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**Functional evaluation of plant defence signalling
against *Fusarium graminearum* and *F. culmorum* in
Arabidopsis floral tissue**

Submitted by

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To the University of Exeter as a thesis for the degree of Doctor of Philosophy in
Biological Sciences, December 2014

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Abstract

Fusarium Ear Blight (FEB) is a globally important floral disease of cereal crops such as wheat, maize and barley. The predominant causal agents of FEB disease of wheat in the UK are *Fusarium culmorum* and *F. graminearum*. Wheat infecting isolates of both of these fungal species infect the floral and silique tissues of the model plant *Arabidopsis thaliana*, providing a tractable model for analysis of factors determining plant susceptibility or resistance to Fusarium infection.

The effect of *F. culmorum* infection on the metabolic composition (metabolome) of *Arabidopsis* pedicel tissue following silique inoculation was investigated in a collection of mutants with altered defence responses to *F. culmorum* and/or other plant pathogens, using a ¹H-NMR/ESI-MS (+/-) triple fingerprinting approach. These mutants showed differing metabolomic fingerprints in the absence of *F. culmorum* infection, as well as differences in accumulation or depletion of metabolites in response to *F. culmorum* colonisation. A number of metabolites were also identified which were induced by *F. culmorum* infection irrespective of plant genotype. Quantitative differences in compound accumulation were also observed between genotypes in the Columbia and Landsberg *erecta* accessions following *F. culmorum* infection.

One of the genotypes investigated was *eds11*, which has enhanced susceptibility to *F. culmorum* floral infection. Mapping of the mutation responsible for the *eds11* phenotype was initiated using an isogenic mapping by sequencing approach. This resulted in a list of potential candidates for the *EDS11* gene.

Additional *Arabidopsis* mutants were investigated for altered defence responses to *F. culmorum* floral infection. Multiple mutant alleles of the *Arabidopsis* homoserine

kinase gene *DMR1* were found to have enhanced resistance to *F. culmorum* silique infection and rosette leaf colonisation, associated with accumulation of homoserine in siliques and delayed leaf senescence. Exogenous homoserine application enhanced resistance in wild type and *dmr1* plants.

Collectively, these findings form a novel contribution to current knowledge of the Fusarium-Arabidopsis interaction. This may have applications for improvement of FEB resistance in cereals.

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List of Abbreviations

µg	microgram
CAPS	cleaved amplified polymorphic sequence
CDPK	Calcium Dependent Protein Kinase
cfu	colony forming unit
Col-0	Columbia - 0
CWDE	cell wall degrading enzyme
CYMMIT	Centro Internacional de Mejoramiento de Maíz y Trigo (International Centre for Improvement of Maize and Wheat)
d.f.	degrees of freedom
<i>dmr</i>	<i>downy mildew resistant</i>
DNA	deoxyribonucleic acid
DON	deoxynivalenol
dpi	days post inoculation
ds	double stranded
ECP	extracellular protein
<i>eds</i>	<i>enhanced disease susceptibility</i>
EMS	ethyl methanesulfonate
ESI	electrospray ionisation
ET	ethylene
FAD	Fusarium-Arabidopsis Disease
FEB	Fusarium Ear Blight
FFA	free fatty acid
GABA	γ-aminobutyric acid
GC	gas chromatography
HCA	hierarchical cluster analysis
HR	hypersensitive response
HST	Host specific toxin
JA	jasmonic acid
KEGG	Kyoto Encyclopaedia of Genes and Genomes
kg	kilogram
KGR	kaempferol-glucosyl-rhamnoside
KRR	kaempferol-3,7-dirhamnoside

LC	liquid chromatography
Ler-0	Landsberg <i>erecta</i> - 0
LSD	least significant difference
MAMP	microbe associated molecular pattern
MAPK	Mitogen Activated Protein Kinase
Mb	Mega base
ml	millilitre
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
PAMP	pathogen associated molecular pattern
PCA	principal component analysis
PCD	programmed cell death
PG	polygalacturonase
ppb	parts per billion
PR	pathogenesis related
PRR	pattern recognition receptor
pv.	pathovar
QTL	Quantitative trait locus
<i>R</i> gene	<i>Resistance</i> gene
RNA	ribonucleic acid
ROS	reactive oxygen species
<i>S</i> gene	<i>Susceptibility</i> gene
SA	salicylic acid
SBS	sequencing by synthesis
SED	standard error of the difference
siRNA	small interfering RNA
SLAT	Sainsbury Laboratory <i>Arabidopsis thaliana</i>
SNP	single nucleotide polymorphism
spp.	species
TCA	tricarboxylic acid cycle
var.	variety
WGS	whole genome sequencing
Ws	Wassilewskija

