The role of genes from several different plant defence pathways in resistance to the Fusarium Head Blight causing pathogen, *Fusarium graminearum* in Arabidopsis thaliana

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Contents

Abstract

Fusarium head blight (FHB) causes major crop loss to small grain cereal worldwide. Attempts to breed FHB resistant cultivars have so far been unsuccessful. Expected climatic changes are expected to increase FHB severity paving the way for research into what causes susceptibility. Research has found arthropod activity leads to increased FHB incidence. Here we test *Fusarium graminearum* resistance both with and without arthropod activity. Knock-out mutants were used to examine the resistance of specific genes found within the plant defence pathway. A total of 22 genes were presented by a Multi-Omnics Factor Analysis (MOFA) in relation to causing increased FHB susceptibility. We used the pathogen *Fusarium graminearum* to test resistance of Arabidopsis thaliana plants as well as undertaking gene expression analysis of wheat plants subject to both aphid and *Fusarium graminearum* treatments. In Arabidopsis *Fusarium graminearum* susceptibility was increased due to aphid infestation. The use of mutants enabled specific genes to be associated with either susceptibility of resistance as well as confirming plant hormone signalling pathways. We propose a new interaction between genes found in jasmonic acid signalling (ORA59) and ethylene signalling pathways (ORE1). Further work is required to confirm this pathway including the use of mutants of these two genes.

Fusarium Head Blight- literature Review

Introduction

Fusarium species cause widespread diseases amongst all members of the *Gramineae* family especially in small grain cereals, most notably Wheat plants (Parry et al., 1995). Fusarium head blight (FHB) affects small grain cereals such as wheat and barley, reducing grain quality and yield (Wegulo et al., 2015). The fungal disease negatively impacts the agricultural industry, causing annual losses in excess of 1 billion dollars globally, of which the majority of losses are associated with wheat (Wegulo et al., 2015). Crop losses include lower yield through shrivelled and underdeveloped grains as well as reduction in quality of the grains. Grain quality is diminished through the build-up of mycotoxins (deoxynivalenol and zearalenone), causing grain to become toxic for human and animal consumption (Moonjely et al., 2023).

There are seventeen organisms known to cause FHB, however, five species dominate on cereals in temperate climatic regions, these are; *Fusarium culmorum*, *Fusarium avenaceum*, *Fusarium graminearum*, *Fusarium poae* and *Microdochium nivale* (Parry et al., 1995). Xu et al. (2008) found these species are associated with different climatic conditions. Here we focus on *Fusarium graminearum*, known to prefer warmer/humid conditions.

FHB epidemics of varying intensities have become more common especially in the United States as favourable weather conditions have emerged (Wegulo et al., 2015). Climate change, attributed to changing weather patterns, which is leading to favourable conditions for FHB infection especially around the time of infection (Madgwick et al., 2011). The timing when wheat is most susceptible to FHB infection is identified as Mid anthesis (Miller, 1995), or more specifically growth stage 65 (J.C. Zadoks et al., 1974). Changes in climatic conditions has also been found to increase plant development and some studies suggest the date of anthesis has been bought forward by up to two weeks, further increasing FHB risk (Madgwick et al., 2011).

Unlike other fungal disease of agricultural crops chemical control of FHB is challenging due to the emergence of fungicide tolerant strains of *F. graminearum* as well as a lack of highly resistant wheat varieties (Moonjely et al., 2023). Interestingly, research has found that arthropod interaction with *F. graminearum* can double disease progress. The exact reason behind the increase in infection is not fully understood but it is thought to be either secondary spore dispersal, herbivory actions leading to wounding sites or post-harvest spread of infection between grains (Drakulic et al., 2016).

Changing climatic conditions bring new challenges in controlling FHB outbreaks. Changes in temperature and water availability will cause stress among plants, altering hormonal response and potentially lowering response to infection leading to increased disease severity. It has been reported that warmer night-time temperatures lead to increased mycotoxin accumulation within the grain, specifically deoxynivalenol (Martínez et al., 2022). Highlighting the need for finding genes associated with *F. graminearum* defence.

Sources of Inoculum

The primary source of FHB inoculum arises from the stems and roots of Gramineae plants (Osborne and Stein, 2007). *Fusarium graminearum* species can overwinter saprophytically (Parry et at., 1995) with crop debris considered to be the main source of inoculum (Parry, et al., 1995, Edwards, 2004). Fusarium species can survive and overwinter on colonised crop residue as mycelium (Osborne and Stein, 2007) or as spores (chlamydospores) in soil (Kazan and Gardiner, 2018) (Figure 1). Ascospores develop in spring on crop debris (Vaughan, et al., 2016). These ascospores are forcibly ejected from mature perithecia (Wegulo et al., 2015) in the presence of rain and or wind (Vaughan, et al., 2016)(Keller, et al., 2013). Conidia are produced during wet, warmer conditions and are rain-splash dispersed vertically onto the canopy layer until susceptible heads are reached. The wind dispersed ascospores have been reported to be carried many kilometres from its source before deposition onto susceptible hosts takes place either by gravity or via raindrops during rainfall events (Keller, et al., 2013).

Infection

Infection occurs when ascospores, or conidia (Manstretta et al., 2015) are deposited on anthers where, in the presence of moisture they germinate before entering the florets (Audenaert et al., 2009) (Kheiri et al., 2018). Hyphae can also penetrate through natural openings such as the stomata (Kheiri, et al., 2018). *F. graminearum* initiates biotrophic growth within 12 hours post inoculation (hpi) before switching to necrotrophy from 36 hpi (Brown et al., 2010). The pathogen hyphae secrete a diverse range of enzymes specifically to degrade cell walls in order to enter and absorb nutrients from the plants (Wanjiru, et al., 2002). *F. graminearum* is known to be the most prevalent casual agents in causing FHB infection (Moonjely et al., 2023).

The fungus is capable of developing a dense mycelium in order to invade the lemma, glume, palea and ovary (Kang and Buchenauer, 2002), from here the fungus grows intercellularly through the pith and xylem (Chen, et al., 2022). The spikelet's cells are degraded before infecting other spikelet's through the vascular bundles of the rachilla and rachis (Kheiri, et al., 2018). Fungal growth restricts plat growth in the ear beyond the middle rachis due to the vascular cambium becoming blocked (Chen, et al., 2022). The lifecycle of FHB causing pathogens is illustrated in Figure 1.

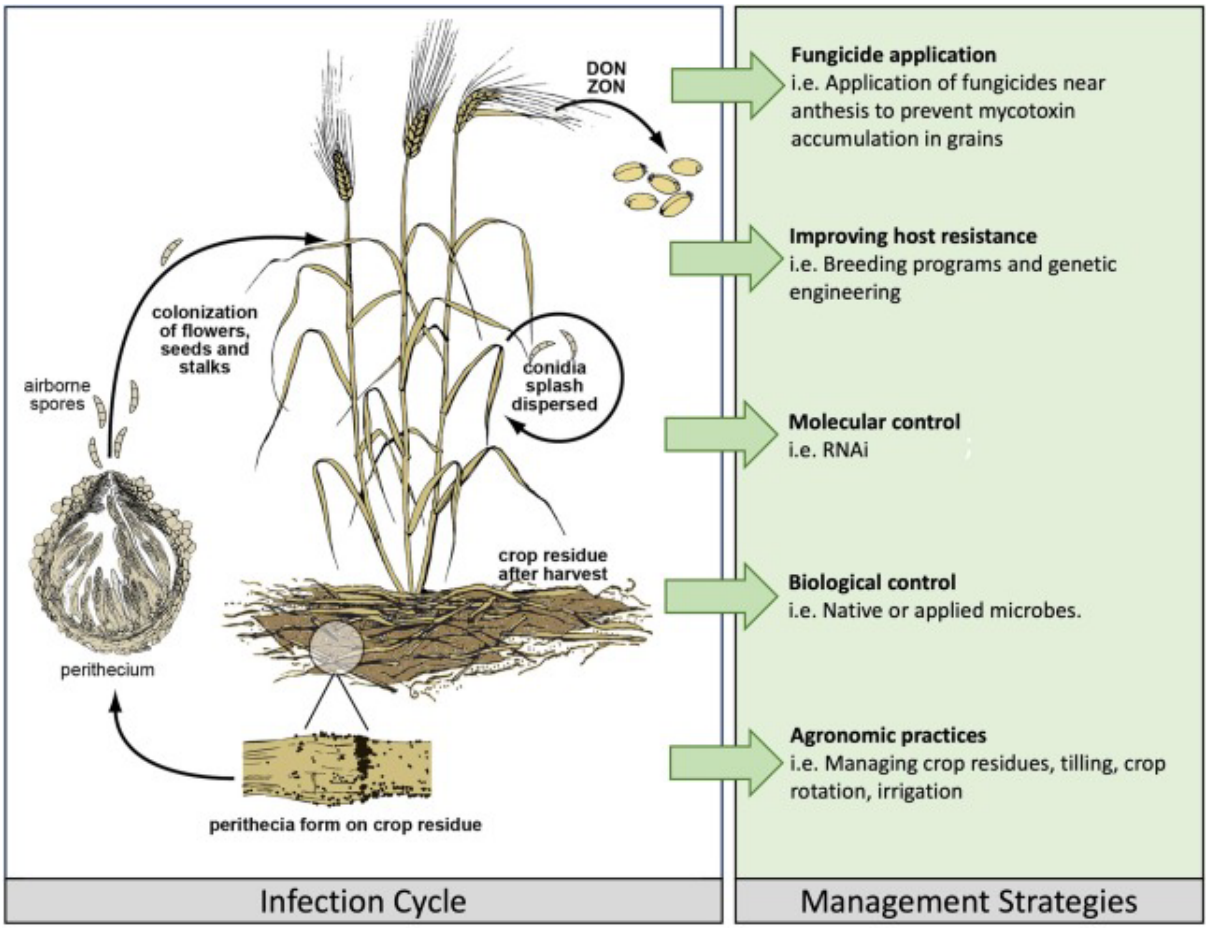

Figure 1; Diagram illustrating the infection cycle of FHB, from over wintering on crop residue to infection of small grains. Diagram taken from (Moonjely et al., 2023). Also illustrated is the management strategies which are explained later in this review.

Symptoms

Initial symptoms of FHB include dark brown, water-soaked spots on the glumes of the infected florets (Kheiri, et al., 2018), glumes may appear shrivelled, if growth is inhibited glumes beyond the infection site may not be developed (Chen, et al., 2022) and infected spikelet's can be completely blighted (Kheiri, et al,. 2018). Infected spikelets turn a pink/orange colour due to asexual fruiting structure from the fungus (Chen, et al., 2022). Inoculum is found on crop debris in black coloured perithecia most noticeable on matured wheat spikes (Chen, et al., 2022).

Effects on yield grain and seed quality

Yield losses due to FHB infection can reach 50-70% in severe cases (Mengesha et al., 2022). The losses stem from lower thousand grain weights (Moradi et al., 2010), as a result of the sterile shrivelled grains or 'tombstones' formed from the discoloured grains which are unsuitable for milling, baking, and malting (Wegulo et al., 2015). This pathogen not only results in the heads of wheat plants becoming discoloured but a detrimental effect on grain yield is also observed (El Chami et al., 2022).

The biggest detrimental affect caused by the fungus is due to the mycotoxin accumulation occuring in the grain of infected heads (Freije and Wise, 2015). These mycotoxins which include deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) pose high health risks to both humans and livestock (Brandfaß and Karlovský, 2006). These mycotoxins are secondary metabolites and are water soluble and can translocate between tissues, leaching can also occur from source tissue (Osborne and Stein, 2007). DON is the most common of the mycotoxins, produced predominantly by *F.graminearum* and *F.culmorum* (Edwards, 2004). Infected grains lose quality through loss of albumin and gluten proteins (Brown et al., 2010) resulting from inhibition of protein synthesis by DON (Freije and Wise, 2015).

The use of infected grain for seed is advised against as not only would the seed offer a primary source of inoculum, but the germination is likely to be lower (Parry et al., 1995). The infected grain, post-harvest, will be degraded as the starch granules, storage proteins and the cell walls are destroyed (Parry et al., 1995).

Resistance

As with many fungal diseases host resistance is the most effective and sustainable strategy in disease control. However, research suggests that no wheat cultivar is immune, most are susceptible, but a few are moderately resistant (Parry et al., 1995). This is despite the wheat cultivar 'Sumai 3' showing the greatest degree of FHB resistance (Bai and Shaner, 2004). The resistance shown by 'Sumai 3' stems from a quantitative trait loci, *Fhb1* which limits the fungal spread from the initial infection site (Sarowar et al., 2019). Breeding work has been centred around 'Sumai 3' but success has been limited due to the variation in pathogenicity of the fusarium isolates (Parry et al., 1995).

Varietal characteristics have been found to aid disease escape with genotypes having dark green heads and leaves being more susceptible. This is the same for genotypes which maintained green heads for longer (Liu and Wang, 1991). Early maturing cultivars were shown to escape disease owing to the grain becoming increasingly harder for penetration via the hyphae (Parry et al., 1995). However, more recent models predict that the effects of climate change will bring forward the date of wheat anthesis and as a result FHB incidence will increase and suggests the incidence of FHB is related to rainfall during anthesis and temperature during the preceding six weeks (Madgwick et al., 2011).

In 1963 Schroeder and Christensen stated there were two components of genetic resistance to FHB, 'Type I' and 'Type II' (Parry et al., 1995). Type I resistance is the resistance against initial infection whereas Type II resistance is resistance to the spread of the pathogen within the host (Semagn et al., 2023). Miller and Arnison (1986) proposed a 'Type III' resistance where the plant is capable of degrading the DON which has accumulated within the grain. Additionally, the same authors proposed a 'Type IV' and 'Type V' which corresponds to tolerance to high levels of DON and resistance to yield reduction, respectively. Type II resistance is regarded as the most effective whereas Type III and Type IV decreased post-harvest losses (He et al., 2016). Type II and Type III have been the most extensively covered due to their association with yield loss and food safety concerns (Wu et al., 2022).

FHB resistance is quantitative and controlled by several quantitative trait loci (QTL) (Bai and Shaner, 2004), for which hundreds have been reported (Buerstmayr, et al., 2009). Seven of the QTL's have been designated to the FHB resistance, complications arise around phenotyping owing to the huge hexaploid wheat genome (Wu et al., 2022).

Control

As agricultural techniques have modernised away from traditional techniques pathogen survival is enhanced (Osborne and Stein, 2007). The agricultural industry is moving away from removing debris via burning or ploughing and more consideration is taken towards soil management. Minimum tillage practices have replaced burning and ploughing of fields post-harvest, this practice has been found to increase FHB incidence (Wegulo et al., 2015). The high value of susceptible crops including maize and wheat has incentivised farmers to increase production, but this has led to shorter rotations (Na et al., 2021) often with maize and wheat grown consecutively, leading to increased inoculum present. Crop rotations with non-susceptible hosts will help to decrease the level of inoculum by depriving the pathogen of suitable substrate for survival and reproduction.

The requirement to feed a growing population has led to the reliance upon inorganic nitrogen fertiliser (Lebender et al., 2014). The impact of inorganic fertiliser, designed to increase crop output can lead to increased FHB incidence through excess nitrogen rich organic matter (Bernhoft et al., 2012). However, some studies counter this by suggesting the use of inorganic fertiliser results in reduced FHB due to a having a healthier plant (Parry et al., 1995). The addition of nitrogen fertiliser leads to increased leaf production which increases water demands from the plants due to increased transpiration rates (Umnajkitikorn et al., 2021). Water stress in susceptible plants is thought to increase FHB incidence (Ferrigo, et al., 2016) leading to explanations why fertilisers cause increased FHB incidence. Irrigation offers a chance to reduce water stress and thus reduce FHB. Irrigation could however aid ideal conditions for FHB spread as a result of increasing humidity as well as attributing to rain-splash spread on inoculum and therefor the effectiveness of irrigation remains unclear (Parry, et al., 1995).

There are no management techniques capable of completely suppressing FHB (Freije and Wise, 2015), however, agronomic traits have found to be strongly linked with FHB resistance (Suzuki, Sato and Takeuchi, 2012). These traits alongside management decisions can have a strong role in controlling FHB. Mesterházy (1995), found that dwarf wheat varieties were severely infected, suggesting plant height has a role in severity of FHB outbreaks owing to the rain splash spread nature of FHB. The same is apparent for ear architecture, Jones et al. (2018) found that the use of an awned wheat variety had no correlation to FHB incidence despite suggestion awns aided the infection process. The authors here found spikelet density to favour disease development within the ear.

Wheat yields are strongly associated with the semi dwarf wheat cultivars which enable production of more fertile tillers and higher grain yields than taller wheat varieties (Daoura et al., 2014). These yield boosting traits have a strong correlation with FHB. Mesterházy

(1995), found that dwarf wheat varieties were severely more infected, suggesting plant height has a role in severity of FHB outbreaks. Tiller numbers have also been found to increase the severity of FHB infection due to increases in plant density (Jones et al., 2018). Despite higher tillers numbers leading to higher rates of infection, grains from the tillers have been shown to have lower DON accumulation as a result of delayed development of the ears in comparison to the main stem (Gautam, Halley and Stein, 2012).

Jones et al. (2018) also found a longer flag leaf aided spread of FHB as a result of more frequent contact between leaves and ears of neighbouring plants which aided horizontal spread. This morphological trait though is vital to final yield with a positive correlation between flag leaf size and thousand grain weight, panicle weight as well as other yield determining traits (Liu et al., 2018). This makes breeding for a smaller flag leaf to boost FHB control unrealistic.

Weed control has shown to have impacts on FHB infections. Edwards (2004) found FHB incidence to be greater in the presence of high weed populations. Weeds varying form grassweeds (Lager and Wallenhammar, 2003) and broadleaf weeds (Jenkinson and Parry, 1994) have been found to harbour fusarium species. Some studies have shown no correlation between weed densities and FHB (Teich and Hamilton, 1985), this has led to some debating the efficacy of weed control in reducing FHB (Parry, Jenkinson and McLeod, 1995).

Chemical control against FHB is limited with research showing mixed results. Some triazoles applied at mid-anthesis led to a 52% reduction of incidence (Blandino et al., 2006) leading to a decrease in FHB and DON accumulation (Edwards et al., 2001). Some research suggests strobilurins can cause increases in DON levels due to activity of the strobilurins on microorganisms within the wheat ear (Pirgozliev et al., 2003). Despite fungicides having an observed reduction in FBH the mycotoxin levels within the grain may not necessarily decline (Edwards, 2004).

Overall, the majority of research applauds ‘good agricultural practice’ as the main form of control against FHB (Mielniczuk and Skwaryło-Bednarz, 2020) (Edwards, 2004). A combination of the choice of cultivar and good weed control may offer the best form of control, however environmental factors also play a role in FHB incidence. Suzuki et al., (2012) found lower levels of sunlight during the growing season as well as lower temperatures between ear emergence and anthesis increased the level of DON in grains.

Role of insects in FHB epidemics

Literature is limited in investigating the role of insects in FHB infection (Edwards, 2004). Alternate literature highlights how insects such as mites carry spores of fusarium (Parry et al., 1995) (Miller et al., 1998). The impacts of insects on FHB work similarly to water stress. The presence of insects increases the stress within the plant which can facilitate fungal infection and mycotoxin accumulation (Ferrigo et al., 2016). This risk of insect induced stress peaks during the reproductive stage (Ferrigo et al., 2016) highlighting how FHB poses its greatest infection threat during anthesis. Stress is induced, as well as insects providing wound sites for which the fusarium conidia utilise as entry points (Avantaggiato et al.,

2002). Two fusarium causing species, *F.langsethiae* and *F. graminearium* have shown to spread quicker and produce more toxins under insect infestation (Drakulic et al., 2015). Furthermore Drakulic et al., (2015) found that the longer the period of aphid colonisation prior to inoculation with *F. graminearum* , there was a greater amount of pathogen DNA accumulated.

The period during which wheat is susceptible to FHB coincides with increased activity grain aphids (*Sitobion avenae*are) (Drakulic et al., 2015). The attractiveness of the wheat to the aphid can be influenced by volatiles released from the plant (De Zutter et al., 2012). The volatile emissions are altered by both abiotic and biotic stress. In the case of FHB once infection has occurred, the plant becomes less attractive to the English grain aphid. The production of secondary metabolites such as DON can alter the ability of feeding and reproduction stages of aphids (Drakulic et al., 2015). The same authors also found the presence of *F. graminearum* decreased the fitness of the aphid to the extent that aphids had a significantly higher mortality rate (59.4%) when feeding had taken place on FHB infected ears compared to FHB free ears (36.5%). Presence of aphids had a huge effect on disease levels and consequent mycotoxin levels, a 5-fold increase in DON accumulation occurred in infected plants when aphids were present. Host plants will also release volatile organic chemicals (VOCs) which will differ dependent of the health status of the plant. It is believed that due to mortality upon feeding on *F. graminearum* infected plants the aphids have developed a behavioural adaptation in order to evade infected plants due to the release of VOC's (Drakulic et al., 2015).

F. langsethiae and *F. graminearium* have been shown to spread quicker and produce more toxins under insect infestation (Drakulic et al., 2015). Furthermore Drakulic et al., (2015) found that the longer the period of aphid colonisation prior to inoculation with *F. graminearum*, there was a greater amount of pathogen DNA accumulated. The aphids were not believed to carry inoculum for FHB but the presence of aphid feeding damage increased the spread of the disease as well as enabling pathogen colonisation (Drakulic et al., 2015). Research by Bagga (2008) highlighted the importance of aphid control as this research in India found that FHB incidence decreased by up to 31% when insecticides were used to control aphid populations. This further highlighted the impact in which aphids have on FHB incidence and severity.

Arthropod activity and its effect on FHB susceptibility is interesting due to potentially having both direct (spore dispersal) and indirect (plant damage) impacts on susceptibility to FHB. The presence of insects can increase the stress within the plant which can facilitate fungal infection and mycotoxin accumulation (Ferrigo et al., 2016). This risk of insect induced stress peaks during the reproductive stage (Ferrigo et at., 2016) highlighting how FHB poses its greatest infection threat during anthesis. In addition to increasing stress on the host, insects can provide wound sites for which the fusarium conidia utilise as entry points (Avantaggiato et al., 2002). Host weakening by arthropods as well as the alteration of plant defence pathways both play indirect roles of increasing FHB susceptibility. Importantly a negative cross talk between the SA pathway and the JA pathway could explain how plant defence may be reduced due to arthropod activity (Drakulic et al., 2016)

Hormonal regulation of defence to herbivores and *Fusarium graminearum*

Plant hormones play a significant role in plant immunity especially abscisic acid (ABA) (Cao, Yoshioka and Desveaux, 2011). Research shows ABA can increase the susceptibility of *fusarium oxysporum* in arabidopsis (Anderson et al., 2004). However, ABA does influence defence against necrotrophy and biotrophy by enhancing jasmonic acid (JA) signalling and suppressing salicylic acid (SA) pathways (Torres-Zabala et al., 2007). It has been confirmed that JA signalling is associated with FHB resistance (Xiao et al., 2013). The timing of SA activation has shown to be highly relevant in FHB resistance with an early activation associated with FBH resistance (Wu et al., 2022). SA works by reducing germination and growth efficiency of *F. graminearum*. Makandar et al., (2010) found that SA was critical in controlling *F. graminearum* infection in Arabidopsis through using mutants which were deficient in salicylic acid induction. This SA deficient mutant experienced higher *F. graminearum* disease pressure.

The Ethylene (ET) pathway is thought to be naturally activated in resistant wheat but not in susceptible cultivars (Foroud et al., 2019). *EIN2* is a central regulator of ET signalling (Ma et al., 2022) and research conducted by Chen et al., (2009) suggests mutants with EIN2 silencing reduced FHB symptoms implying importance of ET signalling in FHB susceptibility. However, Wu et al., (2022) suggested the ET signal pathway may not associated with FHB resistance. SA signalling is believed to be active early in the stages of infection followed by ET signalling and JA signalling (Ding et al., 2011).

Auxin too is thought to play a role in FHB susceptibility (Ma et al., 2022). Su et al., (2020) found during F. *graminearum* infection the auxin receptor gene, TaTIR1 was downgraded and the knockdown of this gene also increased FHB resistance.

Physical defence from the plant itself stem mainly from cuticle thickening, as well as cell wall lignification and modification (Walker et al., 2024). This acts to increase defence against cell wall degradation from enzymes released by *F. graminearum* (Kikot, Hours and Alconada, 2009). Hence why lignin biosynthesis has been of interest for researchers (Walker et al., 2024).

The small underlying FHB defence response from the SA pathway is likely to attenuated by JA induced defence pathways against insect attack. This may increase FHB susceptibility. Other ways that insects can interfere with the defence pathway is through proteins released upon feeding (Drakulic, Bruce and Ray, 2016), in particular effector molecules (Bos et al., 2010) designed to disable defence responses, and so increasing the possibility of secondary attack.

Plants have evolved and created complex defence mechanisms which are capable to rapidly initiate once changes in environment are recognised (Li et al., 2006). Plant defence mechanisms are highly sophisticated pathways which the plants use to defend itself from attack. Defence starts with preformed structural barriers on the leaf surface once the pathogen passes through the plant will recognise the pathogen attack through microbe-associated molecular patterns (MAMPs). MAMP's are then perceived by specific pattern recognition receptors (PRRs) (Jonathan and Dangl, 2006) which activates initial defence response called pathogen associated molecular pattern (PAMP) triggered immunity (Bürger

and Chory, 2019). Evolution has led to pathogens developing specialised effector proteins to overcome PAMP triggered immunity by causing interference with immune signalling in a process called effector-triggered susceptibility (ETS) (Bürger and Chory, 2019). As with pathogen evolvement plants have developed characteristics to limit ETS with plant resistance (R) proteins, (Chisholm et al., 2006) to detect pathogen effector proteins or their modifications to create a second layer of response, called effector-triggered immunity (ETI) (Bürger and Chory, 2019). Figure 2 shows the ways in which plants overcome the presence of pathogens (Bürger and Chory, 2019).

