt to fungicides (Rupp *et al.*, 2017). Another possibility would be that under field conditions B. cinerea infection is coincident with other pathogens or herbivores, so manipulating gene expression through mutating *JAZ6* and *PFP* may have overall negative effects considering the importance of crosstalk when facing multiple pathogens and herbivores (Vos *et al.*, 2015).

Even if crop plants with mutations in *JAZ6* are proven in field trials, it is unknown how long resistance would last before it could be overcome by evolution of *B. cinerea*. For example, the Lm6 *R*-gene only provided resistance to the fungus *Leptosphaeria maculans* in *Brassica napus* in the Eurol host background for 3 years, while resistance was not overcome in the Darmor host background (Brun *et al.*, 2010). The difference between the backgrounds was quantitative resistance was only present in the Darmor host background, so quantitative resistance provided by a *JAZ6* mutation may enable longer lasting resistance when combined with an R gene (Brun *et al.*, 2010). While gene stacking may be a valid approach to impede *B. cinerea*, as previously demonstrated in blight resistant potato, this would require more complementary targets to work with (Jo *et al.*, 2014).

And finally, it would be difficult to convince farmers to grow such plants if there is any kind of yield disadvantage in crops with mutations in *JAZ6*, which could occur due to the growth-defence trade-off, particularly as JAZ proteins mediate the trade-off (Li *et al.*, 2022).

5.7 Priority future work

Determining the content of protein complexes with JAZ6 and PFP is a key expansion of this work. These protein complexes could be investigated using tandem affinity co-immunoprecipitaiton with tagged JAZ6 and/or PFP in Arabidopsis. Stable Arabidopsis lines expressing GFP tagged JAZ6 have recently been made (Wu *et al.*, 2020), and would provide appropriate material for these protein binding experiments. Other alternatives for plant material would be transiently transforming Arabidopsis with JAZ6-GFP or HA-PFP, as performed for transient expression in *N. benthamiana* in this work. Alternatively, CRISPR could be used to edit GFP or HA tags onto JAZ6 and PFP expressed in a native context (Miki

et al., 2018). Further molecular characterisation of the lettuce JAZ proteins by these techniques would advance the understanding of functional conservation of JAZ proteins in crop species.

5.8 Conclusions

Specific objectives were to:

- Predict circadian regulation of JAZ6 (Chapter 2).
- Validate novel protein-protein binding partners of JAZ6, and the specificity of this binding (Chapter 2).
- Examine the role of these novel protein-protein binding partners of JAZ6 involved in plant defence responses (Chapter 3).
- Elucidate the mechanisms of JAZ6 controlling plant defence (Chapter 3).
- Identify JAZ orthologues in lettuce and investigate their potential roles in plant pathogen defence (Chapter 4).

This study elucidated the mechanisms which regulate JAZ6, and how JAZ6 regulates defence. There is evidence for transcriptional regulation of JAZ6 by the evening complex, and post-transcriptional regulation of JAZ6 by JA levels which fluctuate throughout the day. JAZ6 protein-protein binding to PFP was validated in yeast and *N. benthamiana* by three independent techniques. Novel roles were found for *JAZ6* in regulating the transition to flowering in short day conditions, and resistance to biotrophic pathogens. *JAZ6* was found to regulate many genes important in plant defence, notably plant defensins. *PFP* was found to regulate the expression of fewer genes in plant defence, and many of the genes regulated by *PFP* were affected by *JAZ6*. Nine JAZ proteins in lettuce were identified, and their responses to biotic and abiotic stress were characterised as products of cis-regulatory elements conserved between Arabidopsis and lettuce.

Despite the complementarity of JAZ proteins, this work establishes novel roles for JAZ6 and a novel interactor of JAZ6, for which orthologues in crop species are potential targets for

genetic engineering for crop improvement. A major outcome of this work is the verification and characterisation of a novel gene involved in plant defence: PFP.

Many questions remain as the mechanisms of JAZ6 action. These include the reason behind the specificity of regulation of circadian susceptibility to disease by JAZ6, when JAZ5 is highly similar but is not necessary. Also, the functional role of the interaction between JAZ6 and PFP, as they interact additively with respect to flowering time and disease resistance. And it is yet to be determined if the lettuce JAZ genes show functional conservation with Arabidopsis JAZ proteins. Answering these questions will advance our understanding of the mechanisms behind plant defence, which will be vital in the pursuit of improving crops for food security.

Appendices

Appendix 1: Molecular cloning verification PCR primers used in this work:

Primer	Primer sequence
NAC10 cDNA F	TGTGTAACGAGCCGTCCAGATT
NAC10 cDNA R	ACCACCATGCTCATACCCGAAA
WOX12 cDNA F	TGGTCACCTAAACCGGAGCAAA
WOX12 cDNA R	TGGGAAGAGGAAGACCAGAGGA
EIN3 cDNA F	CCCGAGTCATGTCCACCTCTTT
EIN3 cDNA R	CTGTGGGTTGAAGCAGTGACAC
Mybl cDNA F	TGGTCTCGCCCATAAAACACCT
Mybl cDNA R	CAAGATCGAGTCCACCGGAGTT
PHD cDNA F	CCGACCTTACCCTGACCCAAAT
PHD cDNA R	GGCCAGGTTCTGAGTCACTGAT
ZF1 cDNA F	CTTCCTCCTCTACCGTCACGTG
ZF1 cDNA R	GTCACTACCAGCGACGAGTTCT
FHY3 cDNA F	AGTTGGCCCCATTAAGTTCAG
FHY3 cDNA R	TTTGGCTGGGATGTCTACATC
JAZ6 cDNA F	ATGTCAACGGGACAAGCGC
JAZ6 cDNA R	CTAAAGCTTGAGTTCAAGGT
JAZ1 cDNA F	GGCAACTCACGTCAGCCGACAA
JAZ1 cDNA R	CGAGCCACGACATGTTGCCTGT
JAZ8 cDNA F	TGGAACTTCGTCTTTTTCCCACT
JAZ8 cDNA R	GGAGGATCCGACCCGTTTGAGG
YFP NREV I	GCTTCATGTGGTCGGGGTAG
YFP CREV I	GTCGCCGATGGGGGTGTTCTG
YFP NFOR II	CAAGGACGACGGCAACTACAA

YFP CFOR II	AGTCCGCCCTGAGCAAAGA
M13 F	GTAAAACGACGGCCAG
M13 R	CAGGAAACAGCTATGAC
attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCT
attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT
35S 835 F1	TCAGAAAGAATGCTAACCCACAG
35S 1028 F1	CCGGAGATCACCATGGACGACTT

Appendix 2: Genotyping PCR and qPCR primers used in this work:

Primer	Primer sequence
SAIL LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC
WT JAZ6 F	TCGGATTTTCGAGCCAACCC
WT JAZ6 R	GGACACACATCACTGTCACTTC
KO JAZ6	TTTGCAAATGCCCTCATTTAC
SALK LBb1.3	ATTTTGCCGATTTCGGAAC
PHD F	GTACACCAGAGGGGAATGCT
PHD R	ATAACACAACCAGCTGCAGGA
UBQ11 qPCR F	TTCATTTGGTCTTGCGTCTG
UBQ11 qPCR R	GAAGATGAGACGCTGCTGGT
JAZ6 qPCR F	ACAGGGCTGTGGCTAGAG
JAZ6 qPCR R	CTTTCTTGTCCACCTCCATC

Abbreviations and Glossary

35S	Cauliflower mosaic virus promoter
3D RNA-seq	Three day RNA-sequencing analysis
ABA	Abscisic acid
bp	Base pair
B. cinerea	Botrytis cinerea
BiFC	Bimolecular fluorescence complementation
BiFP	BIFC in Planta
BSA	Bovine serum albumin
bZIP	Basic leucine-zipper protein
CaMV	Cauliflower mosaic virus
CATMA	Complete Arabidopsis Transcriptome MicroArrays
cDNA	complementary DNA
CDS	Coding DNA sequence
ChIP	Chromatin immunoprecipitation
Co-IP	co-immunoprecipitation
COI1	CORONATINE-INSENSITIVE 1
Col-0	Arabidopsis thaliana Columbia ecotype 0
CT	Circadian time
DAMP	Damage-associated molecular pattern
DAS	Days after sowing
DE	Differentially expressed
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
DREB	DREHYDRATION - RESPONSIVE ELEMENT BINDING PROTEIN
E. coli	Escherichia coli
EIN3	Ethylene-insensitive3
EIL1	Ethylene-Insensitive3-Like 1
ERF	Ethylene response factor
ET	Ethylene
ETI	Effector Triggered Immunity

FT	FLOWERING LOCUS-T
GA	gibberellic acid
GFP	Green fluorescent protein
H. arabidopsidis	Hyaloperonospora arabidopsidis
HPI	Hours post-inoculation
HR	Hypersensitive response
IAA	Indole-3-acetic acid
JA	Jasmonic acid
JAZ	Jasmonate ZIM-domain
KO	Knockout mutant
L. sativa	*Lactuca sativa*
LB	Lysogeny broth
LD	Long day
limma	Linear models for microarrays
MAMP	Microbe-Associated Molecular Pattern
MAPK	Mitogen-activated protein kinase
MS	Murashige & Skoog
MS	Mass spectrometry
NASC	Nottingham Arabidopsis Stock Centre
N. benthamiana	*Nicotiana benthamiana*
NB-LRR	Nucleotide binding leucine rich repeat
OE	Overexpressor mutant
ORA59	octadecanoid-responsive AP2/ERF 59
ORF	Open reading frame
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PDF1.2	PLANT DEFENSIN 1.2
PIF	PHYTOCHROME-INTERACTING FACTOR
PPI	Protein-protein interaction
P. syringae	*Pseudomonas syringae*
PRR	Pattern recognition receptors

PTI	PAMP-triggered immunity
RGL2	RGA-LIKE2
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
RT-PCR	Reverse transcription PCR
SA	Salicylic acid
SD	Short day
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOC	Super optimal broth with catabolite repression
UBQ11	Ubiquitin 11
UTR	Untranslated region
Voom	Mean-variance modelling at the observational level
WT	Wild-type
Y2H	Yeast two-hybrid
YFP	Yellow fluorescent protein
ZT	Zeitgeber time

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