1 Introduction

1.1 The Threat of Pathogens to Global Food Security

Pathogens of crop plants pose a major threat to global food security, and to our ability to meet the challenge of sustainably feeding 9 billion people by the year 2050 (FAO, 2009). Not only does more food need to be grown on existing agricultural land, but this needs to be done with less energy-intensive inputs such as chemical fertilisers and pest and disease control agents, in the face of additional challenges posed by factors such as climate change (Godfray *et al.*, 2010).

Climate change, along with international travel and trade, is contributing to the global spread of pathogens of both plants and animals, especially fungal pathogens (Fisher *et al.*, 2012, Bebber *et al.*, 2014, Bebber *et al.*, 2013). Furthermore, climate change associated environmental perturbations such as temperature changes and altered rainfall patterns will likely alter the outcome of interactions between plants and microbes which already co-exist in agricultural systems (Chakraborty and Newton, 2011, Francl, 2001).

Existing methods to control plant diseases rely heavily on the use of chemical inputs, such as the use of pesticides to control insect pests and manage their activity as virus vectors, and fungicides to control diseases caused by fungal pathogens. The sustainability of these control measures is in question for a number of reasons, such as the potential environmental damage they cause, the energy required to produce them, inherent and emerging pathogen and pest resistance to existing chemistries, and the lack of development of novel chemistries (Fraaije *et al.*, 2007, Deising *et al.*, 2008, Fan *et al.*, 2013, Phillips McDougall, 2013, Government Office for Science, 2011). Use of these chemistries may also be limited by bans such as that currently imposed in the EU on neonicotinoid pesticides, and new legislation such as the EU

plant protection products directive (2009/128/EC). It is therefore imperative to find new, durable ways of controlling crop diseases while sustainably intensifying agricultural practices.

1.2 Pathogens of wheat

Cereals account for the majority of crops grown and consumed worldwide, with wheat being the most widely grown crop in the world and comprising a fifth of the global calorific intake (Curtis, 2002). Bread wheat (*Triticum aestivum*) is the predominant crop grown in the Northern hemisphere, with approximately 1.8 million hectares grown each year in the UK. Wheat is consumed directly by humans, and also used as livestock feed. Pathogens affecting wheat yield and grain quality therefore have a significant impact on global food production. It is estimated that without crop protection measures, wheat yields would be reduced by up to 50% by crop pests and pathogens. Even with the use of crop protection strategies these losses are estimated to amount to 29% of the potential yield (Oerke, 2006).

While cereal crops are susceptible to a small number of diseases caused by viral and bacterial pathogens, the majority of significant pathogens of wheat and other small grain cereals are fungi (Strange and Scott, 2005, Attwood, 1985, Curtis, 2002, HGCA, 2014). This is particularly true in regions of high productivity and intensification (Oerke, 2006).

Every part of the wheat plant is potentially susceptible to infection by fungal pathogens (Figure 1.1). The roots are susceptible to diseases such as take-all caused by *Gaeumannomyces graminis* var. *tritici*, (Figure 1.1a) and root rot by *Cochliobolus sativus*, while *Oculimacula* spp. and *Puccinia graminis* cause eyespot disease and black rust, respectively, on stems. Leaf diseases include brown and

yellow rusts caused by *Puccinia triticina* and *P. striiformis* respectively (Figure 1.1b), powdery mildew caused by *Blumeria graminis*, and Septoria Tritici Blotch (STB) caused by *Zymoseptoria tritici* (Figure 1.1c - formerly *Mycosphaerella graminicola* sexual stage, *Septoria tritici* asexual stage). STB is arguably the most important disease of winter wheat in the UK.