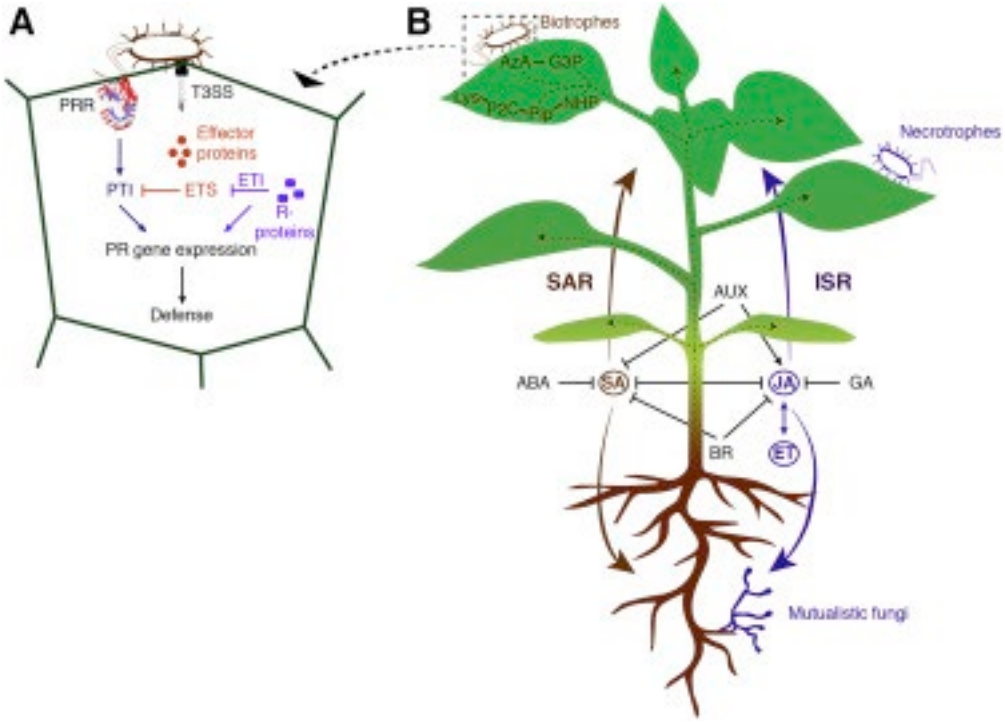

Figure 2; Plant defence response in two different methods of systemic immunity, taken from Bürger and Chory, (2019). (A) Initail defence response: Pattern recognition receptors (PRRs) which recognise microbe associated molecular patterns (MAMPs) leading to PAMP triggered immunity (PTI). Type III secretion system (T3SS) are used by the pathogenic bacteria to eject effector proteins causing effector triggered susceptibility (ETS). The plant can overcome this with R proteins leading to effector triggered immunity (ETI).
(B) Systemic acquired resistance (SAR) is established against biotrophic pathogens and is controlled by salicylic acid (SA) (left hand side in brown). Induced systemic resistance (Right hand side in blue) is maintained by jasmonic acid (JA) and ethylene (ET).
After initial infection plant response depends upon the nature of the pathogen, biotrophic activity results in SA activation whereas necrotrophic pathogens activate defence response via JA signalling (Bürger and Chory, 2019). However, these defence pathways are known to work antagonistically with each other with the induction of one leading to suppression of the other (Ullah et al., 2022). Other plant hormones such as ABA, ET, gibberellins (GA) and auxin (AUX) fine tune the defence pathway (Bürger and Chory, 2019).

Salicylic acid (SA) defence

As previously stated, SA is important in defence against biotrophic pathogens (Bürger and Chory, 2019) and produce defensive properties against hemitropic pathogens (Kunkel and Johnson, 2021) (Fernandes and Ghag, 2022). And as such provides basal resistance against *F. graminearum* (Makandar et al., 2012). SA is derived from the metabolite chorismite and preferably produced via the isochorismate pathway (Wildermuth et al., 2001).

During infection SA accumulates in the infected leaf (Bürger and Chory, 2019) some research has shown that SA in the infected leaf peaked three hours post infection (Makandar et al., 2012). Once in the leaf SA is converted to its volatile derivative methylsalicylate (Fernandes and Ghag, 2022). SA accumulation within the leaf is perceived by the non-expressor of pathogenesis related 1 (NPR1) gene (Fu et al., 2012) which is the main regulator of SA signalling (Bürger and Chory, 2019). NPR1 monomers localise in the nucleus, dimerize, and interact with TGA transcription factors which bind to that pathogenesis related (PR) gene promoters resulting in activation of PR-1 and other defence genes (Fernandes and Ghag, 2022).

Cross talk between hormones is significant in the SA defence pathway as previously mentioned SA has an antagonistic relationship with JA. SA however, does work synergistically with ET (Fernandes and Ghag, 2022). Both JA and ET as well as ABA work to regulate SA (Zander, et al., 2014).

Jasmonic acid (JA) defence

Differing from SA, JA works to defend against necrotrophic pathogens (Bürger and Chory, 2019) as well as helping in wound response (Turner et al., 2002). The time period between inoculation and hormone response for JA was found to be slower than SA. Makandar et al., (2012) found that JA peaked 12 hours post infection. Upon infection, signals trigger a phosphorylation cascade leading to JA biosynthesis (Carvalhais et al., 2013).

JA is synthesised upon the release of a systemin, a polypeptide signal molecule consisting of 18 amino acids (Ruan et al., 2019). Systemin is hydrolysed from prosytemin, from here systemin is transported via the apoplast with the cell surface receptor (SR160) to activate JA signalling (Ruan et al., 2019). JA signalling can also be activated by oligosaccharides of which the signalling pathway is similar to systemin (Stratmann and Ryan, 1997).

Later in the infection process the polypeptide AtPEP1 is again hydrolysed from the protein PROPEP1. The AtPEP1 attaches to the receptor PEPR1 on the plasma membrane which will activate JA signalling (Ruan et al., 2019).

In *Arabidopsis thaliana* there are three synthesis pathways each in different reaction sites: chloroplast, peroxisome and cytoplasm (Ruan et al., 2019) required for synthesis of jasmonates. In the chloroplast 12-oxophytodienoic acid (12-OPDA) or deoxymethylated vegetable dienic acid (dn-OPDA) are synthesised from unsaturated fatty acids which are then converted to JA in the peroxisome (Ruan et al., 2019). In the cytoplasm however, a range of chemical reactions takes place to produce a class of fatty acids and are collectively known as jasmonates (JAs) such as methyl ester (MeJA) and isoleucine conjugate (JA-Ile) (Ruan et al., 2019).

As well working antagonistically with SA however, this crosstalk does lead to the production of defence related genes (Fernandes and Ghag, 2022). JA pathway is tightly regulated by SET, SA and GA, the latter two of which form antagonistic relationships with JA (Fernandes and Ghag, 2022).

Ethylene (ET) defence

The other key plant hormone acting in defence of pathogen attack is ET. Here the hormone regulates programmed cell death, a key factor in response to environmental stresses (Pennell and Lamb, 1997). ET is produced to ward off pathogens after recognition of conserved microbe-associated molecular patterns (MAMPs) (Shekhawat et al., 2022), and ET is known to be produced in large quantities in early in the infection process (Bouchez et al., 2007).

The ET signalling cascade is initiated by binding of ethylene to its receptors (Hua and Meyerowitz, 1998), including ethylene response 1 (ETR1), ethylene resistant (ERS1), ethylene response 2 (ETR2), ethylene resistant 2 (ERS2) and ethylene insensitive 4 (EIN4) (Bleecker et al., 1988). These act to remove the block of constitutive triple response1 (CTR1) on EIN2 (Zhang et al., 2009). This causes a release of EIN2 which in turns activates an additional transcription factor (TF), EIN3/EIN3-like1 (Chao et al., 1997) which regulates downstream defence. This is illustrated in Figure 3 (Binder, 2020).

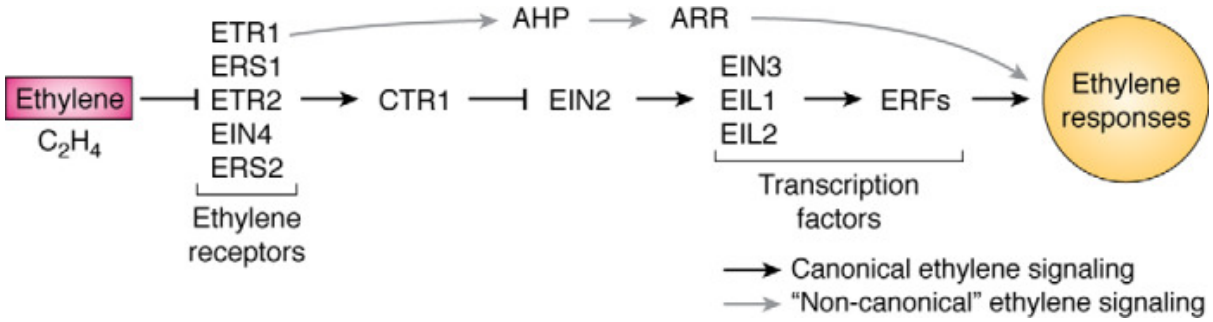

Figure 3: simplified model of ethylene signalling. Illustrates ethylene receptors, the protein kinase CTR1, and EIN2 signalling to transcription factors EIN3, EIL1 and EIL2. In turn these signal to other transcription factors (ERFs), leading to ethylene responses. This is known as the canonical signal pathway. The non-canonical pathway is shown in grey and is supported by more recent evidence where ETR1 signals to histidine-containing AHPs and then to ARRs to modulate responses to ethylene (Binder, 2020)

ET biosynthesis pathway takes place via only two committed enzymatic reactions (Pattyn, Vaughan-Hirsch and Van, 2020). The first step is completed by the enzyme ACC synthase (ACS) where the substrate *S*-adenosyl-l-methionine (SAM) is converted to ACC and 5'-methylthioadenosine (MTA) (Adams and Yang, 1979). ACC is converted to ethylene, CO2 and cyanide by the ACC oxidase (ACO) enzyme in the second reaction (Hamilton, M Bouzayen and Grierson, 1991).

Gaps in knowledge

Plants cope with stresses and environmental changes such as cold, drought and herbivory attack (Erayman et al., 2015). This process is fine-tuned, however, the need to feed an increasing population has caused a monoculture on a huge scale allowing pathogens to evolve to increase infection strength. Plant defence pathways have been described however what is not clear is the effect arthropod infestation has on these pathways and why it leads to increased FHB incidence. We set out to find genes likely to be involved in this process.

Candidate genes

The knowledge of plant defence pathways allowed for multiple candidate genes to be selected for experiments to be run in search for genes likely to aid *F.graminearum* resistance. The majority of these genes are found within signalling pathways for their corresponding hormone.

Jasmonic acid signalling pathway

JA has perhaps the largest number of regulating transcription factors as a result of the many different synthesis pathways. Figure 4 shows a simplified model of the signalling pathway (Ruan et al., 2019).

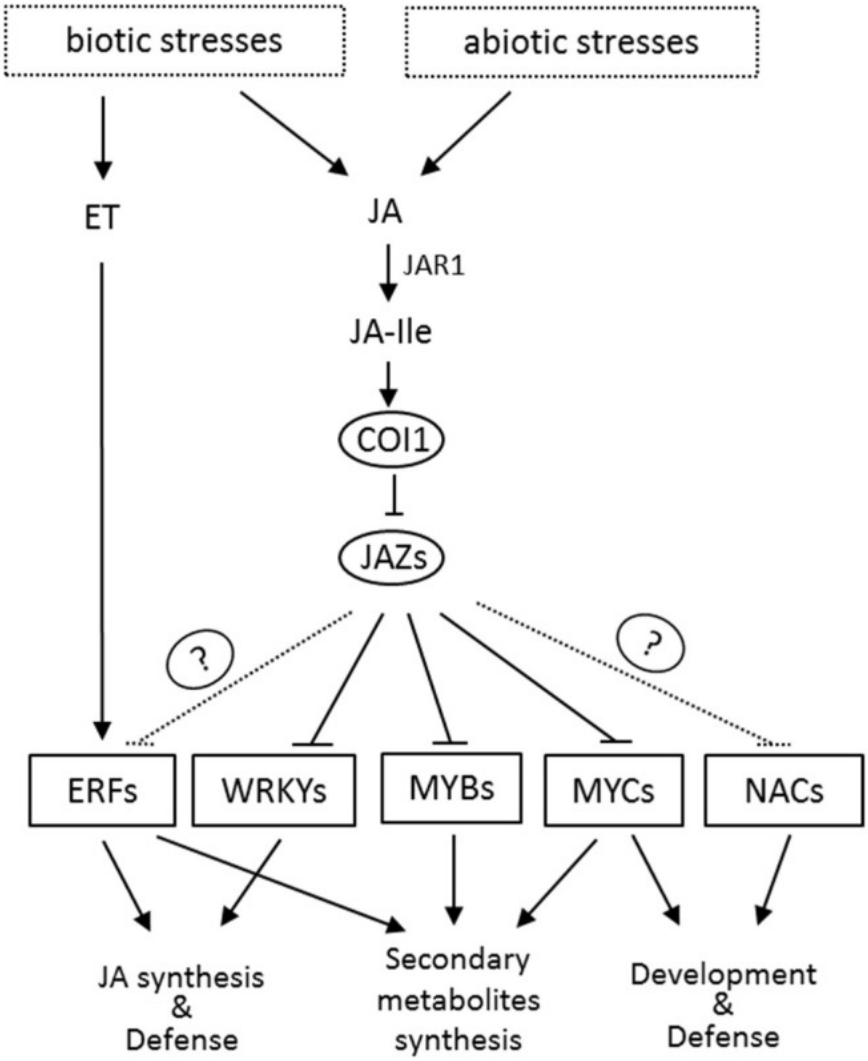

Figure 4: Regulation network of jasmonic acid signalling pathway. JA induced by biotic and abiotic stresses which is converted to JA-Ile by JAR1. JAR1 is perceived by its receptor COI1 which triggers the degradation of JAZ repressors which releases downstream transcription factors and regulation of responsive genes in various processes. Question markers indicates an adaptor protein which is yet to be identified (Ruan et al., 2019)

A total of seven candidates were selected to experiment upon in the JA pathway: JAZQ, MYB20, MYC2, PDF1.2, VSP2, ANAC019 and ANAC055. As shown in Figure 4 JAZQ is highest up in the signalling pathway and degradation of this is required for the transcription factors further down the pathway to be activated (Ruan et al., 2019). MYC2 is a regulatory protein known as being a major mediator of JA signalling as well as crosstalk with ABA and ET (Hong et al., 2012). MYC2 also upregulates AHP6, an inhibitor of the cytokinin pathway and thus negatively regulates cytokinin defence processes (Jang, Yoon and Choi, 2020)

The same authors found MYC2 is also connected to the GA signalling pathway. Upon the degradation of JAZ, MYC is activated which promotes both the plant defensin gene (PDF1.2) and the vegetative storage protein 1 and 2 (VSP1, VSP2) (Fernandes and Ghag, 2022). PDF1.2 is associated with pathogen defence, whereas VSPs are associated with insect

defence. PDF1.2 was found in Arabidopsis leaves upon fungal attack, both in inoculated and uninoculated leaves (Manners et al., 1998). It was originally believed to be induced as part of the SA signalling pathway however Manners et al., (1998) found PDF1.2 to react to treatments of methyl jasmonate but not SA. Fernandes and Ghag, (2022) claimed that *Fusarium oxysporum* resistance was ascertained to PDF1.2. Both VSPs are acid phosphatases, where they are involved in plant defence and flower development (Chen et al., 2012). In arabidopsis VSPs are involved in resistance to insect attacks and pathogens (Chen et al., 2012). Both VSP1 and VSP2, induced by methyl jazmonate and insect feeding. More in known about VSP2, it is an anti-insect protein by delaying development and increasing mortality of feeding insects (Liu et al., 2005) (Chen et al., 2012).
MYB20 is a transcriptional factor to which its main function is to activate lignin biosynthesis genes (Geng et al., 2019). MYB Tfs can directly bind to JAZ proteins, indicating the release from JAZs to activate their target genes (Ruan et al., 2019) (Figure 4). MYB TFs participate in responses to stress as well as stomatal movement.

ANAC019 and ANAC055 form part of the NAC family. This family of transcription factors consist of a target-binding domain at the N-terminus and a highly versatile C-terminal domain that interacts with other proteins and have a large range of functions (Han et al., 2023). Both of these NACs are also involved in the cross talk between JA and SA (Ruan et al., 2019). ANAC019 is more involved in the SA-JA crosstalk as well as only being upregulated late on in pathogen infection (Fernandes and Ghag, 2022). Signalling pathways which result in expression of these NACs is shown in Figure 5.

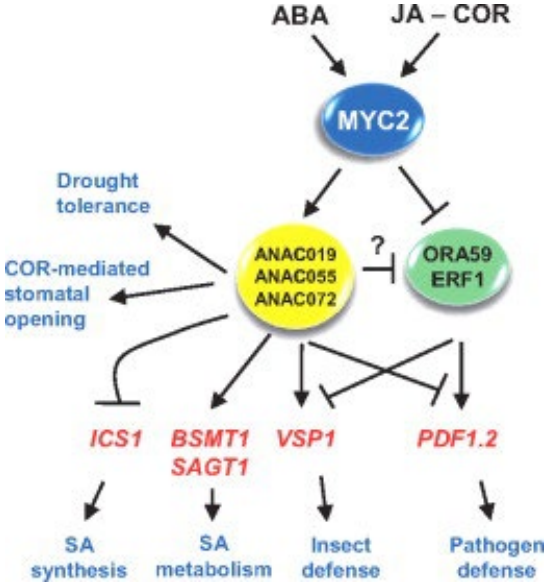

Figure 5; Diagram showing the process leading to expression of the NAC domain containing ANAC019, ANAC055 and ANAC072 as well as resulting expression. Taken from Kazan and Manners (2013).

Ethylene signalling pathway

Figure 3 above illustrates the ET signalling pathways through EIN2 and EIN3 leading to expression of ETHYLENE RESPONSE FACTORS (ERFs). Within this pathway also includes numerous NAC transcription factors expressed due to EIN3. Figure 6 shows ET signalling pathway downstream of EIN3.

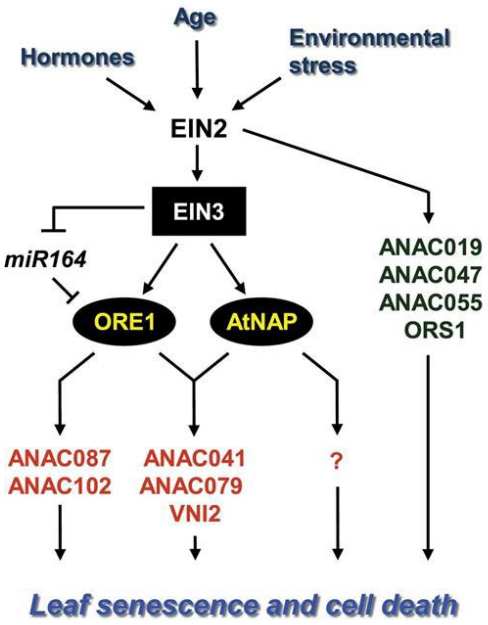

Figure 6; ET signalling pathway downstream of EIN3. Model taken from (Kim et al., 2014)

Hormonal defence regulations to arthropods and Fusarium in Arabidopsis/wheat

In response to biotrophic attacks Auxin as well as ABA, ET and JA see expression reduced (Gilroy and Breen, 2022) (Macioszek et al., 2023). Interesting, is the change in hormone expression in response to necrotrophic attacks due to the previously mentioned hemi-biotropic nature of *F. graminearum* where during attack the pathogen changes from biotrophic to necrotrophic. In response to necrotrophy attacks the hormones which saw expression reduced in biotrophic attacks are increased in necrotrophic attacks. GA and SA expression is also switched between biotrophic and necrotrophic attacks (Gilroy and Breen, 2022) (Macioszek et al., 2023).

Table 1; Resultant plant hormone action in response to different type of plant stress caused by different types of attack.

Hormone	Resistance to biotrophs	Resistance to necrotrophs	Resistance to Herbivores
Abscisic acid (ABA)	**Reduces**	**Increases**	**Reduces/Increases**
Auxin (IAA)	**Reduces**	**Increases**	**Species/tissue specific**
Ethylene (ET)	**Reduces**	**Increases**	**Reduces/Increases**
Gibberellins (GA)	**Increases**	**Reduces**	**Reduces**
Jasmonic acid (JA)	**Reduces**	**Increases**	**Increases (chewing insects)**
Salicylic acid (SA)	**Increases**	**Reduces**	**Increases (sucking insects)**

The primary role of ABA in defence against pathogen invasion is through regulating stomatal closure, an invasion pathway often used by pathogens. ABA works antagonistically with SA and synergistically with ET resulting in increased susceptibility to biotrophic pathogens and increased resistance to necrotrophic pathogens (Gilroy and Breen, 2022). A key role ET plays

in pathogen resistance is reducing pathogen defence resulting in ethylene being crucial for pre-invasion defence (Gilroy and Breen, 2022). GAs identified as growth regulators they are important in pathogen resistance (Gilroy and Breen, 2022). MAMP- triggered immunity inhibits auxin signalling in the absence of this auxin signalling results in supressing SA biosynthesis, altering responses to biotrophs (Gilroy and Breen, 2022). JA has a crucial role to play as a signal mediator in defence against herbivorous insects and necrotrophic pathogens (Macioszek et al., 2023). Upon perception of necrotrophic fungi signal transduction through secondary messengers (e.g., reactive oxygen species, ROS) triggers plant resistance responses leading to JA biosynthesis and activation of a JA-dependent signalling cascade including a set of transcription factors (TFs) and following over-expression of defense-related JA marker genes such as, e.g., plant defensin (e.g., *PDF1.2*) (Macioszek et al., 2023). SA's role in defence ranges from forming initial recognition of the pathogen, immune response such as cell wall strengthening and production of secondary metabolites (Mishra et al., 2024). SA is also a key regulator of systemic acquired resistance (SAR). Elevated levels of SA triggers SAR by coordinated activation of pathogenesis related (*PR)* genes (Mishra et al., 2024), a term given to proteins of a host that are induced only in response to attack by pathogens or by a related event (Bonasera et al., 2006).

MOFA analysis

The multi factor aspect of this research as well as the number of genes which have a possible affect on both disease and insect infestation lead to the need for a multi-omics factor analysis (MOFA) to highlight genes of interest and streamline work efforts. A MOFA promises to produce an improved characterization of biological processes across molecular layers (Argelaguet et al., 2018) enabling certain genes to be disregarded. The MOFA as well as knowledge of plant hormone defence allowed for multiple candidate genes to be selected for experiments to be run in search of genes likely to aid *F. graminearum* resistance.

Arabidopsis

Arabidopsis thaliana has become the most widely studied plant in modern biology despite it providing no nutritional or financial advantages to humans. Instead, Arabidopsis offers a clear view into molecular, cellular, and developmental mechanisms underlying life as a multicellular photoautotroph, Arabidopsis has become a model organism due to its very small gene size, making it easier to perform genetic studies (Woodward and Bartel, 2018).

Arabidopsis has many essential characteristics making an ideal plant to be extensively researched. Arabidopsis is a quick growing, small plant which has the beneficial attribute, in which it flourishes indoors (Woodward and Bartel, 2018). The ability for arabidopsis to grow inside poses a great advantage to limit the effects of unforeseeable weather conditions as well as threats posed from pathogens and herbivory activity to be limited. Figure 7 illustrates the speed at which Arabidopsis grows (Woodward and Bartel, 2018).

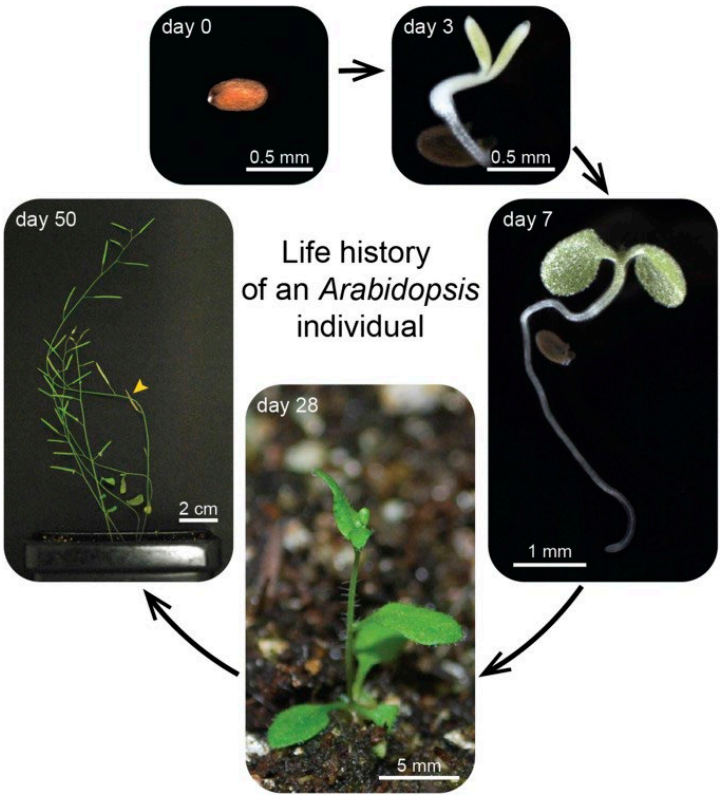

Figure 7; Life cycle of an Arabidopsis plant taken from Woodward and Bartel,(2018). Usually grown in a plant nutrient agar before being stratified at 4° for 48 hrs. Plants are then typically placed in a 22° continuous light growth room. After 3 days the radicle emerges from the testa (seed coat). Green cotyledons, emerging true leaves as well as expanded hypocotyl and elongated roots were apparent by day 7. From about two weeks the lant is transferred to soil, from here flowering typically occurs at 28 days and dry seed pods containing mature seeds are apparent at 50 days.

The aforementioned growth characteristics allows high number of replicates due to high turnover compared to other crops such as wheat which can be between 4-8 months between planting and cutting dependent on variety (Asseng et al., 2020). Despite Arabidopsis being part of the Brassicaceae family and wheat coming from the Poaceae family, research conducted on Arabidopsis is still highly relevant to understanding and improving wheat.

Arabidopsis remains highly favoured by scientists for physiological, biochemical, genetic, and molecular investigations owing to its compact, well characterised genome which is relatively simple to cultivate and manipulate (Ferjani et al., 2023). Ease of genome cultivation stems from remarkable size, in fact Arabidopsis has one of the smallest plant genomes at only 135 mega base pairs (Sims et al., 2021). This small genome allows for the creation of mutants which have had specific genes knocked out. Here we have obtained 22 *Arabidopsis thaliana* mutants with a range of the previously mentioned genes knocked out and tested the phenotype. The different phenotypes were tested for the effect of aphids, both survival and development as well as susceptibility to *F. graminearum.* Phenotypes were also tested to see if the aphid altered susceptibility to *F. graminearum.*

Gene expression in Arabidopsis

Arabidopsis has a wide range of ecological relationships and adaptations to abiotic environments, it responds quickly and efficiently to environmental cues, including light, daylength, vernalisation, nutrient, and water levels (Shimizu and Purugganan, 2005). It is also susceptible to pathogens and insect herbivory (Shimizu and Purugganan, 2005). The expression of genes can have an influence on the relationship between the genotype and the phenotype (Cortijo et al., 2019) owing to the hormone cross talk. Examination of gene expression in Arabidopsis has the advantage as a result of being an inbreeding species meaning heterozygosity is extremely low (Abbott and Gomes, 1989). Widespread variability in gene expression has been observed between day and night in Arabidopsis (Cortijo et al., 2019). Cortijo et al., (2019), found up to 1,358 highly variable genes in Arabidopsis which enhanced several processes involved in responding to biotic and abiotic stresses as well as in the response to endogenous and exogenous signals most notable in genes associated with lipid transport and fungus response.

Hypotheses

We set out to find how gene expression is altered in response to both aphids and *F. graminearum* in an attempt to find the genes which are responsible for increased FHB incidence in response to arthropod activity. We perform gene expression analysis of two wheat varieties with multiple treatments as well as phenotyping experiments on *Arabidopsis thaliana* mutants using aphids and *F. graminearum* spores. We will conclude with gene expression analysis of wildtype Arabidopsis in response to aphids and *F. graminearum*.

We hypothesis that through gene expression we will observe examples of hormonal cross talk especially within ET and JA signalling pathways. It is also within this antagonistic cross talk we expect to observe changes in *F. graminearum* resistance due to the influence of aphids. We hypothesise that resultant effects of aphid infestation will lead to upregulation of genes responsible for aphid resistance specifically *VSP1.* This upregulation of the insect defensive gene will likely, at some point in hormone signalling lead to downregulation of genes involved in *F. graminearum* defence, hypothesised to be *PDF1.2* or an ET responsive gene such as *RAP2.12* or *ANAC087*.

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Methodology

Gene Function

Within this work a variety of genes were worked upon. These genes, identified by a MOFA were tested with the aim to learn more about functions of the gene in response to both aphid and *F.graminearum* treatment. The MOFA offers a method for discovering the principal sources of variation in this plant defence pathway as the plant-initiated defence response. The MOFA allowed for a select few genes to be highlighted as genes of interest in what otherwise would be a huge selection pool of genes. The genes highted by the MOFA consisted of genes known to be involved in both *F. graminearum* infection and aphid infestation. This process allowed for work to be streamlined into specific genes of interest in for the aim of this experiment. In total 22 genes were selected from many different plant defence pathways all with varying functions within the plant. The functions of each gene were obtained from TAIR.Arabidopsis (Arabidopsis.org, 2024) and presented in Table 2.

Table 2; Gene function of all genes used across all experiments. Genes were selected based on their weighting in the MOFA. Gene function found on TAIR.Arabidopsis.

Gene	Function
ANAC019	Encodes a NAC transcription factor , induced by drought, high salt, and abscisic acid.
ANAC055	Encodes an ATAF-like NAC-domain transcription factor that doesn't contain C-terminal sequences shared by CUC1, CUC2 and NAM. The mRNA is cell-to-cell mobile.
ANAC087	NAC domain containing protein 87
DELLA	Encodes a DELLA protein. DELLA proteins restrain the cell proliferation and expansion that drives plant growth. Negative regulator of the response to GA in controlling seed germination. GA triggers the degradation of RGL2 protein in a process blocked by both proteasome inhibitors and serine/threonine phosphatase inhibitors. Rapidly degraded in response to GA. Regulates GA-promoted seed germination. Involved in flower and fruit development.
EIN2	Involved in ethylene signal transduction. Acts downstream of CTR1. Positively regulates ORE1 and negatively regulates mir164A,B,C to regulate leaf senescence. A maternally expressed imprinted gene. The mRNA is cell-to-cell mobile.
JAZQ	JAZ genes play an important role in regulating the adaptation or defence of biotic and abiotic stresses in different species. For example, the higher-order mutant jazQ of Arabidopsis enhanced the biosynthesis of anthocyanins and glucoside sulfate, defence against insects (Song et al., 2022)
MYC2	Encodes a MYC-related transcriptional activator with a typical DNA binding domain of a basic helix-loop-helix leucine zipper motif. Binds to an extended G-Box promoter motif and interacts with Jasmonate ZIM-domain proteins. MYC2 interacts with EIN3 and EIL1 to repress hook curvature and resistance to Botrytis cinera. Its transcription is induced by dehydration stress, ABA treatment and blue light via CRY1. Positive regulator of lateral root formation. Regulates diverse JA-dependent functions. Positively regulates flavonoid biosynthesis, resistance to insects, and response to oxidative stress.

	Regulates other transcription factors, and negatively regulates its own expression. For example it binds to and regulates the expression of NST1. Its stability is modulated by PUB10 through polyubiquitination.
PDF1.2	Encodes an ethylene- and jasmonate-responsive plant defensin. mRNA levels are not responsive to salicylic acid treatment; although jasmonate and salicylic acid can act synergistically to enhance the expression of this gene. Belongs to the plant defensin (PDF) family.
VSP1	Encodes an acid phosphatase similar to soybean vegetative storage proteins. Gene expression is induced by wounding and jasmonic acid.
EIN3EIL1	Ethylene-insensitive3-like1 (EIL1) The mRNA is cell-to-cell mobile.
GSTF11	Encodes glutathione transferase belonging to the phi class of GSTs.
GSTF12	Encodes glutathione transferase belonging to the phi class of GSTs. Mutants display no pigments on leaves and stems. Likely to function as a carrier to transport anthocyanin from the cytosol to tonoplasts.
LAC7	putative laccase, a member of laccase family of genes (17 members in Arabidopsis).
LAC8	Putative laccase, a member of laccase family of genes (17 members in Arabidopsis).
RAP2.12	Encodes a member of the ERF (ethylene response factor) subfamily B-2 of ERF/AP2 transcription factor family (RAP2.2).
SWEET17	SWEET (for SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS) and SUC/SUT (for Sucrose transporter/Sugar transporter)-type transporters are responsible for transfer of Suc from the phloem parenchyma into the sieve element companion cell complex for long-distance translocation
UGP2	UDP-glucose pyrophosphorylase, functions redundantly with UGP1 for starch biosynthesis during pollen development
YUC10	Encodes a member of the YUC family that is expressed in the root apex and is ethylene inducible in the root
ABI3	Essential for seed maturation. Regulator of the transition between embryo maturation and early seedling development. Putative seed-specific transcriptional activator. ABI3 is a central regulator in ABA signalling and is unstable in vivo. Based on double mutant analyses, ABI3 interacts genetically with both FUS3 and LEC1 and is involved in controlling accumulation of chlorophyll and anthocyanins, sensitivity to abscisic acid, and expression of the members of the 12S storage protein gene family
MYB20	Encodes a transcriptional regulator that directly activates lignin biosynthesis genes and phenylalanine biosynthesis genes during secondary wall formation.
ABI5	Participates in ABA-regulated gene expression during seed development and subsequent vegetative stage by acting as the major mediator of ABA repression of growth. Plays a role in sugar-mediated senescence.
SHS1	Encodes a plastidial nucleotide uniport carrier protein required to export newly synthesized adenylates into the cytosol.

Statistical analysis

Statistical analysis for all experiments were conducted using Genstat® Version 23 for windows (VSN International Ltd, UK). T-tests were conducted on gene expression data in the wheat varieties. Regression analyses were conducted on Arabidopsis phenotyping data using a general linear model. Significant levels were set at $P<0.05$ across all statistical analysis, highlighting all statistical significant results from all experiments.

Reference:

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Selected wheat gene expression in plants exposed to single or dual *F. graminearum* and aphid attacks

1- Introduction

Plants have evolved defence mechanisms against pest or pathogen attacks in an attempt to minimise detrimental damage to tissues and organs. The three main phytohormones involved in defence signalling and regulation are ethylene (ET), salicylic acid (SA) and jasmonic acid (JA), modulating defence pathways to encode for specific plant responses. Upon pest or pathogen detection phytohormones are regulated to form defence which has been researched extensively (Bürger and Chory 2019) leading to some phytohormone signalling pathways being found.

In response to herbivory attack, plant defence pathways work through through various morphological, biochemical and molecular mechanisms, which aim to affect feeding, growth or survival of herbivores (War et al., 2012). These mechanisms act either directly, through morphological characteristics (thick leaves, hairs and thorns) or toxic chemicals such as terpenoids, phenols and alkaloids aiming to kill or reduce development of herbivores(War et al., 2012). Indirect response includes release of volatiles to specifically attract natural enemies (War et al., 2012).

In contrast, plant defence responses against pathogen attack range from innate immunity to immune response. Innate immunity involves formation of physical barriers to infection such as rigid cell walls and waxy cuticles (Nishad et al., 2020). Systemic signals are released from infected cells forming initiation of plant immune response. The first branch of immune response is MAMP-triggered immunity (MTI). MTI is induced upon recognition of MAMPS, resulting in rapid calcium influx, reactive oxygen species (ROS) accumulation cell wall alteration and defence gene expressions (Nishad et al., 2020). Gene expression in response to MTI is of significant interest given the complexity of hormone signalling pathways within plants.

F. graminearum infection causes wheat to activate immune response interactions (Tu et al., 2023), resulting in changes in gene expression especially pathogenesis related proteins, including chitinase and β-1,3-glucanase (Dmitriev et al., 2017). The same is thought of genes related to the ET and JA signalling pathway, which as stated in the numerous cross talk interactions which many of the plant hormones have. Using a quantitative polymerase chain reaction (qPCR) machine allows for gene expression to be quantified. This machine allows a 'real-time' measurement of the concentration of the desired DNA during a PCR. Here the use of fluorescent dyes which bind to the DNA of interest as such the amount of fluorescence is proportional to the quantity of DNA present. The fluorescence is picked up on a camera and the accompanying computer software processes the timing and quantity of DNA expression (Dymond, 2013).

ET is known for its role in both senescence and plant stress response especially to drought, flooding and pathogen attack. ET is perception is initiated by the binding of ET to receptors

which leads to *EIN2* positively regulating the transcription factors *EIN3, EIL1, EIL2* which in turn upregulate *ERFs* (Ethylene response factors). Kim et al, (2014) proposed Figure 6 as the ET signalling pathway which leads to defensive proteins and secondary metabolites. ET also initiates hypersensitive response, involving programmed cell death at the infection site in an attempt to limit spread of infection (Bouchez et al., 2007).

SA is involved in biotic attacks, especially necrotrophic pathogens. SA has a large impact upon hormone cross talk, known to surpress genes associated with the JA signalling pathway such as *VSP1, VSP2* and *PDF1.2.*

As stated, JA regulates the plant response to both necrotrophic pathogens and herbivores. This pathway is the most intrinsic with many factors effecting expression of genes downstream in the pathway. In the presence of JA, *JAZ* proteins are degraded, releasing the transcription factor, *MYC2 which coordinates JA and ET defence against both herbivores and pathogens. MYC2* negatively regulates ET response by suppressing *EIN3* needed for defence against necrotrophic pathogens. The full pathway proposed by Kazan and Manners (2013) is shown in Figure 5 whereby *MYC2* acts to upregulate the NAC domain causing upregulation of further genes downstream leading to JA response. Conversely, MYC2 acts to downregulate ORA59 ultimately leading pathogen defence.

The impact of aphid infestation prior to *F. graminearum* infection has been shown to increase host susceptibility to FHB (Drakulic et al., 2015). and the inclusion of treatments which involve indirect interactions between *F. graminearum* and the aphid was expected to reveal how the aphid influences the defence of the host to *F. graminearum*. The hypothesis was that genes involved in ET signalling would be upregulated under *F. graminearum* due to its known role in plant resistance to fungal pathogens (Guan et al., 2015), in contrast to genes forming part of JA signalling pathways which regulate response to the aphid. It may be in the JA pathway where interactions are present due to the potential of either organism altering expression and changing expression of genes downstream of *MYC2*.

However, it maybe that genes downstream of *MYC2* and associated with JA response may see decreased expression resulting from antagonistic cross talk with SA. This cross talk has been suggested as a node for pathogen manipulation to increase infection (Hou and Tsuda, 2022).

To give indications to which genes are expressed due to either *F. graminearum* or aphid attack gene expression analysis took place to figure which pathways are expressed in response to each organism. Wheat varieties were used that exhibited varying resistance to both pest and pathogen. Sumai exhibits strong resistance to FHB (Bai and Shaner, 2004) whereas Gallant exhibits higher resistance to aphids. . In contrast to each other Sumai is more susceptible to aphid infestation than Gallant which itself is susceptible to FHB. Using this knowledge of resistance and susceptibility knowledge can be expanded to indicated which genes are potentially used by either organism to help prosperity. As well as this, indications may be given as to the strength of cross talk between plant signalling pathways.

1.2- MOFA

A multi-omics factor analysis (MOFA) provides an improved characterization of biological processes across molecular layers (Argelaguet et al., 2018). The aim of this MOFA was to create an indication which genes are involved in increasing susceptibility to FHB as a result of pest activity. This MOFA was undertaken prior to this research starting and results presented in an attempt to find out how the pest (aphid) suppress immunity to benefit the pathogen (*F. graminearum*). Figure 8 shows results from the MOFA. For this research interest is in second column labelled Fg. Results indicate genes of interest are found within Factor 1 and Factor 2. Table 3 shows the genes selected by the MOFA used within all experiments here.

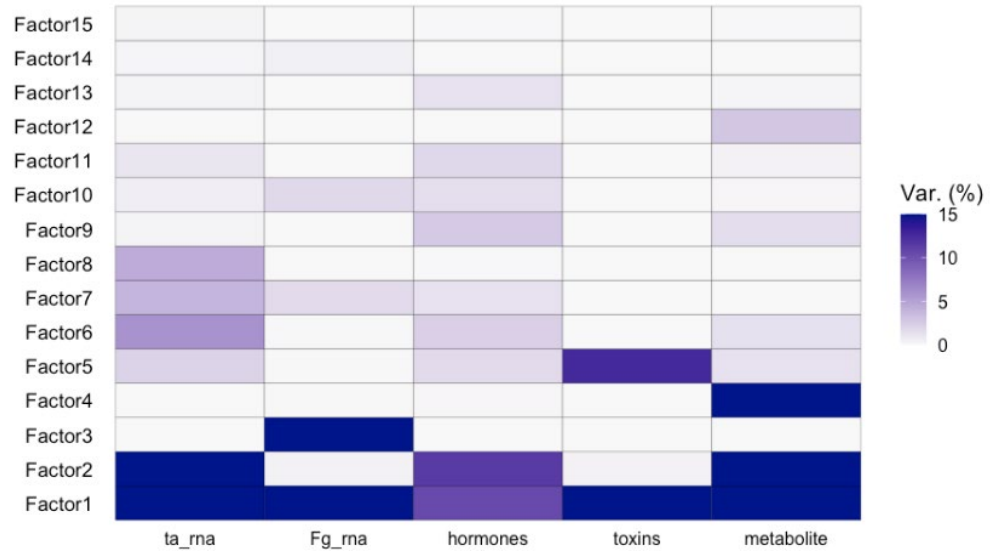

Figure8; Multi-omics Factor Analysis. Darker boxes signify greater variation and suggests factors of interest. The first column indicates wheat RNA, and the second column indicates fusarium RNA. DNA was taken 24 hours after treatment in Sumai3 (GS65).

Table 3; Genes and transcript identified by the MOFA including weights and orthology confidence. Separate transcripts could not be obtained for RAP, GSTF, LAC, UGP and SWEET.

Variety	Gene name	Transcript	Weight	Scale Weight	Orthology confidence 0=low, 1=High
Sumai	RAP2.2 and 2.12	TraesCS6A02G097700	0.43727872	0.447717	0; 0
Sumai	VSP2	TraesCS4A02G431200	0.26062435	0.266846	0; 0
Sumai	YUC10	TraesCS5B02G216000	0.86383092	0.884452	1
Sumai	SHS1	TraesCS6B02G210000	0.90394288	0.925521	1
Sumai	GSTF11 and 12	TraesCS7D02G479100	0.5585928	0.571927	0; 0
Sumai	LAC7, LAC8	TraesCS3A02G452400	0.58345181	0.59738	0; 0; 0
Sumai	LAC7, LAC8	TraesCS6A02G058300	0.52100974	0.533447	0; 0; 0
Sumai	EIN2	TraesCSU02G131600	0.60644841	0.620925	0
Sumai	UGP1 and 2	TraesCS6A02G050100	-0.4015747	-0.41116	0; 0
Sumai	ABI5	TraesCS3A02G371800	0.60323578	0.617636	0
Sumai	ABI3	TraesCS3B02G452200	0.63737065	0.652585	0; 0
Sumai	MYB20	TraesCS7A02G205100	0.44708595	0.457758	0
Sumai	SWEET17 and 16	TraesCS5B02G040800	-0.3010439	-0.30823	0; 0
Sumai	ANAC087	TraesCS7A02G194700	0.26651153	0.272873	0; 0
Gallant	RAP2.2 and 2.12	TraesCS6D02G084900	0.44949239	0.838915	0; 0
Gallant	VSP1+2	TraesCS7D02G053700	0.50393093	0.940517	0; 0
Gallant	YUC10	TraesCS5A02G217200	0.42963678	0.801857	1
Gallant	SHS1	TraesCS6B02G210000	0.52371591	0.977443	1
Gallant	GSTF11 and 12	TraesCS2B02G420800	0.36024997	0.672356	0; 0
Gallant	LAC7, LAC8	TraesCS3B02G489700	0.42525813	0.793685	0; 0; 0
Gallant	EIN2	TraesCSU02G131600	0.14638603	0.273209	0
Gallant	UGP1 and 2	TraesCS6A02G050100	-0.4045625	-0.75506	0; 0
Gallant	ABI5	TraesCS3A02G372200	0.47370943	0.884112	0
Gallant	ABI3	TraesCS3D02G412800	0.44643295	0.833205	0; 0
Gallant	MYB20	TraesCS7A02G205100	0.39898106	0.744642	0
Gallant	SWEET17 and 16	TraesCS5A02G039000	-0.4017825	-0.74987	0; 0
Gallant	ANAC087	TraesCS7B02G094000	0.47822249	0.892535	0; 0

Two transcripts were found for LAC7 and LAC8 (Sumai). VSP (Gallant) is the transcript for both VSP1 and VSP2, whereas for Sumai, the VSP transcript is only VSP2. Some genes seen in Table 3 were found in from the MOFA including genes such as SWEET16+17 and UGP1+2 which are both involved in providing nutrition and so manipulation of these genes by the organism could indicate potential pathways used by the organisms to increase growth. Glutathione S-transferases (GSTs) are proteins which have many functions including stress response and toxicity suggesting the GSTF11+12 gene may see increased expression in response to metabolites released by either the pathogen or aphid in an attempt to manipulate expression to increase survival. Whereas other genes are associated with hormone signalling pathways as shown in Figure 5 and 6. Gene function of all genes used are shown in the materials and methods chapter. Table 4 shows the locus and SALK lines of genes highlighted by the MOFA.

Table 4; Locus, SALK lines and NASC numbers for all genes presented by the MOFA which were used in gene expression analysis.

GENE	LOCUS	SALK	NASC
RAP2.2 and 2.12	AT3G14230, AT1G53910	SALKseq_64808, SALK_047306C	N880929, N662360
VSP2	AT5G24770	SALK_047586C	N677724
YUC10	AT1G48910	SALK_031634C	N69899
SHS1	AT4G32400	SALK_040447	N540447
GSTF11 and 12	AT3G03190, AT35G17220	SALK_014567C, SALK_1131164C	N679343, N673214
LAC7, LAC8	AT3G09220, AT5G01030	SALK_200780C, SALK_006637C	N688009, N673708
LAC7, LAC8	AT3G09220, AT5G01030	SALK_200780C, SALK_006637C	N688009, N673708
EIN2	AT5G03280	CS3071	N3071
UGP1 and 2	AT3G03250, AT5G17310	SALK_100183C, SALK_127440C	N658355, N673861
ABI5	AT2G36270	SALK_013163C	N673861
ABI3	AT3G24650	CS24	NW24
MYB20	AT1G66230	SALK_202624C	N690642
SWEET17 and 16	AT4G15920	SALK_012485C	N659673
ANAC087	AT5G18270	SALK_011502C	N665270

1.3- Candidate genes

Ethylene candidates

The candidates selected from this pathway include *EIN2* and double mutant *EIN3EIL1*. *EIN2* is a central component of the ET signalling pathway its expression activates ET response and allows responsiveness to JA (Alonso et al., 1999). *EIN2* is a main regulator of the ET pathway and as such controls all downstream TFs including *EIN3* and EIL1 (Binder, 2020).

The double mutant acting downstream of *EIN2* are short living proteins which undergo ubiquitination and proteasomal degradation driven by ubiquitin-ligases *EIN3 BINDING F-BOX1 (EBF1)* and *EBF2* (Gagne et al., 2004). The mutant is stabilised upon ethylene released which plays a core role triggering ET directed gene expression (Dolgikh et al., 2019).

Further down the ET synthesis pathway *RELATED TO AP2.12 (RAP2.12)* and *RAP2.2* is a transcription factor which is mainly induced by darkness and can be found in high levels within the roots (Hinz et al., 2010). *RAP2.12* and *RAP2.2* fits under the 'ERF' banner. A NAC is also found within ET synthesis, in this case *ANAC087* expressed in the shoot apex, roots and vascular tissue (Vargas-Hernández et al., 2022) and is involved in leaf senescence (Chen et al., 2023).

Abscisic acid candidates

Although the role of ABA in direct plant defence is thought to vary little two candidates were identified from ABA pathway *ABSCISIC ACID INSENSITIVE 3 (ABI3)* and *ABI5*. ABA typically plays a role in plant growth and development, seed and bud dormancy, stress (Brookbank et al., 2021) and stomatal closure (Lim et al., 2015). Suggestions are ABA in fact has a negative effect on plant resistance to pathogens by suppressing host immune response (Hu and Bidochka, 2021). On top of this ABA deficient plants have been found to have increased stomatal size, increasing potential for pathogen invasion as well as water stress. ABA and *MYB20* from the JA signalling pathway engage in antagonistic cross talk as *MYB20* negatively regulates the ABA mediated stomatal closure (Wang et al., 2021).

Laccase enzyme candidates

Further candidate genes which are involved in leaf morphology are genes encoding for laccase enzymes which are multicopper containing oxidases (Mayer, 2002). *LAC7* and *LAC8* play critical roles in ligin biosynthesis as well as plant development, various stress related responses, xylem sap transport and defence against pest and pathogens (Sun et al., 2022). Lignin biosynthesis traits favour stronger cell wall formation creating a clear barrier against pathogen defence (Sun et al., 2022).

As opposed to providing defence against pathogens laccase enzymes are thought to interact DON. Research suggests that LACs may meet and trap DON in the periplastic space, preventing DON from entering the cell to interfere with the normal metabolism of the cell (Sun et al., 2022). Suggestions are LAC's are able to oxidise DON into less toxic forms to alleviate health issue to human and animals (Sun et al., 2022).

Auxin candidates

Auxin, the main plant growth and development hormone can act as a microbial signal that positively impacts the pathogen (Kunkel and Johnson, 2021). The common form of auxin, indole-3-acetic acid (IAA) has shown to promote disease in many plant-pathogen interactions by increasing virulence (Kunkel and Johnson, 2021).