Floral (ear) diseases of wheat include glume blotch caused by *Parastagonospora nodorum*, and Fusarium Ear Blight (FEB), caused by several species of the genus *Fusarium* (Figure 1.1d). Bunt (*Tilletia spp.*), while currently well controlled by chemical seed treatments, is another potential threat to cereal ears (HGCA, 2014, Curtis, 2002).

While some fungal wheat pathogens, such as the powdery mildews and the rusts, can only survive on living plant tissue, many are soil borne or can grow and survive on crop debris, meaning they can persist for large periods of time in the environment. This is true of the main causal agents of Fusarium Ear Blight (FEB), which are the focus of this thesis. While species of the genus *Fusarium* infect a diverse range of hosts and tissues, *Fusarium* in the context of this thesis refers to the causal agents of FEB, unless otherwise stated.

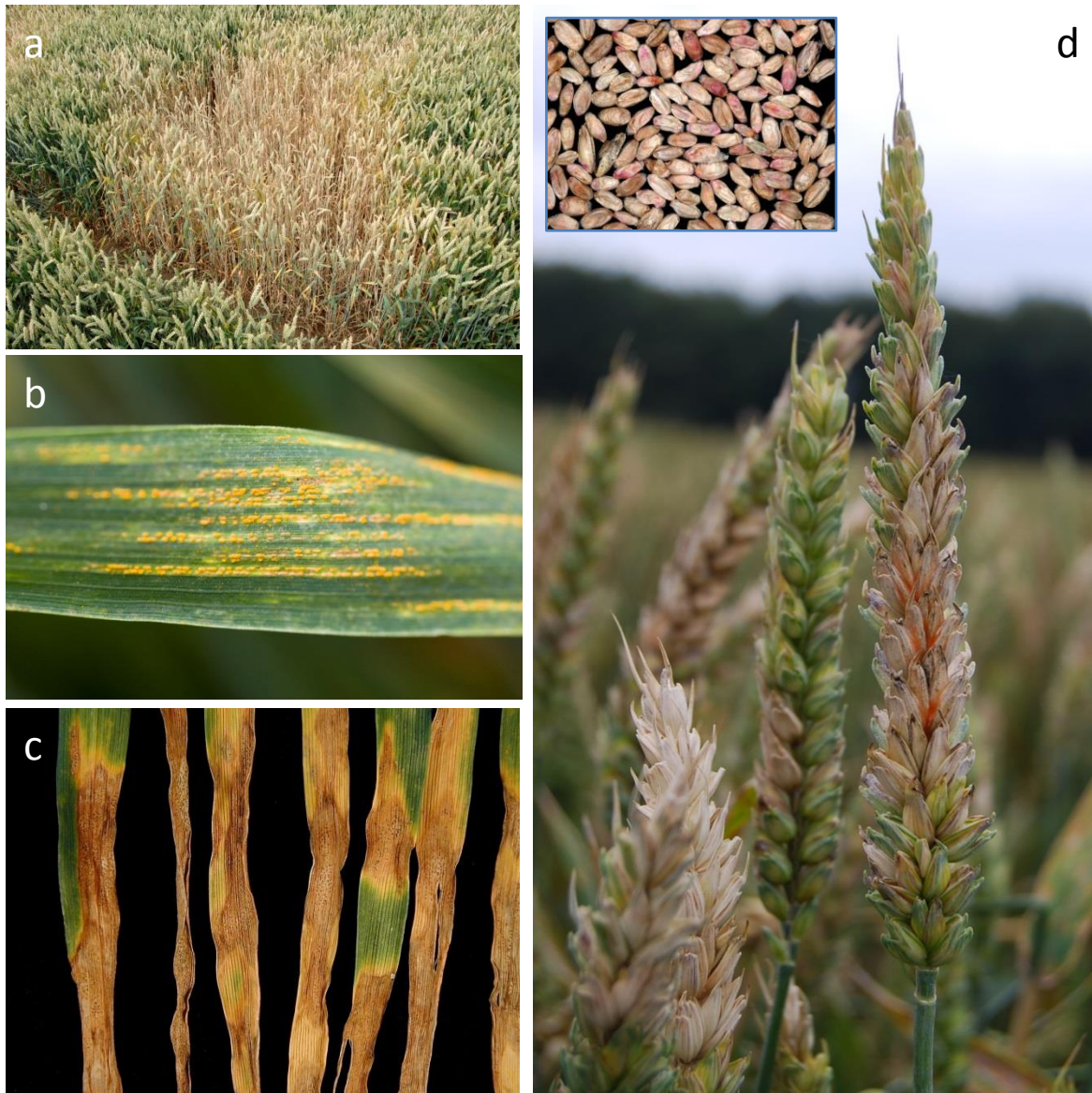


Figure 1.1: Fungal diseases of wheat. a) Take-all patches caused by root infection, b) yellow rust on leaves, c) *Septoria tritici* blotch on leaves, d) *Fusarium* Ear Blight (FEB) caused by infection of floral tissue, resulting in shrivelled, contaminated grain (inset). Photographs courtesy of the Rothamsted Image database and Wheat Pathogenomics team.

1.3 Fusarium Ear Blight

Fusarium Ear Blight (FEB) / Head Scab is a globally important fungal disease of wheat and other cereal crops such as maize and barley. Disease outbreak occurs sporadically, being highly dependent on environmental conditions, but an epidemic can cause 50-70% yield loss. In addition to reducing grain weight and grain quality, the causal species of FEB also produce various mycotoxins, which are harmful to human and animal health (Parry *et al.*, 1995, Doohan *et al.*, 2003, Pestka, 2010, Rocha *et al.*, 2005)

1.3.1 Key species

Seventeen causal agents of FEB have been identified, the most abundant being *Fusarium graminearum* (teleomorph *Gibberella zeae*), *F. culmorum*, *F. poae*, *F. avenaceum* and *Microdochium nivale* (formerly *Fusarium nivale*). These species also cause seedling blight and foot rot when plants are grown from untreated infected seed. *F. graminearum* is the main causal agent of disease in the USA, China and central Europe, being adapted to warmer conditions, while *F. culmorum*, *F. avenaceum*, *F. poae* and *M. nivale* are more common in the cooler maritime regions of Europe (Parry *et al.*, 1995).