Auxin regulates its processes via controlling gene expression via a family of DNA binding AUXIN RESPONSE FACTORS (ARFs) which confer specificity to auxin response through selection of target genes as transcription factors (Li et al., 2016). The selected gene was *YUCCA10 (YUC10*), part of the *YUCCA* protein family which catalyse the rate limiting step for

endogenous auxin biosynthesis (Song et al., 2020). Previous experiments have illustrated the importance of *YUC10* through *YUC10* absent mutant plants producing seedlings without a hypocotyl and root meristem (Song et al., 2020).

UDP-Glucose pyrophosphorylase candidates

UDP-glucose pyrophosphorylase (UGPase) is a key enzyme in carbohydrate metabolism that catalyzes the production of glucose-1-phosphate and UTP to UDP-glucose and pyrophosphate in roots, stems and leaves (Chen et al., 2007). For this two genes were identified, *UGP1* and *UGP2*. *UGP1* is the predominant gene and is closely co-regulated with carbohydrate metabolism genes, late embryogenesis and seed loading (Meng et al., 2009). Chivasa et al., (2013), found that *UGP1* is also involved in programmed cell death, more specifically fumonisin B1 cell death indicating its importance in hypersensitive response to pathogen infection. *UGP2* on the other hand is involved is stress response genes, fertilised flowers and photosynthetic genes (Meng et al., 2009).

Glutathione candidates

Glutathione-S-transferases (*GSTs*) primarily work on glutathione (*GSH*) conjugation, suggesting their involvement in glucosinolate (*GSL*) metabolism (Zhang et al., 2022). GSLs are sulphur rich secondary metabolites and are important defence compounds (Zhang et al., 2022). In fact, research suggests GSH is the main antioxidant involved in plant stress response (Lanubile et al., 2022). Here two genes were selected: *GSTF11* and *GSTF12*.

Fructose transporter candidates

During pathogen infection nutrients move from the plant to the pathogen. Plants try to restrict this transfer by reprograming its carbon metabolism and transport (Chen et al., 2012), during which photosynthetic capacity is reduced (Breia et al., 2021). Chen et al., (2010) first discovered a type of sugar transporters called sugars will eventually be exported transporters (*SWEETs*) which helped explain sugar efflux mechanisms. The same authors found *SWEET* transporters to be classified as uniporters, mediating both uptake and efflux of sugars. Interestingly *SWEETs* have been found to be induced upon pathogen invasion (Eom et al., 2015). From this, two *SWEET* genes were identified *SWEET16* and *SWEET17* which are vacuolar hexose transporters (Eom et al., 2015). *SWEET17* is primarily expressed in root vasculature and meristematic cells of root tip (Valifard et al., 2021). *SWEET16* in contrast has numerous unknowns in terms of transport and activity (Klemens et al., 2013)

Salt hypersensitive candidates

Genes which are mainly associated with salt tolerance were also investigated due them also falling under the umbrella of stress response. The salt hypersensitive response is known to possibly be involved in cross talk with ABA (Inan et al., 2007). For this the gene salt hypersensitive 1 (*SHS1*) was identified.

2- Method

2.1- Treatments

Gene expression was analysed for the genes presented by the MOFA. RNA was collected from wheat plants which had been subjected to six treatments: Control, infected with *Fusarium graminearum*, aphid only, aphid 4 days before inoculation, aphid 4 days after inoculation and *Fusarium graminearum* plus aphid together. Each treatment comprised of five replications. RNA from all treatments were collected from two wheat varieties, Gallant and Sumai. ‘Sumai 3’ is a standard variety used in FHB research due to high levels of

resistance to FHB (Niwa et al., 2014) and a perceived susceptibility to aphid feeding. Gallant on the other hand exhibits much lower resistance to FHB but an increased resistance to aphid feeding.

2.2- cDNA synthesis

cDNA was synthesised using iscript cDNA synthesis kit (Bio-Rad) and diluted down to 20mg/L and stored at -20°C. Quantitative reverse transcription PCR (RT-qPCR) with Sybr Green (Bio-Rad) was conducted using CFX96 Touch Real-Time PCR detection System (BioRad). The primers used are shown in Table 1.

2.3- Gene expression analysis

Quantitative reverse transcription PCR (RT-qPCR) with Sybr Green (Bio-Rad) was conducted using CFX96 Touch Real-Time PCR detection System (BioRad). The primers used are shown in Table 1. Primers were first tested to ensure expression took place to ensure proper melt points. Expression of these genes were tested compared to selected housekeeping genes, which are constitutively expressed (Warshawsky, 2009) in both Gallant and Sumai.

Selected gene transcripts (Table 1) were first tested to ensure expression took place and ensure proper melt points. Expression results were compared against selected housekeeping genes, which are constitutively expressed (Warshawsky, 2009) in both Gallant and Sumai. Here the house keeping genes ADP, CDC and UBI were used to calculate relative expression of each gene.

3- Results

3.1- Gene expression in the ethylene (ET) signalling pathway

These results include only three transcripts used directly linked to the ET signalling pathway, *EIN2, RAP2.2,2.12 and ANAC087*. *EIN2,* furthest upstream in the signalling pathway, was significantly upregulated by aphid infestation compared to the control of *F. graminearum* only treatment in Sumai whilst no significant differences were observed in Gallant (Figure 9). Furthermore, simultaneous aphid and *F. graminearum* attack synergistically increased EIN2 expression compared to individual treatments with each organism.

EIN3 and *ETHYLENE RESPONSE FACTORS (ERFs)* including *RAP2.2,2.12* were also upregulated in Sumai, but not in Gallant, under the influence of the aphid whilst *F. graminearum* treatment resulted in downregulation of these transcripts in both genotypes (Figure 10). Pre-or post-treatment with Sitbion avenae resulted in significant upregulation of *RAP2.2,2.12* in Gallant compared to healthy control, *F. graminearum* or aphid only treated plants.

ANAC087 was expressed in both varieties only in plants which were pre or post *F. graminearum* treated with aphids suggesting that *F. graminearum* infection prior to aphid infestation cause expression when applied in sequence (Figure 11).

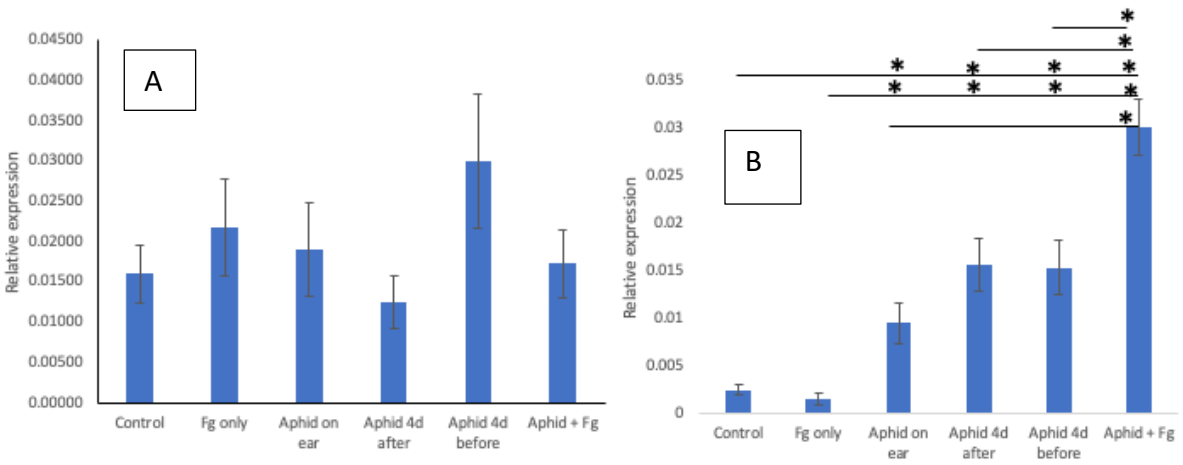

Figure 9; Gene expression graphs for EIN2. A- Gene expression in Gallant (TraesCSU02G131600), no significant differences were observed. B- Gene expression in Sumai (TraesCSU02G131600)i, significant differences are indicated by lines and Asterix, in total 11 differences were found. Significance level set at P<0.05.

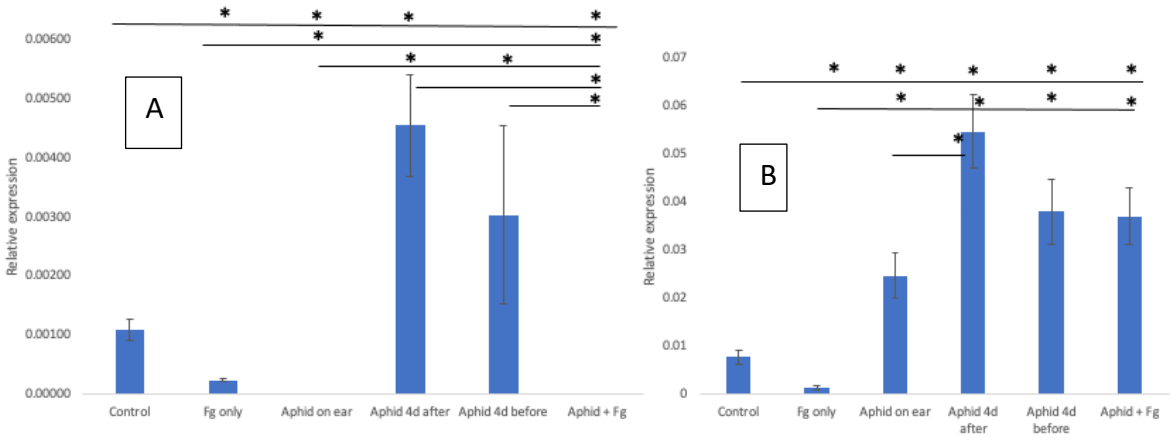

Figure 10; Gene expression in RAP2.2,2.12, transcript encodes for both RAP2.2 and RAP2.12. A- Gene expression in Gallant (TraesCS6D02G084900), significant differences indicated by lines and Asterix. No Expression was observed in aphid on ear and Aphid+Fg treatments. B- Gene expression in Sumai (TraesCS6A02G097700) where expression was seen across all treatments. Significance level set at P<0.05.

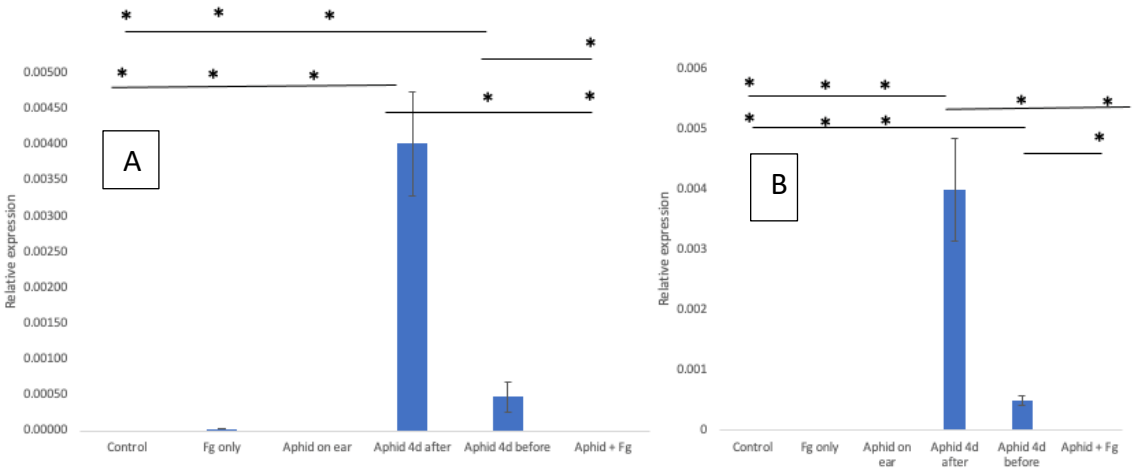

Figure 11; Gene expression in ANAC087. A- Gene expression in Gallant (TraesCS7B02G094000) , lines and Asterix indicates significant differences. No expression was found in either the control, aphid on ear or aphid+Fg treatments. B- Gene expression in Sumai (TraesCS7A02G194700). No expression found in control, Fg only, aphid on ear or aphid+Fg treatments. Significance level set at $P<0.05$.

3.2- Gene expression in the jasmonic acid (JA) signalling pathway

Two transcripts directly linked with the JA signalling pathway were used, *MYB20* and *VSP1*. *MYB20* was significantly expressed in both varieties following Fg infection for 4 days and aphid infestation, however the effect of the 4 days aphid infestation prior to Fg on MYB20 expression was not significantly different to the plants attacked by individual organisms, simultaneous attack or the control (Figure 12).

VSP transcripts showed changes in expression between the two varieties (Figure 8). It must be noted the two primers used for *VSP* expression varied slightly between the varieties. In Gallant expression of *VSP1+2* increased only when aphids were applied 4 days after inoculation (Figure 13). In Sumai, where the transcript coded for *VSP2*, expression was only induced by *F. graminearum* inoculation followed by aphids, suggesting that the aphid suppressed the expression of this transcript.

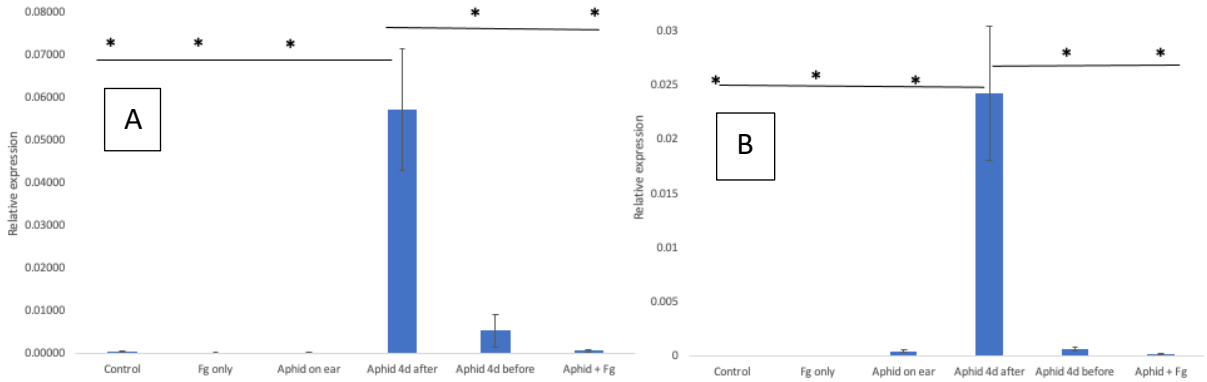

Figure 12; Gene expression for MYB20. A- Gene expression in Gallant (TraesCS7A02G205100), significant differences indicated by lines and Asterix. B- Gene expression in Sumai (TraesCS7A02G205100), no expression was found in both the control and Fg only treatment. Significance level set at P<0.05.

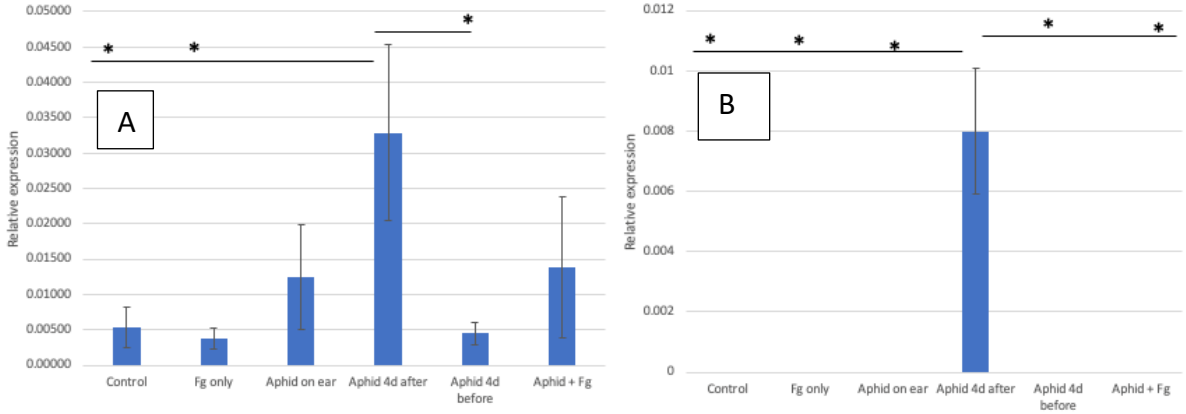

Figure 13; Gene expression in VSPs gene. A- Gene expression in Gallant for VSP1+2 TraesCS7D02G053700. this transcript encoded both genes together significant differences are shown by lines and Asterix. B- Gene expression in Sumai for VSP2 only (TraesCS4A02G431200) where expression was only found in the aphid 4d after treatment. Significance level set at P<0.05.

3.3- Gene expression in the abscisic acid (ABA) pathway

ABI3 and *ABI5* both induced by ABA, *ABI3* expression was significantly increased by prior *F. graminearum* attack compared to aphid attack followed by the pathogen in both varieties (Figure 14). No expression was observed when the two organisms were applied simultaneously in either variety. Similar to ABI3, ABI5 expression increased when plants were treated with aphids after *F. graminearum* inoculation for both varieties (Figure 15). The difference between the two varieties was only apparent in Sumai where no expression was seen for the *F. graminearum* only treatment.

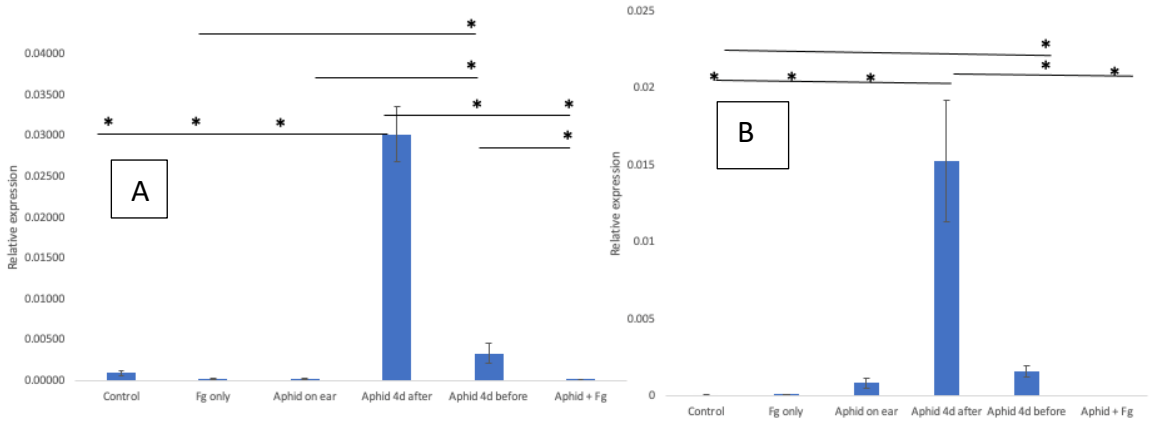

Figure 14; Gene expression in ABI3. A- Gene expression in Gallant (TraesCS3D02G412800), significant differences indicated with lines and Asterix. B- Gene expression in Sumai (TraesCS3B02G452200), no expression found in aphid+Fg treatment. Significance level set at P<0.05.

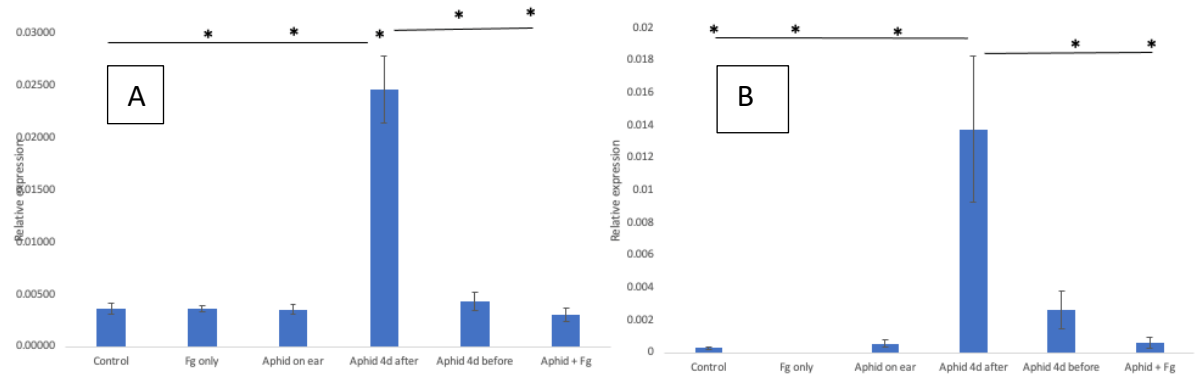

Figure 15; Gene expression in ABI5. A -Gene expression in Gallant (TraesCS3A02G372200), significant differences are shown with lines and Asterix. B- Gene expression in Sumai (TraesCS3A02G371800), no expression was found in Fg only treatment. Significance level set at P<0.05.

SHS1 is upregulated in response to ABA (Inan et al., 2007). No SHS1 expression was found in Sumai, so comparisons between varieties cannot be despite extra reps in an attempt to find expression indicating a possible issue with the transcripts used. In Gallant, no expression was observed in the control or either of the organisms individually. Plants inoculated with *F. graminearum* after aphid feeding increased expression but to a lesser degree than plants which were infected before aphid feeding. No expression was observed when organisms were applied simultaneously (Figure 16).

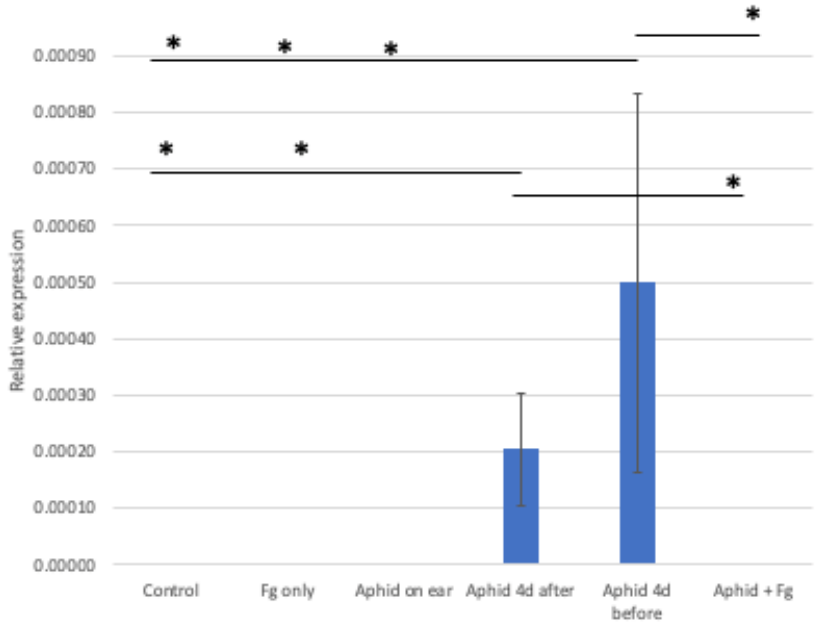

Figure 16; Gene expression for SHS1 in Gallant (TraesCS6B02G210000), despite expression in just two of the treatments the total expression is very low. Significant differences are shown by lines and asterix. No expression was found in Sumai for SHS1 (TraesCS6B02G210000). Significance level set at P<0.05.

3.4- Gene expression in the auxin signalling pathway

YUC10, part of the auxin signalling pathway was significantly upregulated in both varieties in tripartite interactions (Figure 17). The same gene is induced by aphid feeding but not by *F. graminearum* infection in Sumai.

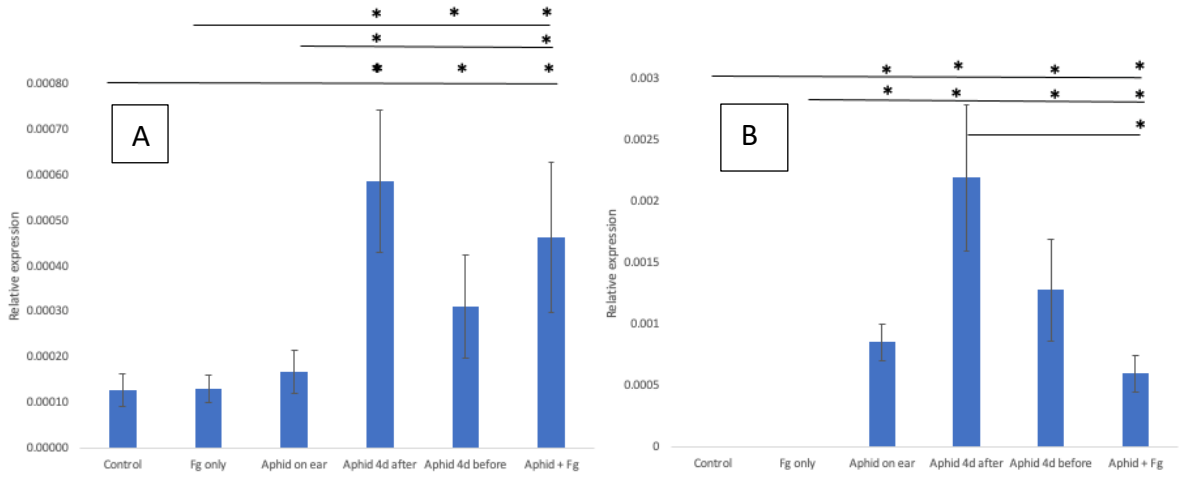

Figure 17; Gene expression in YUC10. A- Gene expression in Gallant (TraesCS5A02G217200), significant differences indicated with lines and Asterix. B- Gene expression in Sumai (TraesCS5B02G216000), no expression in control and Fg only treatments. Significance level set at P<0.05.

3.5- Gene expression in genes associated with nutrition

The *SWEET16+17* genes showed the greatest difference between the two varieties (Figure 18). In Gallant, individual attacks increased gene expression however, when organisms were applied together or in sequence, gene expression decreased. This pattern contrasted with expression in Sumai with upregulation of the gene significantly due to feeding or due to simultaneous aphid-pathogen attack.

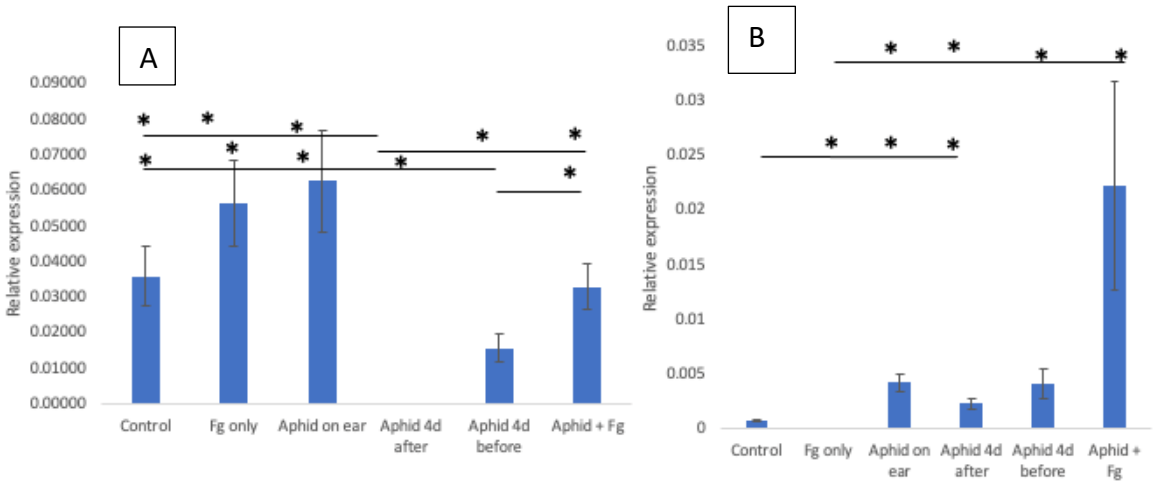

Figure 18; Gene expression in SWEET17,16, transcript used encoded for both genes. A- Gene expression in Gallant (TraesCS5A02G039000), no expression was found in the aphid 4d after treatment, significant differences are shown by lines and Asterix. B- Gene expression in Sumai (TraesCS5B02G040800), no expression was found in the Fg only treatment. Significance level set at P<0.05.

There was one major difference between the two varieties for *UGP1+2* expression. In Gallant, both organisms individually increased expression, however when applied in sequence expression was reduced, diminishing further when plants were inoculated for 4 days prior to aphid feeding. In fact, the plants which were inoculated before aphid feeding showed no expression. In Sumai, expression increased due to the aphid only in both dual and tripartite interactions. When the two organisms were applied in sequence no significant difference was observed. When applied simultaneously expression was greatest, similar to Gallant (Figure 19).

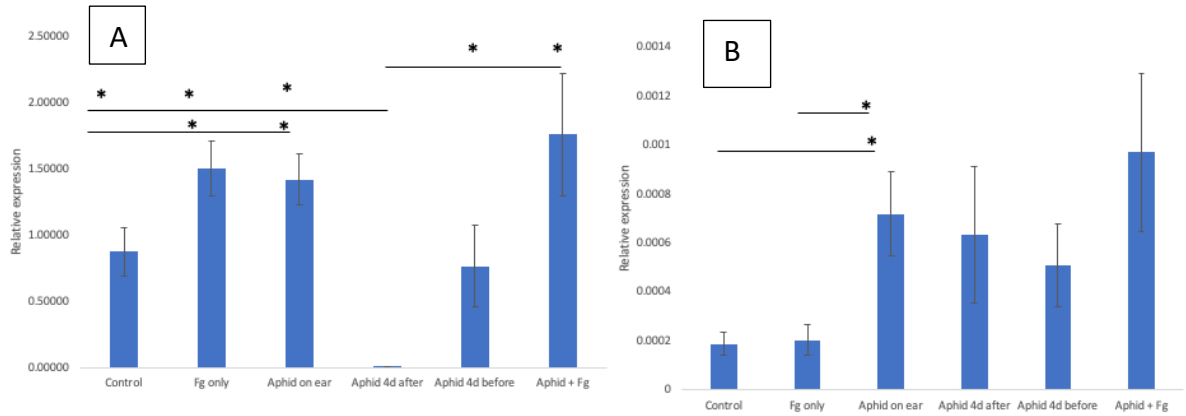

Figure 19; Gene expression in UGP1+2 transcript used encoded for both genes. A- Gene expression in Gallant (TraesCS6A02G050100), significant differences indicted by lines and Asterix. B- Gene expression in Sumai (TraesCS6A02G050100). Significance level set at $P<0.05$.

3.6- Gene expression in sulphur rich secondary metabolites GSTF11+12

GSTF11+12 expression increased when plants were infected before aphid feeding for both varieties but it appeared to show little expression in plants infested by the aphid alone or aphids prior to *F. graminearum*. The only change between varieties was in Sumai, where there was no expression of *GSTF11+12* when inoculated with *F. graminearum* solely (Figure 20). Expression was lowest in plants treated with aphid and *F. graminearum* simultaneously suggesting this gene is most resistance to this treatment.

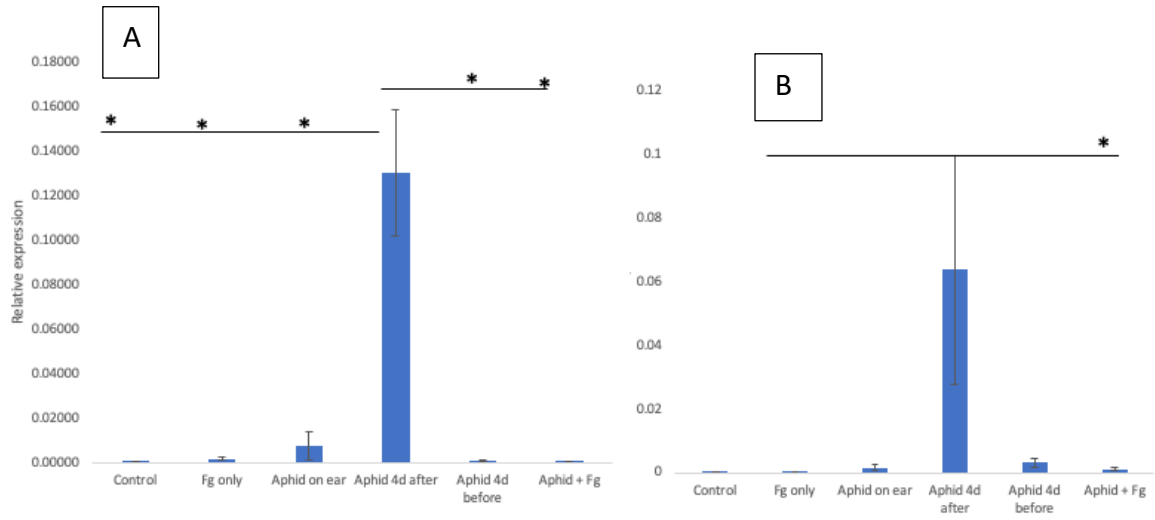

Figure 20; Gene expression for both GSTF11+12 for which the transcript encoded both genes. A- Gene expression in Gallant (TraesCS6A02G050100), significant differences are shown by lines and Asterix. B- Gene expression in Sumai (TraesCS7D02G479100). Significance level set at P<0.05.

3.7- Gene expression in multicopper enzymes, LAC7 and LAC8

For Sumai there were two primers used compared to just one for Gallant. Significant expression was only observed in plants which were inoculated before aphid infestation across both varieties and transcripts (Figure 21) (Figure 22).

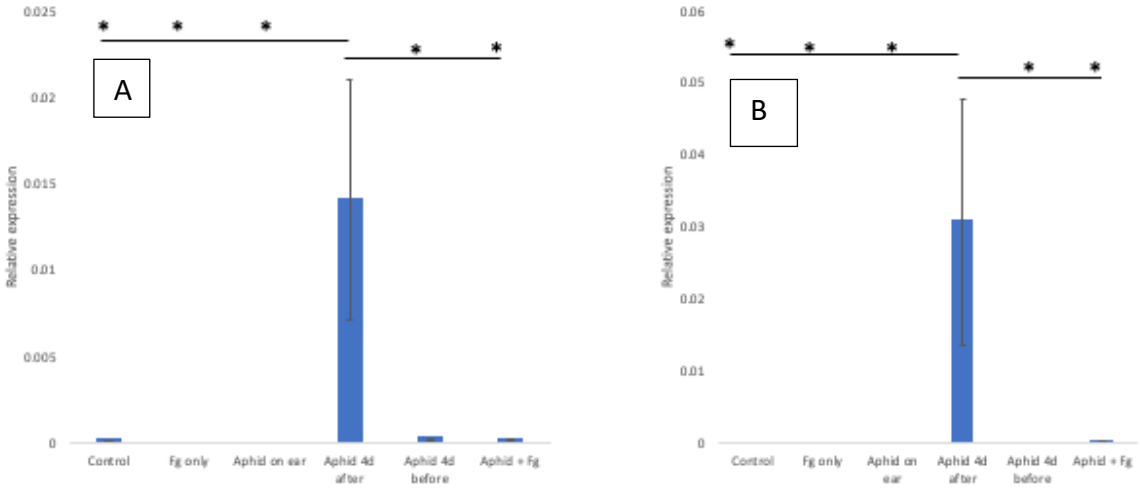

Figure 21; Gene expression for both LAC7,8 transcripts in Sumai. A- Gene expression for the first transcript, significant differences are shown by lines and Asterix (TraesCS3A02G452400). B- Gene expression for the second transcript (TraesCS6A02G058300). Significance level set at P<0.05.

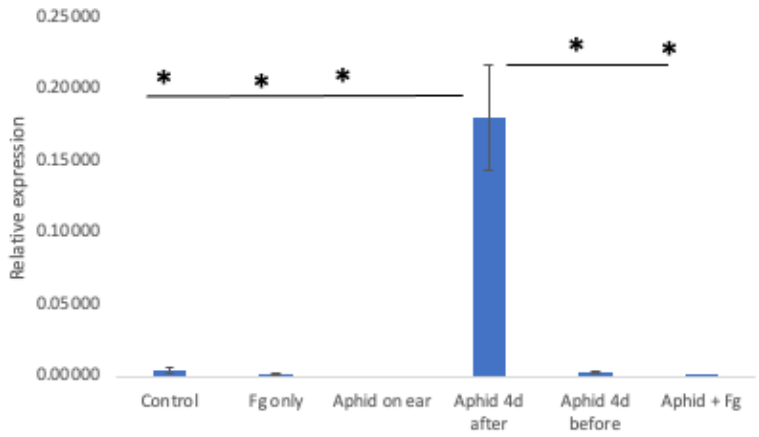

Figure 22; Gene expression graph for LAC7,8 gene in Gallant, only one transcript was available (TraesCS3B02G489700), no expression was seen in the aphid on ear treatment. Significance level set at P<0.05.

Discussion

The major finding from the results is the degree to which the interaction between the aphid and *F. graminearum* increased expression . Defence gene expression was generally greatest in plants which were inoculated before aphid feeding. In the ET signalling pathway, EIN2 is induced early mostly due to the aphid in Sumai leading to upregulation of Rap 2.12 to similar levels as *F. graminearum* infection for 4 days prior to aphid infestation possibly accelerating disease development under aphid influence. In contrast Rap 2.12 is induced mostly due to *F. graminearum* and less in response to aphid feeding in Gallant. In both varieties ANAC087 is induced after 4 days of *F. graminearum* attacking the host prior to introduction of the aphid showing lower expression when the aphid occupies the host first. In the JA signalling pathway, MYB20 is induced following infection of *F. graminearum* for 4 days prior to aphid attack and switching the sequence does not increase expression significantly compared to control or individual attackers. In contrast VSP2 is suppressed by the aphid in Sumai, similar to Gallant with VSP1 and 2 expressed following *F. graminearum* attack first or under aphid influence. In the ABA signalling pathway ABI3 and 5 are again expressed under longer *F. graminearum* infection prior to aphid infestation being less expressed under *F. graminearum* influence alone in Sumai. Auxin response through Yuc10 is associated with both aphid and fusarium susceptibility being mostly expressed highly in tripartite interactions. The opposite is observed in genes involved in nutrition, for Sweet 16,17 and UGP1 and 2 in Gallant where these genes are needed in the initial infection or feeding for the pathogen and aphid, being suppressed in Sumai against individual *F. graminearum* attack indicating that these genes are upregulated under aphid control. GSTF 11 and 12 (sulphur rich secondary metabolites) and LAC 7 and 8 (multicopper enzymes) are generally suppressed by the aphid in tripartite interactions and expressed to Fusarium in later stages of infection in both genotypes. These genes are likely to be important for aphid defence rather than fusarium defence. SHS1 is the only gene expressed more because of aphid influence prior to Fusarium infection in Gallant

The majority of genes experimented upon are those associated with plant hormone signalling. Hormones play crucial roles in regulating plant growth, development and environmental response. Hormones are involved in both antagonistic and synergistic crosstalk leading to changes in expression of the genes involved within the signalling pathway in response to pest and pathogens. Figure 23 indicates known cross talk

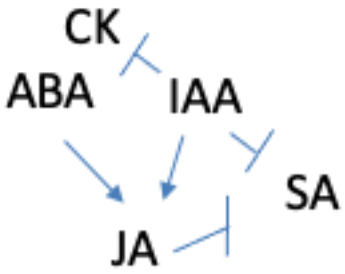

Figure 23; Known crosstalk between plant hormones. Arrows indicate upregulation with baselines used to indicate downregulation.

The ET signalling pathway is involved in antagonistic cross talk with JA signalling. Interaction takes place between *EIN3* and *MYC2* which act downstream in these signalling pathways. *EIN3*, for which no transcripts were found in the MOFA is upregulated by *EIN2*.

EIN2, the furthest up the pathway appears to be upregulated by the aphid, especially in the aphid susceptible variety, Sumai. *F. graminearum* conversely had no effect upon *EIN2* expression. The next step down is *EIN3* and *ETHYLENE RESPONSE FACTORS (ERFs)* of which *RAP2.2,2.12* is grouped among. Upregulation of *EIN2* should lead to increased expression of both *EIN3* and *ERFs* (Binder, 2020). This knowledge would suggest results for *RAP2.2,2.12,* an ET response transcription factor, should be similar to results obtained for *EIN2*, which is confirmed by expression observed in Sumai.

Despite *EIN2* expression showing no difference in Gallant, *RAP2.2,2.12* expression was reduced for aphid and *F. graminearum*. Expression was increased in plants which had been inoculated before aphid infestation. This suggests *RAP2.2,2.12* is involved in aphid resistance response rather than resistance. Changes in resistance could be explained by influences upon *EIN3. EIN3* is upregulated by EIN2 which in turn leads to upregulation of *ERFs* including *RAP2.2,2.12*. Knowledge of cross talk between hormones suggest *EIN3* expression is influenced *MYC2,* a transcription factor involved in the JA signalling pathway. *MYC2* is known to supress *EIN3* (Song et al., 2014). This suppression, in theory would lead to reduced expression of *RAP2.2,2.12* which could be the cause of the changes in expression in *RAP2.2,2.12* compared to *EIN2*.

EIN3, which is upregulated by *EIN2,* in turn, upregulates *ORE1*, another *ERF*, which later leads to *ANAC087* expression (Kim et al., 2014). Despite this pathway suggesting *ANAC087* expression should align with expression seen in both *EIN2* and *RAP2.2,2.12*, results showed neither organism caused expression individually in both varieties. Expression was apparent in the plants which had been inoculated followed by aphid feeding. *EIN3* has another alternate influence on *ANAC087* expression whereby *EIN3* supresses *miR164*, which in turn

supresses *ORE1*. With *EIN3* indirectly promoting and supressing the main promoter of *ANAC087* in theory expression should be similar to *EIN2* which is not the case. Explanations could be that under lower stress conditions *EIN3* preferentially acts to supress *miR164* or acts to expresses other transcription factors as opposed to expressing *ORE1*. Alternatively, under periods of high stress *EIN2* expression would be greater leading to increased *EIN3* expression, altering this potential preferential expression leading to increased *ANAC087* expression. This may offer explanation to why *ANAC087* expression was only observed in plants which had been treated with aphids and *F. graminearum,* but interestingly not when applied simultaneously. There could be other factors such as alternate influencers on any of *ORE1, miR164* or *ANAC087* including the previously stated *MYC2*.

In the JA signalling pathway just two genes were tested upon, at opposite ends of the pathway. *MYB20* is supressed by *JAZ* proteins which are degraded in the presence of JA (Li et al., 2022). Expression of *MYB20* did not change between the two varieties suggesting this gene has negligible effect on either the aphid or *F. graminearum*. Interestingly the aphid appears to act in a way which causes expression to increase when plants are inoculated followed by aphid feeding. *MYB20,* involved in lignin biosynthesis (Geng et al., 2019) is known to supress ABA signalling, a key hormone in stomatal closure in response to stress. Lignin, a key polymer involved in cell wall formation is directly impacted due to pest and pathogen attack and as such, greatest expression was seen when pathogens were present followed by aphids, due to an incubation period of this pathogen

VSP genes are found downstream on the JA signalling pathway and as a result has many more influencing factors than *MYB20. VSP* genes rely upon *MYC2* expression leading to expression of *NACs*, including *ANAC019, ANAC055* and *ANAC072*. These *NACs* lead to *VSP 1 and 2* expression. Unfortunately, the transcripts used varied slightly making comparisons harder, however, *VSP* genes are associated with insect defence (Liu et al., 2005). *VSP* genes are supressed by SA, a key hormone in forming resistance to pathogens (Ding and Ding, 2020) explaining the lack of expression due to *F. graminearum.* The aphid appears to alter this balance as expression was increased in plants which were inoculated first followed by aphid feeding. *PDF1.2* is another gene within the JA signalling pathway which is negatively regulated by SA. *VSPs* and *PDF1.2* are both influenced by *ORA59*. *ORA59* upregulates *PDF1.2* but suppresses *VSPs*. In the presence of SA, *ORA59* is repressed. As transcripts for this gene were not presented from the MOFA, gene expression did not take place, but it appears to be an important gene , as influence upon it is caused by *MYC2* and *EIN3*.

YUC10, expressed in the presence of auxin appears to be upregulated by both the aphid and *F. graminearum* across both varieties. Interestingly, expression in plants treated with *F. graminearum* only seemed to follow the same level as the control. *MYC2* is a main integrator between JA and auxin pathways whereby *MYC2* acts to supress auxin (Kazan and Manners, 2013). This signifies the influence of ET upon auxin through interaction of *MYC2* and *EIN3* Without gene expression data for *MYC2* we are unable to determine the strength of crosstalk.

Two genes encoding for ABA appear to vary in resistance to both pest and pathogen. The greatest expression across both varieties was in plants where aphids were applied after inoculation. This was very similar to *ABI5,* suggesting both of these genes are closely linked.

The fact the greatest expression was observed when organisms were applied in sequence suggest both the aphid and pathogen use the same pathways. In terms of crosstalk ABA is known to upregulate both JA and *MYC2* (Kazan and Manners, 2013). The upregulation of *MYC2* by ABA together with the knowledge that *MYC2* is seen by many to be the major component for hormone crosstalk (Kazan and Manners, 2013), may offer an explanation to why this treatment consistently saw the greatest expression. Previously mentioned, is the negative regulation of ABA by *MYB20*. Expression however is similar between *MYB20* and both *ABI3* and *ABI5*, backing up previous work showing that *MYB20's* interaction with ABA is solely on *ABI1* and *ABI2* (Wang et al., 2021).

UGP genes, essential for sucrose and polysaccharide synthesis are known to be expressed differentially in a variety of organisms with *UGP1* being predominant (Meng et al., 2009). Unfortunately, the only transcript available to use was one which transcribed both *UGP1* and *UGP2*. Expression in Gallant appears to be reduced as a result of aphid feeding after inoculation indicating aphids reduced the previous expression when *F. graminearum* was applied solely. Interestingly this was only the case in the aphid resistant variety (Gallant).

Similar to *UGP, SWEET16+17* genes appear to be downregulated by the aphid in Gallant. In fact, in gallant *F. graminearum* appeared to reduce expression as well. Like with *UGP*, *SWEET16+17* genes are involved in nutrition transport making these two genes highly favourable for both the pest and pathogen to interfere with in order to gain nutrition.

With very little differences seen in the expression of *GSTF11+12* together with the low expression seen for the *F. graminearum* only treatment, it could be inferred that *GSTF11+12* is not acted upon by *F. graminearum* infection. This is further backed up by the similar expression across all treatments in the *F. graminearum* resistance Sumai variety. Similarly low levels of expression in response to the aphid solely suggests this gene is not used in plant defence against both pest and pathogen. However, expression was increased due to the aphid across both varieties in plants which were inoculated beforehand. *GSTFs* have links to both SA (Gullner et al., 2018) and ET pathways (Song et al., 2020). *GSTFs* are known to accumulate during early stages of infection (Gullner et al., 2018), the results however, suggest expression only takes place when plants are infected with *F. graminearum* before aphid feeding.

Laccase genes involved in lignin biosynthesis appear to only be upregulated in plants which were inoculated prior to aphid feeding. Forming part of defence by building a physical barrier to infection expectation would be *LAC7+8* genes are upregulated due to either organism damaging the leaf surface, however it appears that direct interactions between these two organisms and *LAC7+8* expression does not exist. The increase in expression may be a result of the presence of ET which research has suggests causes upregulation of laccase biosynthesis (Ranjbar and Ahmadi, 2016). Results here back this up as greatest expression was seen in the same treatments for both *LACs* and genes involved in ET signalling.

SHS1 is involved in stress response and forms part of the ABA pathway and as such involved in adaptation to multiple stresses (Inan et al., 2007). Expression was only seen when the organisms were applied in sequence and only in Gallant suggesting both the aphid and *F. graminearum* interact causing expression which maybe a result of upregulation

caused by ABA. *MYB20* expression which has previously been discussed to negatively regulate ABA signalling especially in response to salt stress (Wang et al., 2021). *SHS1* corresponding to salt hypersensitive potentially is the point of interaction between MYB20 and ABA signalling which the results support as expression in the treatments switches whereby in treatments that had the two organisms applied in sequence when MYB20 expression was greatest SHS1 expression was reduces and where MYB20 expression was lower, SHS1 expression was higher.

Conclusion

Results show gene expression varies between genes and treatment as indications are given to which genes are involved in individual resistance to either the aphid or *F.graminearum*. Indications are in most genes the two organisms interact leading to greater expression. The majority of the genes show heightened expression in plants which were inoculated with *F.graminearum* before being subjected to aphid feeding.

Genes including *ANAC087, MYB20, GSTF11+12, ABI3, ABI5, LAC7,8* and the *VSPs* showed little to no expression to either organism individually but expression was apparent in plants which were inoculated with *F.graminearum* followed by aphid feeding. *EIN2* and *RAP2.2,2.12* showed increased expression due to the aphid in the aphid susceptible variety Sumai. Whereas *UGP1+2* showed reduced expression in response to the aphid. *SWEET16+17* did not follow the trend whereby greater expression was seen in response to each organism individually and when applied in sequence expression was reduced. *YUC10* was expressed by both aphid and F.graminearum but *SHS1* was only expressed when the organisms were applied in sequence.

This gives an indication as to the specific pathways which are used by the plant to deal with aphid and *F.graminearum* infection. Suggestion from the results are the pathways may overlap when organisms are applied in sequence leading to plants becoming more susceptible to *F.graminearum* infection. Indications are the aphid potentially alters expression either by silencing genes or upregulation of genes, aiding *F.graminearum* infection. Potentially through hormone pathways causing downregulation of alternate pathways and genes.

These results give expression in response to different treatments, but further work is required in order to identify where resistance is caused in the expression pathway. Use of further genes would help indicate and quantify effects of hormone crosstalk such as the effects *EIN3* and *MYC2* upon each other and respective effectors.

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Functional characterisation of genes presented by Multi-Omics Factorial Analysis is response to *F. graminearum* infection and aphid feeding

1- Introduction

The gene expression results in the previous chapter give indications of which genes are upregulated or downregulated in response to aphid and *F. graminearum* interaction. However, due to the vast hormonal crosstalk and signalling pathways it's unclear as to which gene refers to susceptibly and resistance. Here, genes from different hormonal and defence signalling pathways regulated by ethylene (ET), jasmonic acid (JA), gibberellins (GA) and abscisic acid (ABA) were tested. Genes correlating with nutrition supply as well as well leaf morphology were also examined for their influence on susceptibility to both aphids and *F. gramineraum*.