F. culmorum has historically been considered the primary causal agent of FEB in the UK. However, incidence of FEB caused by *F. graminearum* is increasing in the UK (Xu and Nicholson, 2009) (www.cropmonitor.co.uk). This is thought to be due in part to the rise of maize as an important UK crop for animal feed. *F. graminearum* is, incidentally, the most pathogenic of FEB agents, owing to rapid infection and high levels of DON mycotoxin production (Osborne and Stein, 2007, Panthi *et al.*, 2014).

1.3.2 Disease symptoms and development

Infection of susceptible wheat plants occurs via the floral tissue at anthesis. Hyphal growth proceeds throughout the floral tissue of the infected spikelet and into the rachis, allowing the spread of infection to neighbouring spikelets. Research into the infection biology of *F. graminearum* by Brown *et al.* (2010) has revealed that initial colonisation occurs via the intercellular spaces, during an extensive symptomless phase. Intracellular growth and associated plant cell death are only observed during the later stages of infection. It is therefore predicted that *F. graminearum* has both a biotrophic and necrotrophic infection stage (Dean *et al.*, 2012), with important implications for plant defence strategies (see section 1.4.2). Macroscopically, infection symptoms begin with discoloration and the appearance of brown spreading lesions at the base of the glume, with subsequent bleaching of all or part of the ear. Pink fungal growth is visible as hyphae emerge from the host tissue producing new conidia (Parry *et al.*, 1995, Osborne and Stein, 2007) (Figure 1.1d).

1.3.3 Host and tissue specificity

Fusarium Ear Blight is restricted to cereal species, namely wheat, barley and maize. However, FEB causing species of *Fusaria* have also been isolated from non-cereal crops such as sugar beet and potato (Scherm *et al.*, 2013, Burlakoti *et al.*, 2007, Estrada Jr *et al.*, 2010). In addition, cereal infecting *Fusarium spp.* have been shown experimentally to infect the floral tissue of other plants including *Arabidopsis*, tobacco and tomato (Urban *et al.*, 2002). Importantly, infection is limited, for the most part, to the spikes of cereals, and the flower and silique tissue of *Arabidopsis*. *Fusarium* does cause infections of root/stem tissue such as crown rot and seedling blight, but only during senescence is the fungus able to colonise leaf tissue. This tissue specificity, and therefore the division of different plant organs into host and

non-host, may be important for identifying the genetic and molecular mechanisms of successful fungal colonisation and the basal plant defence mechanisms which inhibit infection.

1.3.4 Environmental conditions favouring infection and sources of inoculum

FEW infection is favoured by a warm and humid environment at plant anthesis. A minimum of 15°C and 24h rainfall may be required for infection, but the longer the rainfall period, the lower the required temperature (Parry *et al.*, 1995, Doohan *et al.*, 2003). Individual *Fusarium* species have distinct optimum temperatures for colonisation, with *F. graminearum* and *F. culmorum* infection most successful at 25°C and 20°C, respectively. Betaine and choline present in pollen are considered to be an important nutrient source during initial colonisation (Strange *et al.*, 1974, Li and Wu, 1994).

Fusarium spp. can grow saprophytically on dead plant material, and can therefore overwinter on crop debris. Low tillage systems increase the likelihood of infection as inoculum is allowed to build up on the soil surface and sexual sporulation can occur on the exposed crop debris. Repeated drilling of the same cereal species also increases the likelihood of infection, as does growing maize crops within the wheat rotation (Dill-Macky, 2008, Dill-Macky and Jones, 2000, Landschoot *et al.*, 2013). It is thought that the primary mode of short distance *Fusarium* dispersal and translocation to wheat ears is via rain splash from soil and leaf surfaces onto ears, which is why epidemics favour wet weather at anthesis (Osborne and Stein, 2007). However, movement by wind is likely to be important for long distance spore dispersal (Prussin *et al.*, 2013).

1.3.5 Mycotoxin production

Fusarium is damaging to crops not only in terms of reduced grain quality and yield but also due to the production of mycotoxins, which are required for successful colonisation. These include zealarenone (ZEA), fumonisins, trichothecenes and moniliformin. Both *F. culmorum* and *F. graminearum* produce the class B trichothecene deoxynivalenol (DON) and its acetylated derivatives 15-A-DON and 3-A-DON. Mycotoxins pose a severe health threat to both humans and animals (Doohan *et al.*, 2003, Bai and Shaner, 2004, Rocha *et al.*, 2005, Audenaert *et al.*, 2014). There are therefore strict limits on acceptable levels of DON mycotoxin in grain. The current EU limits are 750µg/kg (ppb) for pasta and cereals, with a stricter limit of 200ug/kg for baby foods. In 2008, 10% of the UK wheat crop was rejected due to exceeding legal mycotoxin limits. Even in non-epidemic years, testing for mycotoxins adds to production costs.

1.3.6 Control of FEB

Reactive treatment of FEB with fungicides is at present not effective enough to lower mycotoxin concentration to an acceptable level for consumption. Since the disease has an early symptomless infection stage, treatment must occur before symptoms have been observed in the field to successfully prevent colonisation. This requires either prophylactic treatment, which may be costly and unnecessary, or advanced forecasting systems, which are not always accurate (Shah *et al.*, 2013).

Furthermore, *F. graminearum* is inherently resistant to azole fungicides due to triplication of the target site (Fan *et al.*, 2013), and exposure to some azoles may in fact lead to increases in DON mycotoxin production (Audenaert *et al.*, 2010).

Cultural control methods include removal of crop debris after harvest, and rotation of crops to prevent inoculum build up. Although reduced tillage increases *Fusarium*

prevalence, it is an important agricultural practice offering economic benefits including prevention of soil erosion and retention of soil moisture. Therefore increased tillage, while reducing FEB incidence, would have negative impacts elsewhere (Dill-Macky, 2008).

1.3.7 Plant resistance to FEB

Control efforts now focus heavily on marker-assisted breeding for resistance. In wheat, the genotype providing the greatest known resistance is the Chinese cultivar Sumai 3. Resistance to *Fusarium* in this cultivar is quantitative trait loci (QTL) based, with well-known QTLs located on chromosome 3BS, 6B and 5A and another on 7A (Zhou *et al.*, 2010, Jayatilake *et al.*, 2011, Buerstmayr *et al.*, 2009). In addition, a QTL contributing to resistance in the CIMMYT spring wheat cultivar Catbird has been identified on chromosome 7DS (Cattivelli *et al.*, 2013). So far the molecular identity of the genes underlying any of the resistance QTLs has not been reported although efforts are ongoing in several laboratories for over two decades.