Arabidopsis thaliana plants are susceptible to both aphids and *F. graminearum,* leading to the hypothesis that some genes within this experiment are associated with susceptibility to both organisms apart from two genes *VSP1* and *PDF1.2*. These genes form part of JA response where *VSP1* genes are known to be involved in insect defence and *PDF1.2* involved in pathogen defence (Kazan and Manners, 2013). *MYC2*, a transcription factor responsible for tight regulation of JA response leads to preferential upregulation of *VSP1* (Kazan and Manners, 2013). *PDF1.2* requires crosstalk from the ET signalling pathway (Kazan and Manners, 2013). JA signalling genes are known to act in response to herbivory action (Kloth and Dicke, 2022), whereas ET signalling genes act in response to pathogen attack (Gilroy and Breen, 2022). Changes to *F. graminearum* resistance post aphid feeding is likely to be a result of hormone crosstalk and as such we hypothesise that gene expression will be altered in both JA and ET signalling pathways. Here gene expression in both defence pathways is testede

2- Materials and Methods

Three sets of knockout mutants were subject to experiments to test response to *Fusarium graminearum* and aphids both individually and in combination. Genes to target were identified from the MOFA analysis described in the gene expression chapter. Each experiment was repeated twice, and Table 5 shows the list of mutants used in each experiment. Two genetic backgrounds were used, DELLA is the only mutant used from the *Landsberg erecta* (Ler) background, all other mutants are derived from the *Columbia* (Col-0) genetic background. Statistical analysis was carried out using Genstat® Version 23 for windows (VSN International Ltd, UK). Regression analyses were conducted on all data using a general linear model. Differences between mutants and the wildtype were considered significant at $P<0.05$.

Mutants in the first two sets which gave results of interest lead to the creation of a third set of mutants to be tested in subsequent experiments. The third set underwent identical treatments as the with the first and second set with leaf samples being collected from the third set to conduct gene expression analysis.

Table 5; List of Arabidopsis mutants used across three experiments.

1st set of mutants	2nd set of mutants	3rd set of mutants
Col-0 (Wildtype)	*Col-0 (Wildtype)*	*Col-0 (Wildtype)*
Ler (Wildtype)	*ein3eil11*	*anac087*
anac019	*gstf11*	*ein2*
anac055	*gstf12*	*ein3eil11*
anac087	*lac7*	*vsp1*
della	*lac8*	*lac8*
ein2	*rap2.12*	*myc2*
jazq	*sweey17*	*ugp2*
myc2	*ugp2*	*myb20*
pdf1.2	*yuc10*	*abi5*
vsp1		

Mutants were subject to three treatments, aphid only, *F. graminearum* only and aphid followed by *F. graminearum* inoculation after 7 days.

The experiments allowed assessment of aphid survival and susceptibility to *F. graminearum*. Susceptibility to *F. graminearum* was tested both post aphid feeding and in the absence of aphid influence the aphid influence on defence prior to *F. graminearum* infection.

2.1- Plant growth conditions

All wildtype and mutant lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC) were grown by directly placing seeds on compost in 10cm diameter plant pots. Initially the pots were covered in a plastic sleeve to maintain humidity. Plants were grown in Conviron controlled environment rooms at 20°C in 16-hour light 8-hour dark conditions (Liao et al., 2015). Humidity was set at 70% and light levels at 250µmol m^2 s^{-1} Plants were transplanted at 7-10 days growth into 3x4 potting trays at six plants per tray (Figure 1). Each experiment consisted of either 10 or 11 different wildtype or mutant plants, with six replicates of each, organised in a randomised block design.

2.2- Aphid infestation

First-instar *Myzus persicae* aphids also known as green peach aphid were collected from the University of Nottingham aphid stocks. Aphids were placed within clip cages on the plants. Five aphids were applied per plant. Plants were left under normal conditions. Seven days after application aphids were removed and, any nymphs found were excluded. Aphid survival was calculated as a percentage of the original number of aphids applied on each plant. Figure 24 illustrates the experimental set up.

Figure 24; Example of the plant set up used in all experiments with the clip cages confiding aphids on the plants.

2.3- Pathogen inoculation

All wildtype and mutant plants were inoculated with *F. graminearum* after about 21 days of growth. Plants which had been subject to aphid attack were inoculated with *F. graminearum* same day as the aphids were removed. Fusarium spores (isolate 212 from the University of Nottingham isolate collection) were grown on Potato Dextrose Agar plates (PDA; Sigma-Aldrich,UK) in a 20°C incubator for 5 days. Fungal spore solution was collected by flooding the PDA plate with 1ml of distilled water, the plates were gently scraped, and the spore suspension was removed and diluted to $1x10^{-5}$. Spore infiltration was conducted using a needle-less syringe on the abaxial surface of Arabidopsis leaves (Makandar et al., 2010). Two leaves from each plant were inoculated and marked.

2.4- Disease assessments

Disease assessments were conducted at seven days post *F. graminearum* inoculation. Each inoculated leaf was visually assessed and the percentage area of leaf showing disease symptoms recorded. Examples of infected leaves where disease assessments were taken are shown in Figure 25.

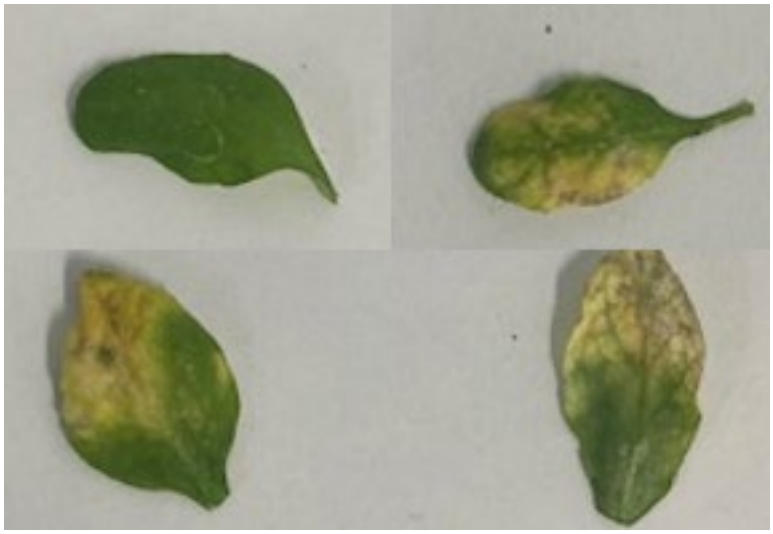

Figure 25; Examples of leaves taken from plants 7 days after inoculation with *F. graminearum.* The four different leaves highlight the variance between disease symptom appearance. Top left= 0% disease, top right= 40%, bottom left= 30%, bottom right= 50%.

3- Results

3.1- *F.graminearum* resistance in Columbia (Col-0) wildtype

As previously mentioned, the presence of aphids prior to feeding has been shown to increase wheat susceptibility to *F. graminearum* (Drakulic et al., 2016). Using the experimental standard wildtype of *Arabidopsis thaliana, Columbia* (Col-0), this hypothesis was tested.

Results showed that aphid feeding prior to the pathogen significantly increased *F. graminearum* infection ($P<0.001$). Disease was doubled due to aphid treatments prior to *F. graminearum* inoculation. Leaves inoculated with *F. graminearum* only, showed 9.83% leaf area disease compared to 19.17% on leaves from plants which were inoculated after aphid infestation. Confirming *F. graminearum* severity increased as a result of aphid feeding prior to infection. From here the knockout mutants were experimented upon, searching for genes which alter *F. graminearum* resistance due to the aphid.

3.2- *F.graminearum* responses in the first set of Arabidopsis mutants

3.2.1- Aphid susceptibility

Aphid susceptibility of Arabidopsis was assessed using aphid survival. In total, six mutants *(anac019, anac087, ein2, jazq, myc2, pdf1,2)* showed significantly lower aphid survival compared to Col-0 (Figure 26). Whereas *vsp1* mutants showed increased aphid survival, indicating *VSP1* is important in plant defence against aphids. *anac055* mutants showed a small reduction in survival however this was not significant compared to the wild type ($P= 0.077$).

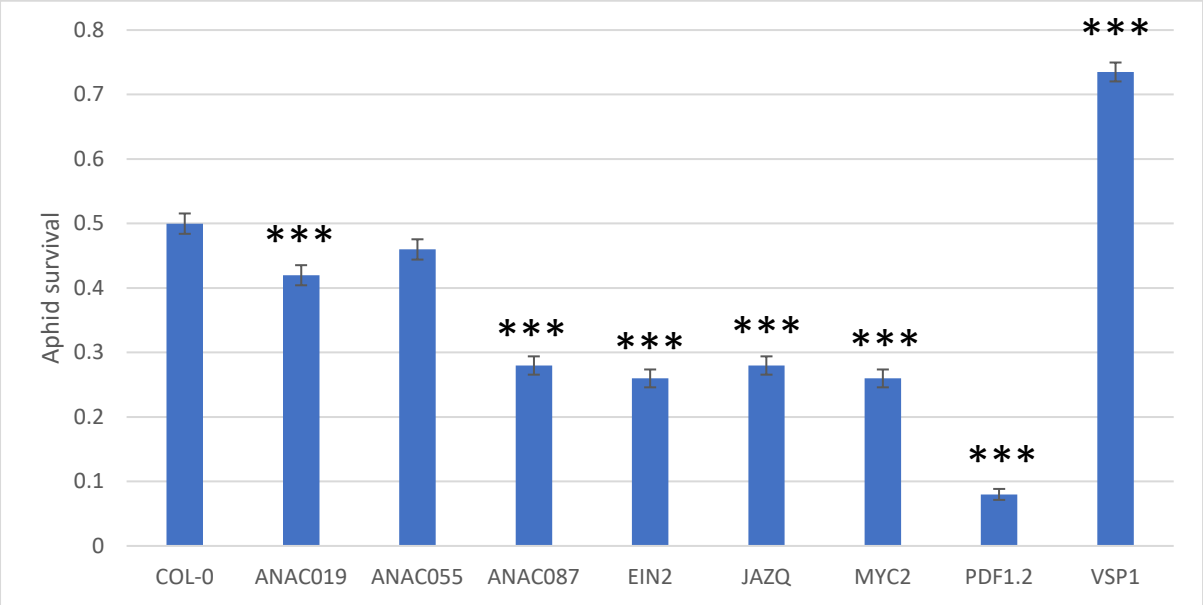

Figure 26 ; Aphid survival of mutants coming from the COL-0 background for the first set of genes. '***' indicates significant differences where P<0.001. All mutants are compared to the COL-0 at P<0.05.

3.2.2- *F. graminearum* infection

Results showed all mutants had significantly reduced disease symptom expression compared to the wildtype (Col-0) (Figure 27). This indicates all genes in this set, when functional are associated with increased susceptibility to *F. graminearum*. Mutants in this set range are from genes associated with JA signalling *(JAZQ, MYC2, ANAC019, ANAC055, VSP1 and PDF1.2)* and ET signalling (*EIN2 and ANAC087*). Suggesting the JA-defence pathway at ET signalling are involved in increased susceptibility to the pathogen.

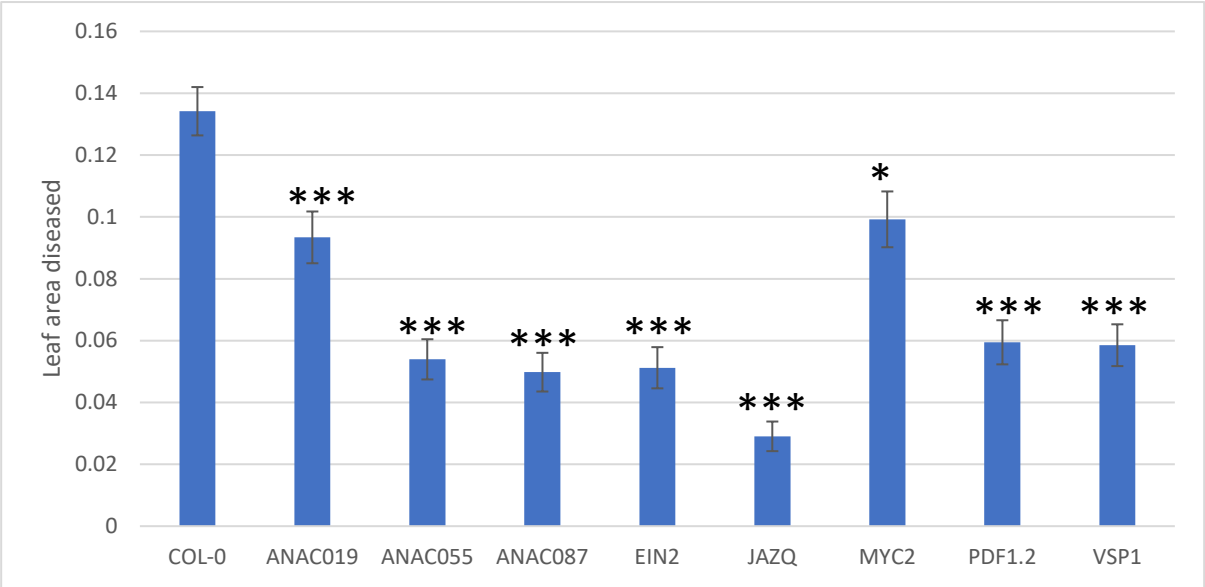

Figure 27; Disease assessment results from the first set of mutants. Data the combination of data from both reps. '***' indicates significant differences where P<0.001. '**' indicates significant difference where P<0.01.

3.2.3- Susceptibility to *F.graminearum* post aphid feeding

As a result of aphid feeding prior to inoculation we observed that resistance responses to *F. graminearum* changed in some mutants (*anac019, anac087, vsp1*) (Figure 28). Specifically, the *vsp1* mutants previously showing resistance to *F. graminearum* showed susceptibility compared to the wildtype (Col-0). *anac087* mutants exhibited greater levels of disease due to the aphid but results were not significant. *anac019* mutants were no significantly different to the wildtype Col-0 despite showing decreased disease levels in *F. graminearum* only treatments. ANAC019 is grouped within the same NAC domain as ANAC055 (Kazan and Manners, 2013) and so differences between these mutants for resistance were surprising.

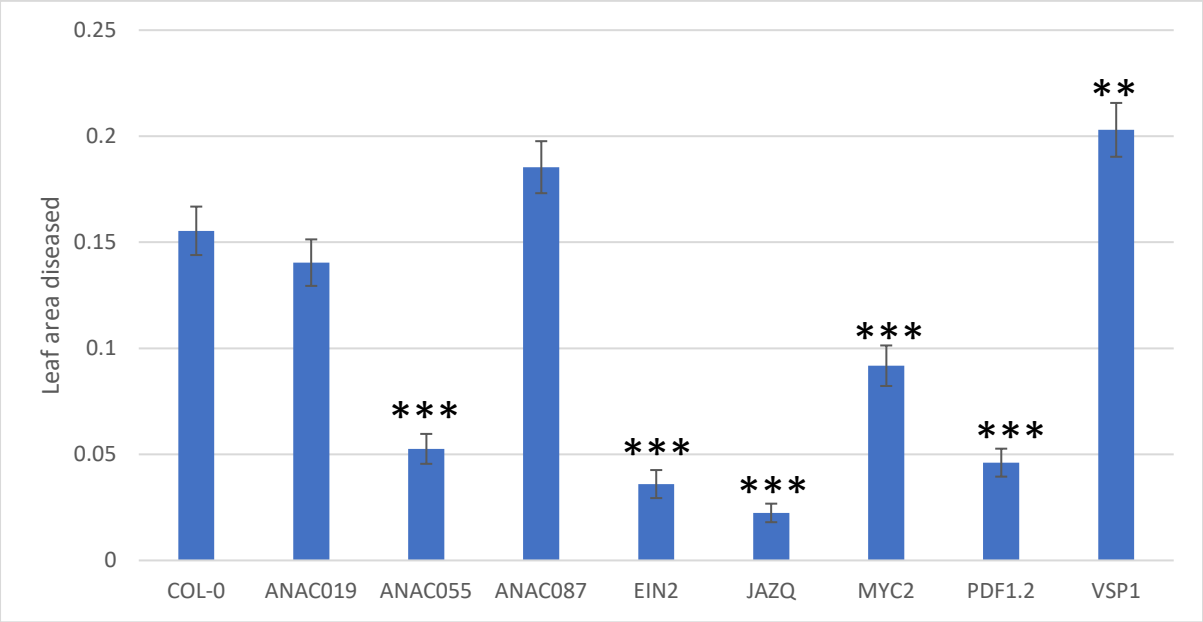

Figure 28; Infection post aphid feeding disease assessment for the first set of mutants in the COL-0 background. Using COL-0 as the control significant differences are indicated by the asterix. '***' indicates P<0.001, '**' indicates P<0.01. Significance level set at p<0.05

3.3- *F. graminearum* resistance in the second set of Arabidopsis mutants

3.3.1- Aphid survival

Mutants in this set contained knocked out genes found within ET signalling pathways (*EIN3, EIL1* and *RAP2.12*) (Binder, 2020), as well as genes known to be upregulated due to ET (*LAC7* and *LAC8*) (Daneshi and Ahmadi, 2014). Also found in this set were genes linked with providing nutrition (*SWEET17* and *UGP2*) (Guo et al., 2013, Meng et al., 2009) as well as genes involved in detoxification of noxious compounds and protection against oxidative damage (*GSTF11* and *GSTF12*) (Zhang et al., 2022). *YUC10* is associated with auxin signalling (Yu et al., 2022). Here we found six mutants which showed significantly lower aphid survival in comparison to the wildtype (Col-0) (Figure 29). The *gstf12* mutants showed increased aphid survival, with *yuc10* mutants showing no significant change. All other mutants significantly decreased aphid survival.

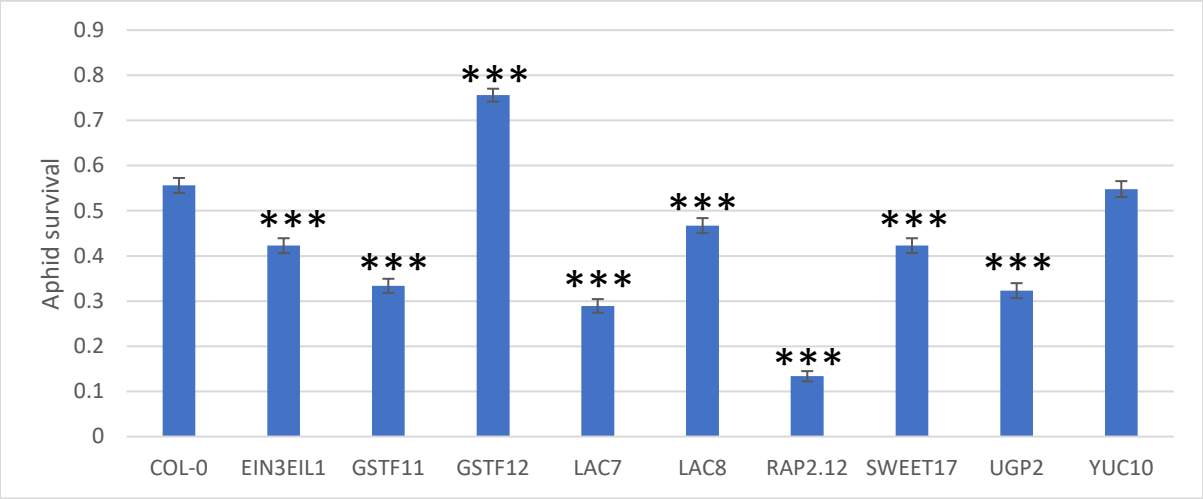

Figure 29; Aphid survival for the second set of mutants. all results are compared to the COL-0 as control. '***' indicates significant differences where P<0.001. . Significance level set at P<0.05.

3.3.2- *F. graminearum* infection

This set highlighted five mutants in which we observed significantly reduced *F. graminearum* infection (Figure 30). Only the *gstf11* mutants showed significantly increased levels of disease, potentially linking the functionality of this gene to *F. graminearum* resistance. Interestingly, *gstf12* showed no significant difference compared to Col-0, despite being closely linked to *GSTF11*. Similar to the first set, mutants of with genes involved in ET signalling and transcription showed decreased disease levels compared to Col-0.

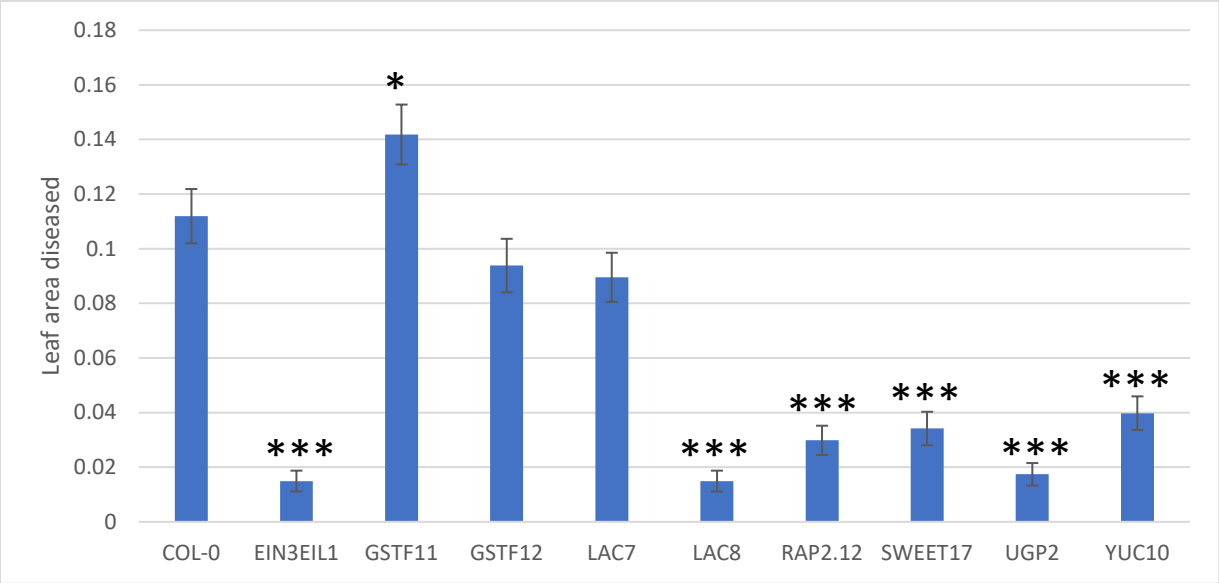

Figure 30; Disease assessment results from the second set of mutants. COL-0 is the control, '***' indicates significant difference where P<0.001. '*' indicates significant difference where P<0.05. Significance level set at P<0.05.

3.3.3- *F. graminearum* susceptibility post aphid feeding

Similar to the first set we again observed responses in mutants to *F. graminearum* resistance change following aphid feeding (Figure 31). Most notable was the *sweet17* mutants which had previously shown reduced disease level compared to Col-0 in the *F. graminearum* only experiment. However, following aphid infestation the *sweet17* mutants showed increased disease levels suggesting the aphid suppressed plant defence. *ein3eil1, gstf11* and *lac8* mutants showed no significant difference despite *gstf11* mutants previously increased disease levels.

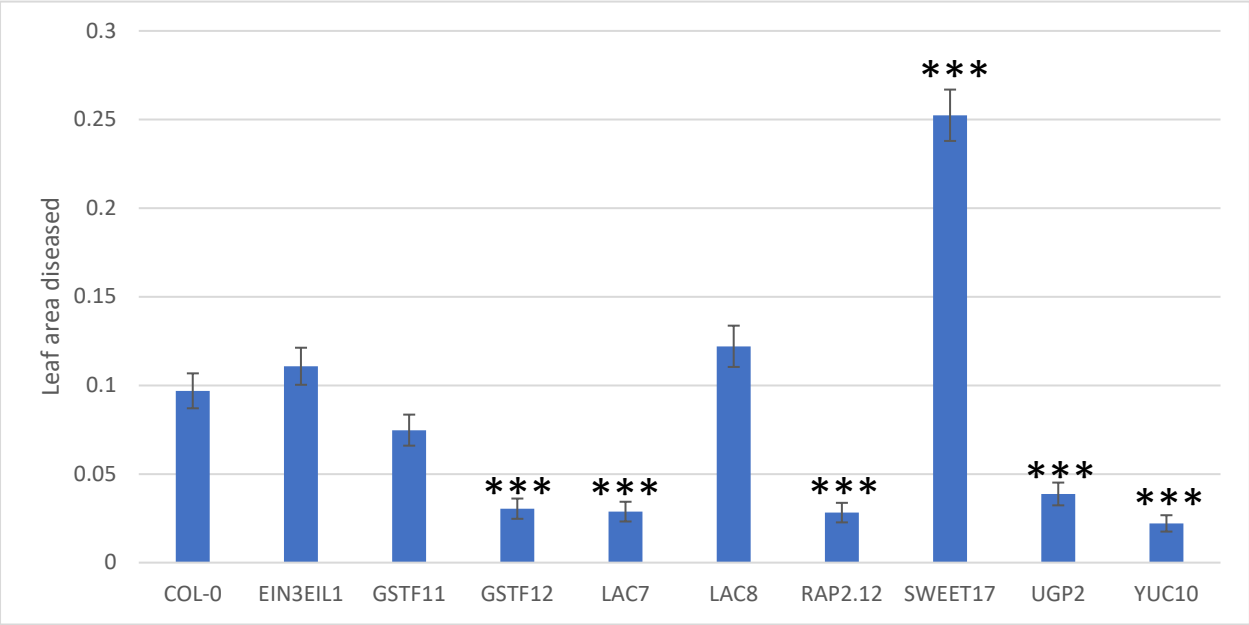

Figure 31; Infection post aphid feeding disease assessment for the mutants in the second set of mutants. Using the COL-0 as the control significant differences are shown by the asterix. '***' indicates P<0.001. Significance level set at P<0.05.