Natural resistance in wheat to FEB has so far been grouped into two main types and several other types: Type I confers resistance to the plant by preventing initial infection by the fungus. Type II confers resistance to subsequent internal colonisation throughout the rachis and the infection of additional spikelets. This form of resistance, while important if *Fusarium* incidence is low, is less useful if inoculum levels are high enough to allow discrete infection of several spikelets per ear.

Additional resistance types identified in a few wheat germplasms include prevention of high DON accumulation. Reduced DON accumulation may, however, simply be a direct result of Type I or Type II resistance. Furthermore, highly susceptible varieties may contain less DON in harvested kernels (grains), as the infected kernels fail to

develop and are therefore not harvested. These additional types of resistance are therefore difficult to formally assess (Bai and Shaner, 2004).

The search for genetic material which confers resistance to FEB included landraces and crop wild relatives. Landraces with increased FEB resistance often come with undesirable agronomic traits such as low yields and small heads, as seen in Sumai-3. These traits may or may not be genetically linked to increased resistance, and may therefore be difficult to separate during breeding programmes. The multigenic nature of resistance, combined with polyploidy in modern wheat varieties, also impedes conventional breeding efforts.

In summary, management of FEB disease on wheat presents a global challenge, as the epidemiology and infection biology of the causal agents make it hard to control by chemical and cultural methods. Existing sources of host resistance are complex and poorly understood, leading to a requirement for further research into finding and / or developing additional sources of resistance.

1.4 The molecular basis of plant-pathogen interactions

The interaction between plant pathogens and their hosts and the determinants of disease development have been widely studied, especially using the model plant organism *Arabidopsis thaliana*. Pathogens of *Arabidopsis* used in these systems range from the biotrophic bacteria *Pseudomonas syringae* and *Xanthomonas campestris* and the biotrophic oomycete *Hyaloperonospora arabidopsidis* through to the necrotrophic ascomycete fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola*. Key aspects of pathogen recognition and defence activation by plants, and evasion of detection or defence by pathogens, are discussed below.

1.4.1 Pathogen recognition

Plants, as sessile organisms which interact closely with their environment, are exposed to a huge number of microbial organisms. However, few of these plant-microbe interactions result in disease. This is because plants possess a number of both passive and inducible defence mechanisms which prevent invasion by would-be pathogens. While some plant defences are preformed, others are triggered by the recognition of conserved microbial molecules, known as Microbe / Pathogen Associated Molecular Patterns (MAMPS/PAMPS), by pattern recognition receptors (PRRs) in the plant cell membrane (Schwessinger and Zipfel, 2008). PAMP triggered immunity (PTI) is achieved when a plant is able to recognise these molecules and activate defence mechanisms which block invasion (Boller and Felix, 2009, Zipfel, 2014). Examples of PAMPS include bacterial flagellin, which is recognised by the plant PRR FLS2 (Gomez-Gomez and Boller, 2000), and the fungal cell wall component chitin, which is recognised by CERK1 (Miya *et al.*, 2007, Wang *et al.*, 2008). Successful pathogens must therefore overcome PTI, by blocking recognition of PAMPS and/or manipulating induction of subsequent defence responses. This is broadly understood to be achieved via the secretion of small molecules known as effectors (Giraldo and Valent, 2013, Wang *et al.*, 2014a). For example, some fungal pathogens possess effectors containing LysM domains which bind chitin, blocking its recognition by CERK1 and therefore evading detection (Lee *et al.*, 2013, de Jonge *et al.*, 2010, Marshall *et al.*, 2011). Plant susceptibility mediated by effectors is known as Effector Triggered Susceptibility (ETS).