3.4- *F. graminearum* resistance in mutants formed from LER background

DELLA was the only gene presented by the MOFA from a different genetic background (Ler). *DELLA* forms part of the GA signalling pathway (Eckardt, 2007). Here, *della* mutants were found to exhibit increased resistance to both the aphid and the pathogen shown by reduced aphid survival (Figure 32) and *F. graminearum* infection (Figure 33), respectively. This indicates functional *DELLA* genes are associated with susceptibility to both the pest and pathogen. Knowledge of *DELLA's* role GA signalling pathways indicates GAs are not involved in resistance to either aphids or *F. graminearum*. This aligns with previous work completed by Gilroy and Breen (2022).

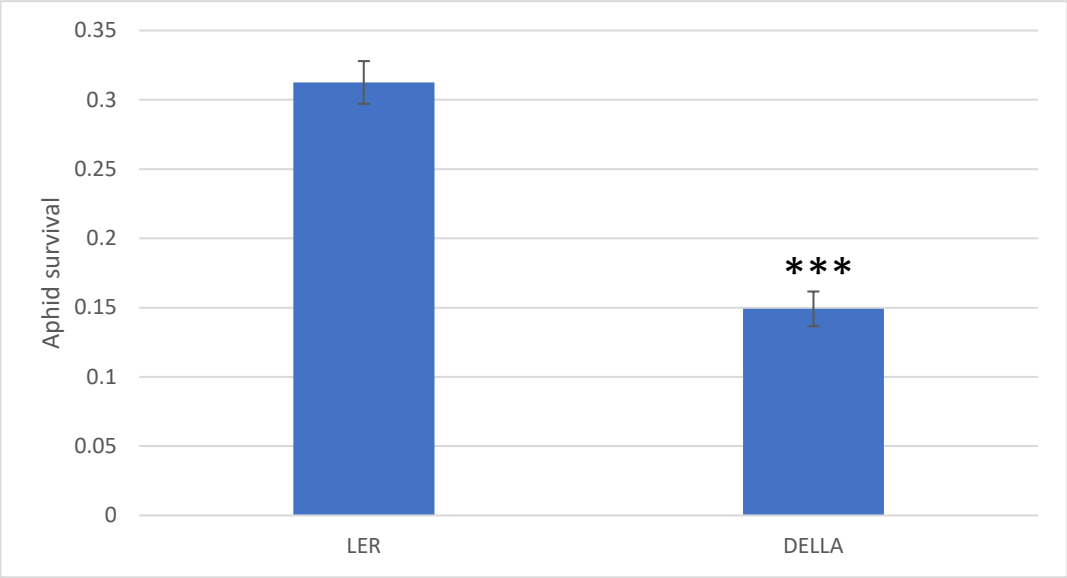

Figure 32; Aphid survival for both the DELLA knockout mutant and the wildtype LER Arabidopsis plants. the results were significantly different highlighted by '***' illustrating a p-value <0.001. Significance level set at P<0.05.

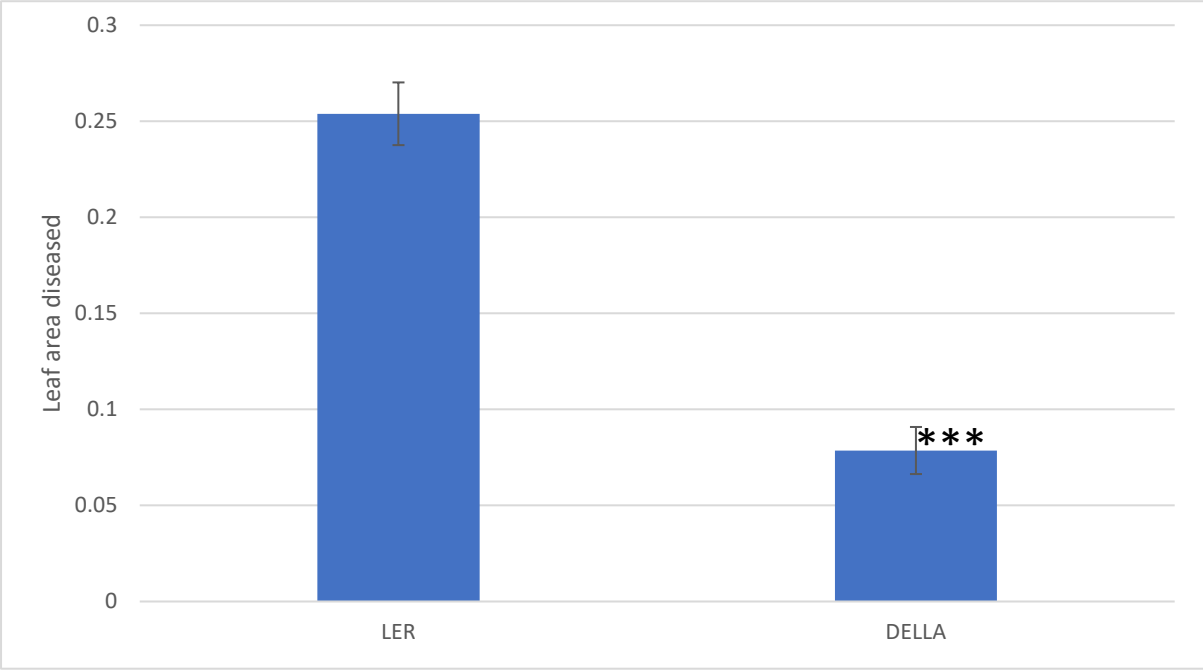

Figure 33; Disease assessment results showing that DELLA knockout mutants significantly reduced disease in comparison to LER. '***' indicates a significant difference where P<0.001. Significance level set at P<0.05.

Interestingly, the aphid appeared to suppress plant defence as when inoculated after aphid feeding *della* mutants showed increase of disease symptoms by *F. graminearum* (Figure 34), suggesting interactions between the two organisms are likely to be modulated by GAs.

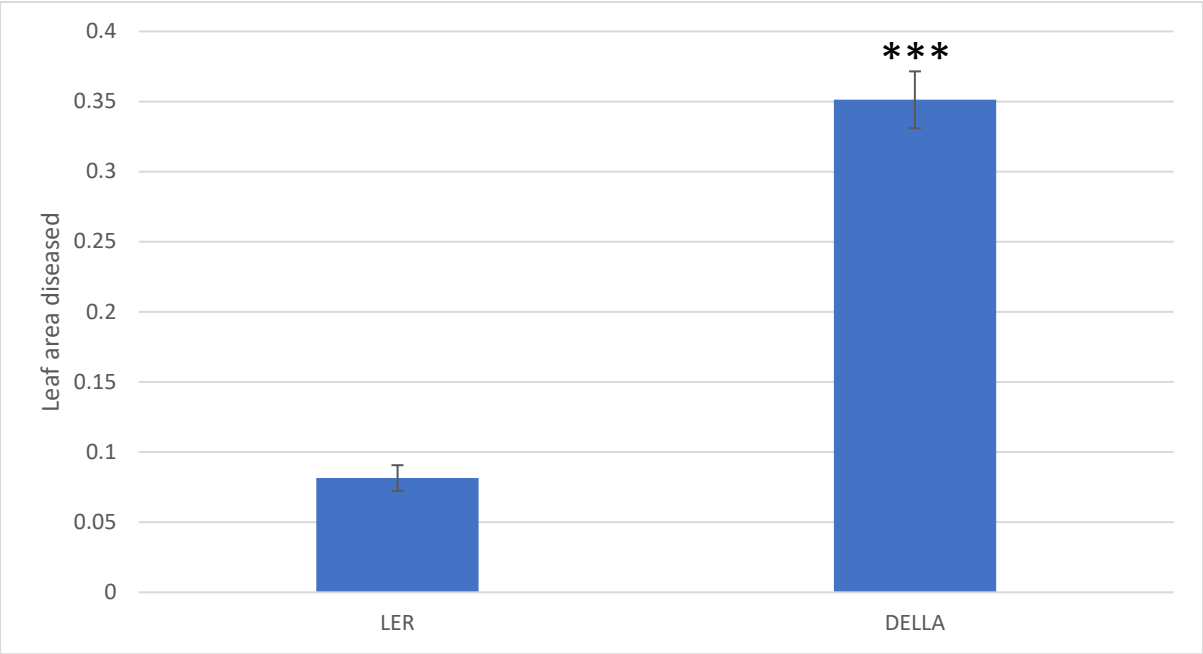

Figure 34; Infection post aphid feeding disease assessment for the mutant in the LER background. Using LER as the control significant differences are indicated by the asterix.

3.5- *F. graminearum* resistance in the third set of knockout mutants

Results from the first two sets of mutants were analysed and mutants which showed changes in pathogen response were included in a third set of experiments with the aim of collecting RNA to examine gene expression. These plants were subject to the exact same treatments. Two previously untested mutants, *myb20* and *abi5* were also included.

3.5.1- Aphid Survival

The two new mutants, *myb20* and *abi5* significantly reduced aphid survival (Figure 36). In comparison to previous experiments, *ein2* and *ugp2* mutants produced identical results. *anac087, pdf1.2* and *myc2* mutants continued to show significantly reduced aphid survival, aphid survival in *vsp1* mutants was still greater than Col-0, however, the difference was not statistically significant at $P<0.05$. The *ein3eil1* mutant was no longer different to the wildtype, despite still showing to reduce aphid survival.

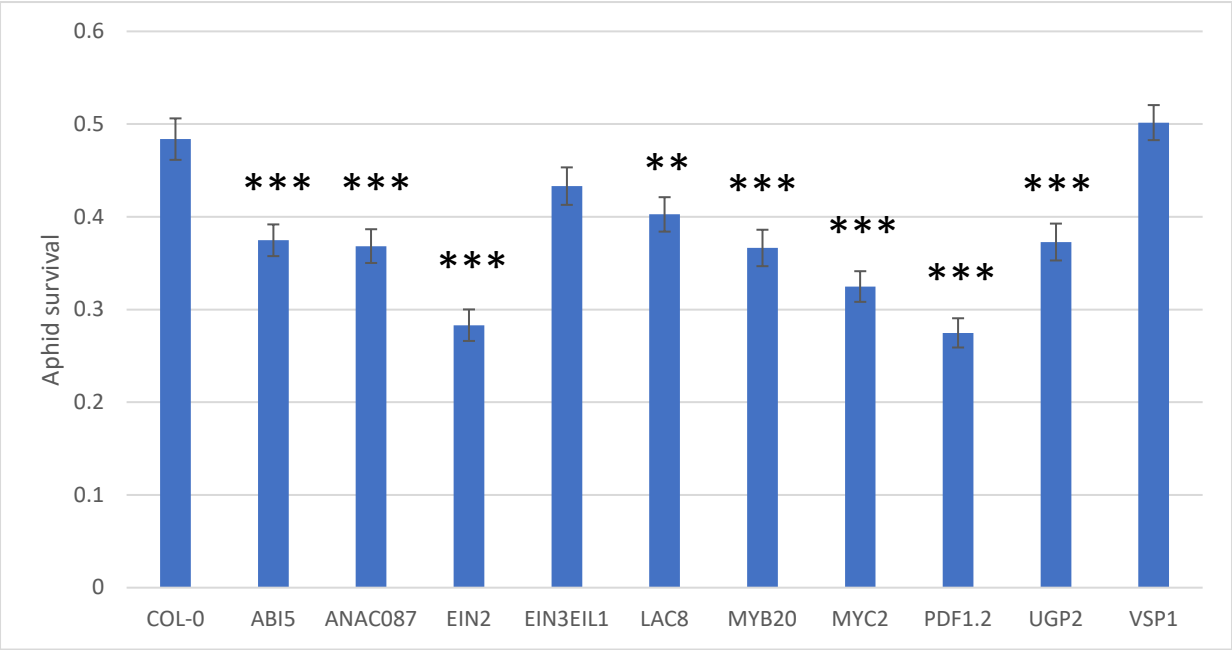

Figure 36; Aphid survival results from the third set of mutants. In comparison to the first two sets both EIN3EIL1 and VSP1 failed to reproduce significantly different results. Significant differences are indicated by the asterix where '***' denotes significant difference where P<0.001 and '**' where P<0.01. Significance level set at P<0.05.

3.5.2- F. graminearum infection

All mutants showed significantly greater resistance to *F. graminearum* infection (Figure 37) as observed previously. *abi5* and *myb20* followed the same trend among mutants with exhibiting reduced disease levels. *MYB20*, involved in JA signalling is downregulated in the presence of stabilised *JAZ* proteins (Pauwels and Goossens, 2011), and plays a role in forming a physical barrier to infection via lignin biosynthesis (Geng et al., 2019) *ABI5* is involved in ABA signalling (Collin et al., 2021) which in turn leads to upregulation of *MYC2* (Kazan and Manners, 2013).

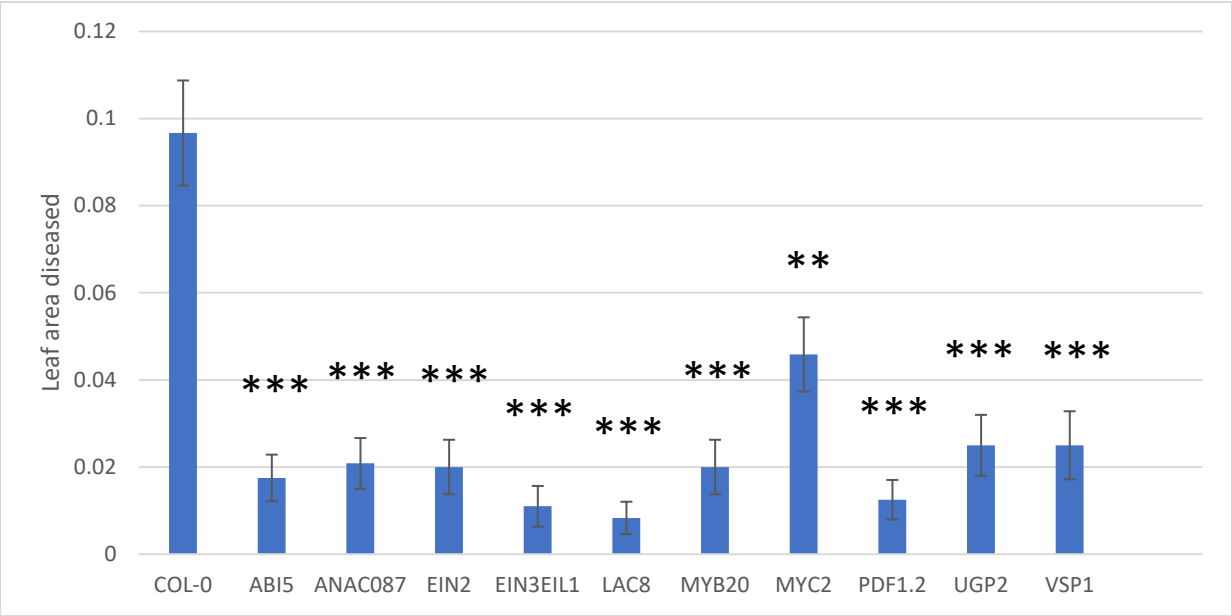

Figure 37; *F. graminearum* disease assessment results for the third set of mutants. All mutants agree with previous findings. '***' indicates significant difference compared to the control

3.5.3- *F.graminearum* susceptibility post aphid feeding

Given knowledge that aphid infestation increases disease susceptibility the overall results from this experiment were expected. In this experiment disease was greater across all mutants than previously seen (Figure 38). *abi5* and *myb20* showed significantly greater *F. graminearum* infection compared to Col-0*. myc2, pdf1.2, ugp2* mutants showed significantly reduced infection whereas *vsp1* still showed significantly increased *F. graminearum* disease symptoms. Previously *anac087*, *ein3eil1* and *lac8* mutants showed no significant differences, these results showed similar trends, however this time they were found to exhibit significantly greater *F. graminearum* disease symptoms compared to Col-0*. ein2* mutants showed a small reduction in disease which wasn't found to be significant, despite previously showing increased resistance to *F. graminearum* infection.

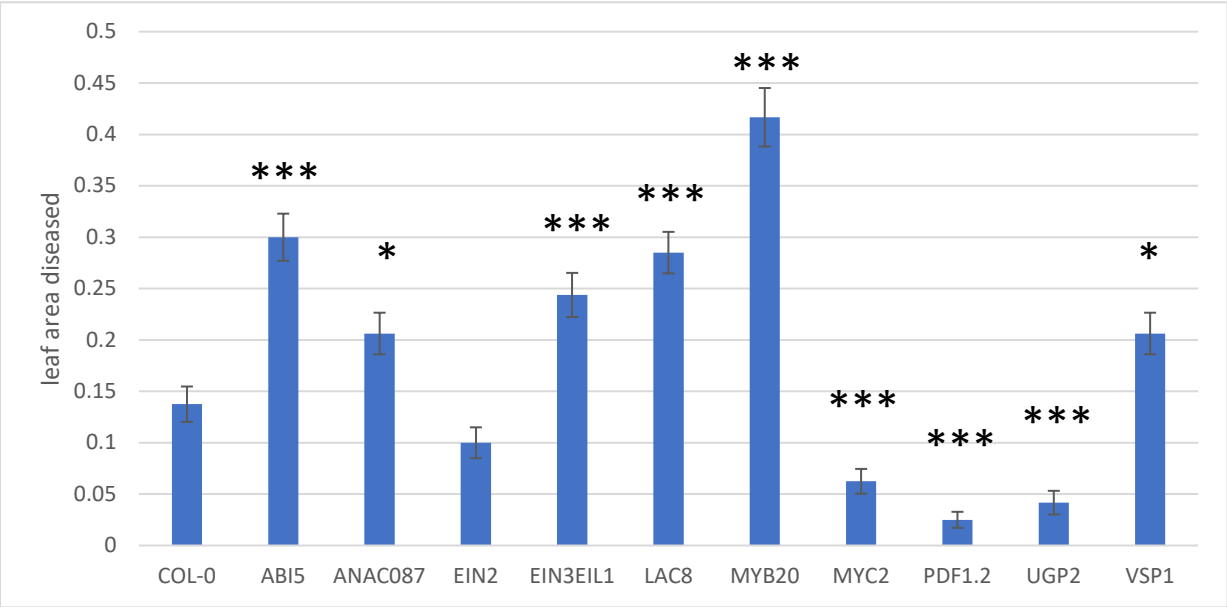

Figure 38; Disease assessment results post aphid feeding on the third set of mutants. Significant differences are indicated by the asterix. '***' indicates P<0.001 and '*' P<0.05. Significance level set at P<0.05.

4- Discussion

EIN2 is a crucial component of ET signalling and modulated EIN3 driven transcription. In the absence of ET *EIN2* is repressed by the *CRT1* protein kinase and ET receptors such as *EIN3EIL1* are degraded in the nucleus (Chang, 2016). Upon ET detection *CTR1* is no longer activated enabling *EIN2* release. *EIN2* release causes stabilisation of *EIN3EIL1* through inhabiting the protein translation of F-box proteins *EBF1/2* (Figure 3)

Our results showed that EIN2 functionality is required for susceptibility to both organisms. This was expected due to *EIN2* working upstream of other genes involved in ET signalling and transcription considered key regulators of ethylene response which help shape ethylene response (Dolgikh et al.,2019). The double mutant *ein3eil1* is fully ethylene insensitive (Dolgikh et al.,2019) and our results show that in plants treated with aphids first, ET insensitivity leads to increased resistance to *F. graminearum*.

EIN3 shape ET response through interactions with genes located downstream in ET signalling. *ETHYLENE RESPONSE FACTORS (ERF's)* including *RAP2.12* and *ORA59* are directly upregulated by *EIN3* (Dolgikh et al., 2019). *RAP2.12*, regulated plant response to hypoxia stress (Kozmacz et al., 2014) however, the mutant was resistant to pathogen infection indicating the gene functionality will be increasing susceptibility. The other *ERF*, *ORA59* standing for *OCTADECANOID-RESPONSIVE ARABIDOPSIS 59* is considered to act as an integrator between the ET and JA crosstalk and is key in ensuring the expression of *PDF1.2* (Yang et al., 2021). *PLANT DEFENSIN* gene, *PDF1.2* is a pathogenesis-related gene, which is known for its actions against necrotrophic invaders (Kazan and Manners, 2013). However, our results indicate *PDF1.2* is associated with susceptibility to all treatments. *PDF1.2*

participates in cross talk with SA with SA acting to decrease *PDF1.2* expression (Kazan and Manners, 2013).
It is possible that in the *pdf1.2* mutants SA may contribute to plant defence fitting with reduced disease symptoms in *pdf1.2* mutants.

Kim et al., (2009), found a trifurcate feed forward pathway in which EIN3 directly enhances expression of the *NAC* protein *ORE1* (Kim et al., 2014). In this pathway *EIN3* also directly suppresses the expression of *miR164* which itself negatively regulates *ORE1* (Li et al., 2013). This suggests *EIN3* is vitally important in *ORE1* expression due to simultaneously controlling expression of *ORE1* and the negative regulator *miR164* (Kim et al., 2014). *ORE1* activates *ANAC087* in which results here found ANAC087 corresponded to *F. graminearum* resistance only after aphid feeding. The trifurcate feed forward pathway found by Kim et al., (2009) is illustrated in Figure 6.

EIN3 shares an antagonistic relationship with MYC2 whereby they repress each other which antagonistically regulates wounding or pathogen responsive gene expression (Zheng et al., 2017). *MYC2*, a JA activated transcription factor is the main point for interaction between the ET and JA signalling pathways (Song et al., 2014). Here we found *MYC2* to be susceptible to all treatments, confirming that this transcription factor is vital for response to pathogen and herbivore attack. MYC2 interacts with many genes downstream in JA response (Figure 5) making it highly likely that potential resistance maybe found downstream of MYC2.

EIN3 and *MYC2* interact antagonistically upon expression of *ORA59*, *MYC2* acts to repress expression (Kazan and Manners, 2013). *MYC2* regulates a NAC-domain including the transcription factors *ANAC019* and *ANAC055* (Kazan and Manners, 2013). These *NAC's*, have been proven to positively regulate *VSP* as well as negatively regulate *PDF1.2* (Bu et al., 2008). Results from the two *nac* mutants suggest susceptibility to both aphids and *F. graminearum*. Inoculation post aphids resulted in *anac019* mutants showing no difference compared to *COL-0*. Along this *MYC2* controlled pathway aphid resistance is only apparent only in *VSP1.*

MYC2, known to be acted upon by two other hormones, GA and ABA antagonistically. GA influences *MYC2* through *DELLA* proteins. GA's role within the plants involves regulating growth and development which is achieved through degradation of *DELLA* proteins (Gomez et al., 2023). *F. graminearum* resistance switched in the *della* mutants. *DELLA* genes were found to change to resistance when inoculated after aphid feeding. Explanation for this change stem from *DELLA* proteins repressing *MYC2* expression (Kazan and Manners, 2013), and in their absence *MYC2* expression is enabled. This will have a twofold affect whereby both *ANAC019* and *ANAC055* will be leading to expression of *VSP1* which results show this could be the site of resistance to *F. graminearum* infection post aphid feeding. However, greater *MYC2* expression will lead to greater expression of *VSP1's* repressor, *ORA59*, suggesting resistance may be located elsewhere.

GA and JA signalling pathways interact through *JAZ* proteins. At low GA levels *DELLA* proteins reduce *JAZ* expression resulting in a reduction of repressive effects that *JAZ* has on *MYC2* (Hou et al., 2010). When *DELLA's* inhibitory effects on *JAZ* are prevented, *JAZ* proteins

bind to *MYC2,* inhibiting *MYC2* function upon dependent JA signalling outputs (Hou et al., 2010), such as *EIN3, ORA59* and both *ANAC019* and *ANAC055*.

JAZ proteins directly interact with the lignin biosynthesis transcriptional factor, *MYB20* (Makandar et al., 2012), a defence mechanism used against pest and pathogens. Research completed by Geng et al. (2019) found in the absence of *MYB20* caused growth defects as well as reductions in lignin biosynthesis. Our results indicate *MYB20* corresponds to susceptibility to the aphid but resistance to *F. graminearum* after aphid feeding. This is likely a result of the aphids causing upregulation of JA signalling due to wound sites caused by aphids, leading to upregulation of MYB20 in an attempt to heal these wounds. Wounds can be a potential infection site but quick and potentially excessive expression of MYB20 leads to lignin helping to heal this wound as well as potentially reducing pathogen susceptibility by exhibiting greater cell wall defence.

RAP2.12, part of the ERFs are transcription factors upregulated by *EIN3. RAP2.12* has been found to regulate central metabolic processes to sustain growth, development and anoxic resistance in plants (Melanie Verena Paul et al., 2016). These results suggest *RAP2.12* corresponds to susceptibility to all organisms experimented with.

ABA is known to promote stomatal closures which in turn mediates pathogen infection by regulating the size of stomatal aperture which the pathogen could use as an entry point (Collin et al., 2021). This is backed up by these results where *abi5* mutants much like *myb20* mutants shows an increase in disease only when *F. graminearum* inoculation is preceded by aphid feeding as the combination of aphid feeding puncture hole and lack of stomatal closure creates more points for infection. ABA has been shown to promote the degradation of *JAZ* proteins and in turn upregulate JA biosynthesis (Collin et al., 2021). However, if *JAZ* proteins are not degraded, they have been shown to repress *ABI5* (Collin et al., 2021). Other research suggests ABA also increases expression of *MYC2* (Kazan and Manners, 2013), and as such the previously mentioned *VSP1* gene will be expressed further. This pathway is backed up by the results for *ABI5* and *VSP1* mutants only showing results corresponding to resistance when plants were infected post aphid feeding.

Auxin is known to have different effects on disease, dependent on the type of pathogen. In necrotrophic fungi, greater amounts of auxin leads to decreases disease susceptibility, whereas in biotrophic pathogens auxin promotes disease (Kunkel and Johnson, 2021). *F. graminearum* being hemibiotrophic (Xu et al., 2022) whereby the fungi exhibits both necrotrophic and biotrophic stages. These disease assessments undertaken are in a relatively short time frame so expectancy would be for *YUC10,* involved in auxin biosynthesis (Hentrich et al., 2013) to be associated with susceptibility. Results backup this hypothesis as *yuc10* mutants showed lower disease compared to the control across both *F. graminearum* experiments. *YUC10* appears to have no effect on aphid survival. Auxin has numerous transcription factors including *PLETHORAs (PLTs)* which are supressed by *MYC2* (Jang et al., 2020). This highlights the cross talk between auxin and JA, more specifically the role of JA in regulating auxin levels. However, with *MYC2* corresponding to susceptibility to all organisms used in this experiment it seems unlikely that this pathway is of significance to

any changes in resistance caused by the aphid. Auxin can have both positive and negative effects on ET signalling. Positively, auxin has the ability to increase the stability of *EIN3*. However, negative effects include during periods of high auxin concentrations ET can be stimulated which can inhibit root and shoot elongation (Strader et al,. 2010).

The two *LAC* genes as with *MYB20* are involved in lignin biosynthesis however the two LAC genes gave contrasting results. *LAC7* seemingly suggests susceptibility to *F. graminearum* due to the aphid, whereas *LAC8* functionality indicates resistance increase due to aphids owing to its role in lignin biosynthesis. Research suggests that laccase biosynthesis can be upregulated by ET (Ranjbar and Ahmadi, 2016). This knowledge could explain why some of the genes upstream in the ET signalling pathway failed to show resistance *F. graminearum* especially after aphid feeding.

Plant glutathione S-transferases (*GSTs*) have been found to be induced in early phases of bacterial and fungal infections and some *GSTs* are activated by SA specifically *GSTF11* (Gullner et al., 2018). This knowledge binds well with results where *GSTF11* corresponds to susceptibility. This susceptibility may not be due to the gene but also where *GSTF11* is upregulated in the presence of SA which will have negative effects on *VSP1* which we have found to be important in *F. graminearum* resistance after aphid feeding. Research has shown links between *GSTF12* and the ET pathway whereby plant mutants lacking the *ETR1-1* gene (ET receptor mutant) showed increased accumulation of *GSTF12* (Song et al., 2020) indicating potential suppressing effects ET has upon *GSTF12*. Some *GSTs* respond to environmental stimuli such as pathogen and pest attack (Gullner et al., 2018) offering potential explanations for the resistance to aphids shown in these results.

UGP2, involved in UDP-glucose production which is used in sucrose and polysaccharide synthesis proved in these results to be susceptible to both the aphid and *F. graminearum.* Results are probably understandable due to *UGP2* providing sugars which both aphids and the pathogen will use to provide nutrition to survive and develop. Research has found that *UGP2* is not influenced by either ethylene or ABA (Ciereszko and Kleczkowski, 2006). *SWEET17* also involved in providing sucrose to both the aphid and pathogen which explains why our results represent *SWEET17* showing susceptibility to aphid and *F. graminearum*.

5- Conclusions

A major takeaway from these phenotyping results is the vast extent of hormone crosstalk found within plants. The full extent of affect transcription factors from the GA signalling pathway have on the final expression of genes which are yet to be fully examined however we can say they are affected in some way. The major influencer in this cross talk between GA, JA, ABA and ET is *MYC2*. Interestingly *MYC2* main influence on other transcription factors and genes is to supress apart from both *ANAC019* and *ANAC055*. This is significant as this pathway leads to upregulation of *VSP1* a gene shown by these results to show resistance to the aphid. *MYC2* acts to increase *VSP1* regulation through supressing *ORA59* which is significant due to suppressive actions shown by *ORA59* upon *VSP1.*

Unfortunately, we had no access to an *ora59* mutants as similar to *MYC2* it is a crucial integrator in hormone crosstalk, particularly between ET and JA as well as SA and JA. Despite working to supress *VSP1, ORA59* acts to increase *PDF1.2*, known to be expressed during fungal infection, however, results showed *PDF1.2* to correlate with susceptibility to both *F. graminearum* and the aphid. *PDF1.2* may not show suppressive effects upon the fungus due to presumed elevated SA level, however SA also suppresses *VSP1*. VSP1 however did show expected results, potentially linking to differences in which SA supresses both of these.

Like with *ORA59* we were unable to gain access to *ore1* mutants which lies between *EIN3* and *ANAC087* in the ET signalling pathway. Both *EIN3* and *ANAC087* saw susceptibility change to resistance to *F. graminearum* as a result of aphid interactions making ORE1 a gene of interest. *MYB20* is also a point where susceptibility changes to resistance. All these mutants share a common theme, they are all supressed due to *JAZ* proteins and as such these mutants are expressed in a greater extent when the JA signalling pathway is induced.

MYB20 and *ABI5* are involved in leaf morphology and actions which have highlighted the need for such genes in forming a physical barrier to infection specifically when acted upon by both organisms. The same results were seen for *LAC8* which is involved in lignin biosynthesis. The two genes involved in supplying nutrition both showed susceptibility to aphid and *F. graminearum*. *SWEET17* did show resistance when the two organisms were applied in sequence suggesting *SWEET17* in acted upon by the aphid which in turn reduces infection.

These results give a strong indication as to where both resistance and susceptibility stems from however due to the large complex web of cross talk it is unclear at this stage to confidently claim how resistance is formed or diminished by the aphid. . These mutants are being investigated because they were genes implicated in interactions of the aphid with the plant. RNA is required to be collected from these plants in order to test gene expression within these mutants to form a stronger idea as to where resistance lies.

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Gene expression analysis in selected *Arabidopsis thaliana* mutants in response to varying treatments of aphid infestation and *Fusarium graminearum* inoculation

1- Introduction

Findings from previous chapters in this work showed that susceptibility to *F. graminearum* changed as a result of aphid feeding prior to infection. Hormone pathways form both synergistic and antagonist relationships, most notably the antagonistic cross talk between salicylic acid (SA) and jasmonic acid (JA). SA has been shown to downregulates two main JA responsive genes, *VSP1/2* and *PDF1.2* (Tamaoki et al., 2013).

Previous chapters found that aphids caused upregulation of genes in the ethylene (ET) signalling pathway. *VSP1* expression, induced due to JA was increased due to aphid feeding. In phenotyping experiments the majority of the genes involved in ET signalling were associated with susceptibility to aphids. In this instance *VSP1* was found to show resistance to the aphid. The same mutants were found to show susceptibility to *F. graminearum*, however, *vsp1* mutants switched from susceptibility to resistance to *F. graminearum* infection when inoculated post aphid feeding. *ANAC087* also showed similar changes indicating hormonal response changes in response to aphid feeding. qPCR experiments give quantified gene expression, highlighting pathways of both synergistic and antagonistic cross talk. Gene expression analysis of different mutants allows in depth knowledge of changes in expression due to both removal of key signalling genes and different treatments.

ET is elicited by insect attack and plays an important role in induced herbivore response through accumulation of defensive proteins (Lu et al., 2014). Work on Arabidopsis found ET mutants to be more resistant to some herbivores (Bodenhausen and Reymond, 2007). *VSP1* is induced by physical attack and JA (Kazan and Manners, 2013). Sucking insects trigger SA response (Ali et al., 2024) and SA response acts to downregulate *VSP1* expression, however the *vsp1* mutants showed increased susceptibility to aphids in previous chapters. Figure 5 proposed by Kazan and Manners, (2013), illustrates the downstream network of JA signalling beyond *MYC2.*

MYC2 regulates insect defence including the gene, *VSP1* in antagonistic pattern to pathogen defence. Figure 1 shows there are still questions remaining over the entirety of the pathway in addition to the genetic control of the responses to either attacking organisms. ET and JA have been shown to be involved in antagonistic cross talk through repression of *MYC2* or *EIN3* expression (Song et al., 2014). ET signalling results in direct upregulation of *ORE1* by transcription of *EIN3*. Downstream, *ANAC087* is positively regulated by *ORE1* (Kim et al., 2014).

Here we set out to investigate the expression of marker genes in Arabidopsis mutants on the existing pest-pathogen defence pathways regulated by ET and JA, shown in Figure 5. Mutants and genes were chosen based on their phenotypes to pest, pathogen and pest-pathogen sequence attacks shown in the previous chapters.

2- Method

The third set of mutants were selected in order to test gene expression within the mutants of genes identified in the MOFA (Chapter 1) or previous phenotypic analysis. RNA was collected from the final (3rd) set of mutants which had been subject to four treatments; Control, aphid only, inoculated with *Fusarium graminearum* and Aphid plus *Fusarium graminearum* together. Each treatment had three reps and leaf samples were collected to extract RNA at the same time, seven days after the respective treatments were applied. Expression of the following genes was quantified in the *Arabidopsis thaliana* wildtype Col-0. Genes linked to ET signalling such as *EIN2, EIN3, MIR164a, ORE1* were tested as well as JA signalling genes*, MYC2, ORA59, PDF1.2* and *VSP1.* Description of gene function are found in the previous chapter.

2.1- RNA extraction in Arabidopsis

RNA extraction was completed using a RNeasy plant mini kit (Qiagen, UK). One leaf, (2 cm^2) from each plant was collected and placed into an Eppendorf tube which was immediately flash frozen in liquid nitrogen and stored in a -80°C freezer until extraction. For the extraction small glass beads were added to the Eppendorf tubes before being placed into a TissueLyser to break down the plant tissues before adding the lysis buffer as per the manufacturer's RNeasy plant mini kit protocol. RNA quantification was made using a nanodrop machine. cDNA synthesis was performed as per manufacturer's instructions before being placed in -80°C freezer. The RNA was again synthesised into cDNA and diluted down to 20mg/L and stored at -20°C before being used in RT-PCR assays.

2.2- Gene expression and analysis

cDNA from the wildtype (Col-0) was run in a CFX96 PCR machine (Biorad, UK) with selected primers (Table 6) to assess how gene expression changed in response to different treatments. Expression was compared to a housekeeping gene (Actin2).

Table 6; List of primer design for each gene including both forward and reverse primers.

Target Gene	Primers
Actin2 (Housekeeping)	F- GATTCAGATGCCCAGAAGTCTT R- TGGATTCCAGCAGCTTCCAT
EIN2	F- GGTCTAGAATGAACCAGATATTCATGA R- AGTGCTGCTAACTGCTTCCT
VSP1	F- CTGGTCGTGGTTAGAGTCCG R- TCGATCCGTTTGGCTTGAGT
MYC2	F- CGACGGCGGAGCTGGAGATTTAT R- GATTCGGGTTTTCGGTTATTGTGC
ORA59	F- CTCTGCTTCTACAATTTTTATG R- CTACACATCTATACATGTTTCC
ORE1	F- CTTACCATGGAAGGCTAAGATGGG R- TTCCAATAACCGGCTTCTGTCG
PDF1.2	F- CACATACATCTATACATTGAAAAC

	R- CAGCAAAGAGAACAAGAG
EIN3	F- ACATGGTGGAAGGAAGTT R- TTGCCGCTACTGTTATTG
MIR164a	F- TCAATGCGTTACATATGCTG R- CCATGCCATAGAGTAGATGC

3- Results

Greatest expression of *MYC2* was found in plants which had been inoculated after aphid infestation. *EIN2* expression was low in plants treated with each organism individually with greatest expression seen in plants treated with both organisms. *EIN3* was greatest in response to *F. graminearum* only with the aphid appearing to downregulate expression. *MIR164a* was also found to be downregulated due to the aphid. *ORE1* expression was only recorded in response to aphid only. *PDF1.2* expression was present in plants treated with aphid only, expression was seemingly greater in response to the aphid, however the results produced a large standard error. *VSP1* expression was greatest in response to *F. graminearum* only treatments. *VSP1* expression was decreased due to the aphid. *ORA59* was expressed in low quantities in response to each organism individually, no expression was observed in plants treated with both organisms (Figure 39)

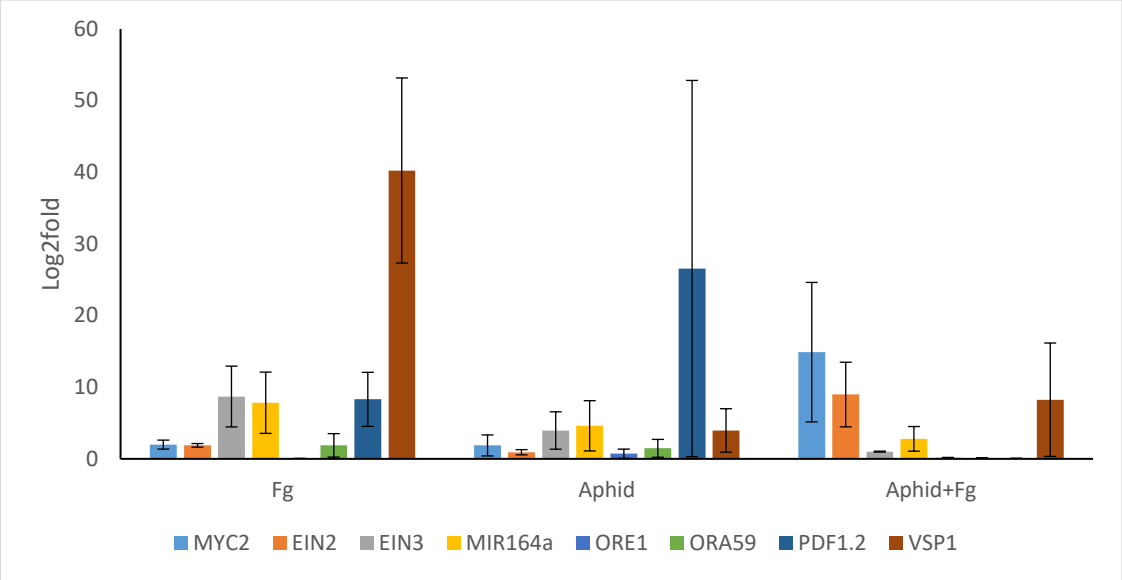

Figure 39; Gene expression in Col-0 plants in response to different aphid and F. graminearum treatments.

4- Analysis

The results show very contrasting results and often do not follow the expected expression pathways based on previous research. The lack of expression of *EIN2* results in attempts to follow the expression pathway harder, especially as resistance to *F. graminearum* is thought to be within this pathway. Additionally, MYC2 is not expressed in large amounts, and given this transcription factor is known to be the master regulator of JA signalling (Kazan and

Manners, 2013) attempts to follow the pathway are tricky. Given both signalling pathways are known to act in respond in plant defence it is surprising gene expression is low for both genes (EIN2 and MYC2) situated upstream in the signalling pathways. Hormonal cross talk which often acts antagonistically may explain the low expression.

Hormone cross talk exists between JA and both gibberellins (GA) and abscisic acid (ABA). GA is widely known for its growth promoting actions (Gilroy and Breen, 2022). Expression of GA is reduced in plant immune response to both necrotrophic pathogens and herbivores (Gilroy and Breen, 2022) suggesting the presence of either organism reduces GA signalling. GA downregulates *MYC2* via *DELLA* proteins (Kazan and Manners, 2013). ABA conversely positively regulates *MYC2* expression (Kazan and Manners, 2013). plant immune response causes increased levels of ABA in response to necrotrophic pathogens (Gilroy and Breen, 2022). In the presence of JA, JAZ proteins are degraded, removing the suppression placed upon MYC2 by JAZ (Kazan and Manners, 2013). Knowledge of this signalling pathway maybe the cause of low expression of MYC2.

Kazan and Manners (2013) stated MYC2 to be the 'Master' in regulating JA response and Figure 1 shows its influence upon other genes and transcription factors, leading to expectation of large MYC2 expression in response to these organisms. Due to the many regulatory functions carried out by *MYC2* there may be suggestions that *MYC2* may be a short-lived (before carrying out regulatory functions) transcription factor which may not be quantified from qPCR results.

MYC2 is crucial in mediating JA insect defence through upregulation of *VSP1* genes (Kazan and Manners, 2013). This part of the pathway is clearly upregulated by *F. graminearum* only, resulting in large VSP1 expression, further backing up the potential for *MYC2* to be a short lived transcription factor as relatively small *MYC2* expression was observed in this treatment. Conversely *MYC2* negatively regulates JA pathogen defence through downregulation of *ORA59* leading to inhibition of *PDF1.2* expression (Kazan and Manners, 2013). ORA59, shown here to only be expressed in response to the organism individually is an additional point of ET and JA antagonism where *MYC2* downregulates expression (Kazan and Manners, 2013) and *EIN3* upregulates expression (Song et al., 2014). This would explain why ORA59 showed no expression in response to aphid and *F. graminearum* treatments, as EIN3 was not expressed in this treatment either. *MYC2* leads to *VSP1* upregulation through a group of NAC's *(ANAC019, ANAC055 and ANAC072*). Kazan and Manners (2013) proposed the *NAC's* downregulate *ORA59* (Figure 5). These results however do not allow us to confirm or deny this proposed downregulation. Lack of ORA59 expression across all treatments maybe explained by downregulatory effects SA has on ORA59. SA also downregulates *VSP1* and *PDF1.2* (Kazan and Manners, 2013)

ORE1 regulation is tightly regulated by *EIN3. EIN3* both directly upregulates *ORE1* and indirectly leads to upregulation through downregulating *MIR164* (microRNAs) which plays vital regulatory roles (Fang, Xie and Xiong, 2014). *MIR164* acts to downregulate *ORE1* but in the presence of *EIN3* this affect is limited (Kim et al., 2014). *ORE1* was only expressed in plants subject to aphid infestation only, which is surprising given the sole positive regulator of *ORE1* is *EIN3* (Kim et al., 2014), yet EIN3 was expressed in greater amounts in response to *F. graminearum*. MIR164a is the only negative regulator of ORE1, however, the changes

seen in MIR164a expression is not mirrored by ORE1. This suggests ORE1 must be influenced by another gene. ORE1 is of significance to here due to its upregulation of ANAC087 (Kim et al., 2014), a gene which we observed *F. graminearum* resistance reduce due to aphid infestation.

5- Conclusion

If *MYC2* is in fact the master regulator and a central node between JA and ET signalling the transcription factor is short lived. By this we propose *MYC2* functions rapidly to regulate downstream genes such as the group of *NACs* leading to *VSP1* expression as well as downregulating *ORA59*.

JA and ABA crosstalk together lead to upregulation of *MYC2* specifically in response to necrotrophic pathogens which simultaneously leads to upregulation of, *VSP1* associated with insect defence, and downregulation of *PDF1.2* associated with pathogen defence.

Our results were not able to confirm the potential link between the *NAC* domain upregulated by *MYC2* and *ORA59*. Suggestions are that another transcription factor influences *ORE1* expression due to expected expression not being apparent in these results. Importance around this is linked to the changes in *F. graminearum* resistance shown in *anac087* mutants in the phenotyping results in the previous chapter.

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Discussion chapter- Further links between JA and ET signalling

Results from all three experiments show that aphid interaction caused increased *Fusarium graminearum* incidence in *Arabidopsis thaliana*. Phenotyping results for the wildtype (Col-0) plants indicated the degree to which *F. graminearum* incidence increased with aphid infestation prior to infection. With this knowledge we set out to find which genes were accountable for the decreased *F. graminearum* resistance in response to aphids.

A number of genes showed reduced *F. graminearum* resistance in response to aphid infestation prior to infection. This change in resistance was best seen in the Arabidopsis phenotyping results. We found *ABI5, ANAC087, EIN3EIL1, LAC8, MYB20* and *VSP1* gene functionality to correspond with *F. graminearum* susceptibility in *F. graminearum* only treatments before becoming resistant due to aphid infestation. Of these genes it was expected that *ABI5, MYB20* and *LAC8* would see resistance increase due to the aphid due to their function in leaf morphology. *ABI5* is involved in stomatal closure (Collin et al., 2021), *MYB20* is crucial in lignin biosynthesis (Geng et al., 2019) as well as *LAC8* (Hiraide et al., 2021). All these help in forming a barrier to infection. The aphid by its piercing and sucking nature will ultimately cause these genes to be upregulated as the plant response to herbivory attack.

EIN3 works upstream of *ANAC08*7 in ET signalling, however this transcription factor is responsible for regulating gene expression further downstream and is not an ERF, unlike *RAP2.12* which showed consistent susceptibility to *F. graminearum*. At *EIN3* ET branches to impact *RAP2.12, MIR164* and *ORE1* along with other ERFs . The resulting effect of *EIN3* is *ORE1* and *RAP2.12* expression and *MIR164a* downregulation. *MIR164* then acts to downregulate *ORE1* constituting in *EIN3* having complete control of regulation of *ORE1*. This is an important fact due to *ORE1* leading to upregulation of *ANAC087* which like *EIN3EIL1* showed *F. graminearum* resistance increased due to aphid interaction. This is despite research suggesting *ANAC08*7 to negatively regulate responses to fungal toxins, specifically fumonisin B1 (Mahmood, 2014). This research was backed up by our results in Arabidopsis mutants in which *anac087* mutants increased resistance to *F. graminearum*. Figure 40 illustrates the complexity of JA and ET signalling pathways.

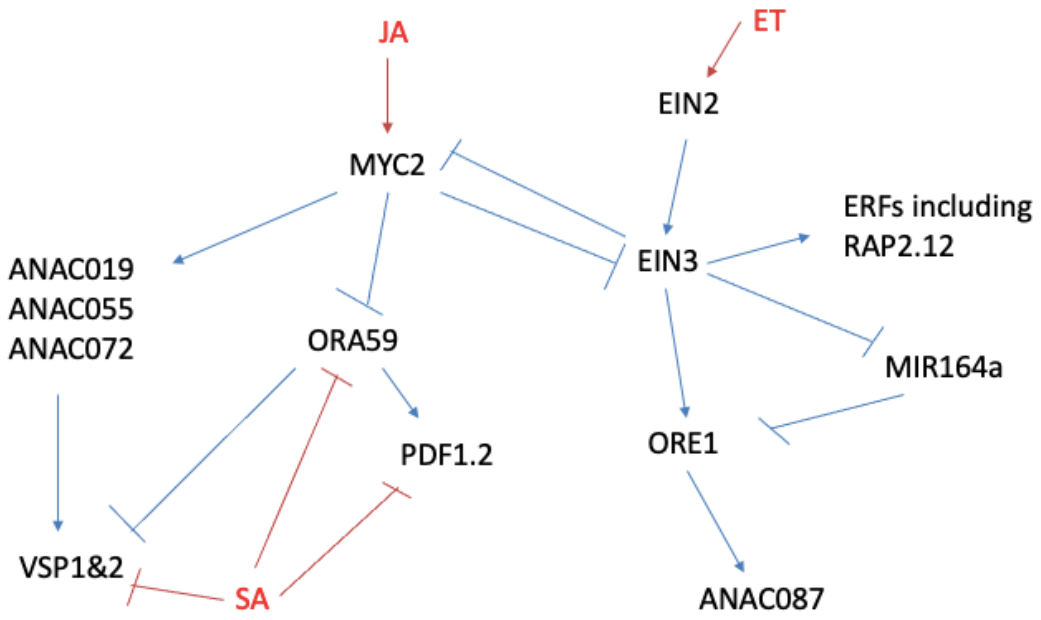

Figure 40; JA and ET signalling pathways. lines and arrows in red indicate direct hormone effects on regulation

On the JA side of the signalling pathway crosstalk shown in Figure 40, *VSP1,* directly regulated by JA*,* expressed susceptibility to *F. graminearum* only treatments but showed increased resistance in response to aphid infestation. Of the two genes at the base of Figure 40 both VSP1 *and* ANAC087 are inversely regulated by MYC2 which is both directly expressed by JA and indirectly suppressed by ET. Interactions between aphid and *F. graminearum* was observed to increase *ANAC087* expression where by greater expression was caused by *F. graminearum. VSP1* however wasn't quantified in this research due to *VSP2* transcripts used for expression analysis in wheat. Arabidopsis expression of *VSP1* showed greatest expression was found in response to *F. graminearum*.

VSP1 is associated with insect defence, which is backed up in this research and as such all treatments which included aphid infestation resulted in resistance for vsp1 mutants. Signalling pathway between JA and ET shown in Figure 6 shows no direct interactions between *ANAC087* and *VSP1*. However, gene expression in Arabidopsis shows even in *ein3* mutants, *ORE1* is expressed indicating this expression may be influence by another gene, potentially linking expression with *VSP1*.

We propose ORA59 and ORE1 interact whereby ORA59 acts to downregulate ORE1 leading to ANAC087 expression under normal conditions. The impact of the aphid however, known to lead to VSP1 expression, would if this link were present to lead to increased ANAC087 due to downregulatory effects of MYC2 upon ORA59. This would ultimately result in reduced downregulation by ORA59 upon ORE1 leading to increased ANAC087 expression leading to increased *F. graminearum* resistance due to aphid interaction. Gene expression in Arabidopsis did find ORE1 was only expressed in aphid only treatment which helps to back up this proposed pathway. However, we did find *ANAC087* to correspond with *F. graminearum* susceptibility.

To confirm this link future work would have to be through the use of ORA59 and ORE1 Arabidopsis mutants and gene expression undertaken. Further work should take place to confirm the potential pathway suggested by Kazan and Manners (2013) between the NAC domain containing ANAC019, ANAC055 and ANAC072 and ORA59 as this may alter ORA59 expression.

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