However, some plants possess the capacity to recognise specific effector molecules via proteins encoded by *Resistance (R)* genes, and this recognition triggers a defence response known as Effector Triggered Immunity (ETI) (Rafiqi *et al.*, 2009,

Hammond-Kosack and Jones, 1997). R proteins often contain a nucleotide binding (NB) domain and leucine rich repeats (LRR), and they are therefore referred to as NB-LRR proteins. Resistance gene mediated immunity was originally described as a 'gene-for-gene' interaction as specific *R* genes were understood to recognise specific effectors (Flor, 1971), however, it is now understood that some *R* genes are involved in recognition of more than one effector, and that recognition of a single effector may sometimes rely on more than one *R* gene (Narusaka *et al.*, 2009, Cesari *et al.*, 2013). Effectors which are recognised by specific plant genotypes and trigger ETI are sometimes referred to as avirulence (avr) factors.

The mechanistic and evolutionary aspects of this 'hide and seek' interplay between plants and pathogens, particularly those with biotrophic lifestyles (see below), are often summarised as the 'zig-zag-zig' model as first described by Jones and Dangl (2006).

1.4.2 Defence activation and signalling

Successful recognition of a plant pathogen leads to activation of defence responses.

There is understood to be substantial overlap in the responses induced by recognition of PAMPs and effectors (Tsuda *et al.*, 2008, Navarro *et al.*, 2004, Tao *et al.*, 2003). These responses include influx of calcium ions into the cell, accumulation of Reactive Oxygen Species (ROS) such as hydrogen peroxide (H₂O₂), induction of Mitogen Activated Protein Kinase (MAPK) and Calcium Dependent Protein Kinase (CDPK) cascades (Wu *et al.*, 2014), deposition of callose, and induction of hormone signalling pathways mediated by plant hormones including Salicylic acid (SA), Jasmonic acid (JA) and its derivatives, and Ethylene (ET). The specific roles of ROS in plant pathogen interactions are discussed further in Chapter 7.

Contrasting responses to biotrophs and necrotrophs

The type of response which will be successful against a pathogen is to some extent dependent on the lifestyle of the pathogen; plant pathogens have traditionally been divided by their infection lifestyle into biotrophs, which derive their nutrients from living host cells, and necrotrophs, which induce host cell death in order to scavenge nutrients. In addition, some pathogens, including *F. graminearum*, are described as having a hemi-biotrophic or 'switching lifestyle', with asymptomatic colonisation of living plant tissue preceding necrotrophy. Plants are thought to deploy different defence strategies against these contrasting infection processes. It is broadly understood that defence against biotrophs is mediated by SA, while defence against necrotrophs is mediated by JA/ET, and that these two signalling pathways are partly antagonistic, as reviewed by Glazebrook (2005), Koornneef and Pieterse (2008), Beckers and Spoel (2006), and Hammond-Kosack and Parker (2003).

The role of SA in plant immunity has been widely studied and is the focus of many review articles (An and Mou, 2011, Vlot *et al.*, 2009, Loake and Grant, 2007, Yan and Dong, 2014). The following provides a brief summary of key aspects of SA mediated defence. SA is understood to act both directly as an antimicrobial compound and also as a signalling molecule. SA signalling is associated with induction of localised programmed cell death (PCD) at the site of infection, known as the hypersensitive response (HR), blocking nutrient uptake and further development of infection, particularly by biotrophic pathogens. SA induced defence is also characterised by the expression of several *pathogenesis related (PR)* genes, some of which are known to have antimicrobial properties (van Loon *et al.*, 2006). Salicylic acid is also involved in the induction systemic acquired resistance (SAR), which

increases resistance in the distal healthy tissues following initially local infection (Durrant and Dong, 2004).

While HR and SA mediated defence helps to block colonisation by biotrophic pathogens, it may in some cases aid infection by necrotrophic pathogens (Govrin and Levine, 2000, Govrin *et al.*, 2006, El Oirdi and Bouarab, 2007). Plants have therefore evolved other methods of limiting infection by necrotrophic fungi, as reviewed by Mengiste (2012). Many of these defences are mediated by JA and ET signalling (Glazebrook, 2005, Spoel and Dong, 2008, Thomma *et al.*, 1998). JA/ET signalling results in the production of antimicrobial compounds such as defensins, coumarins and thionins which limit pathogen growth (Penninckx *et al.*, 1998, Eppele *et al.*, 1995, Sun *et al.*, 2014, Thomma *et al.*, 2002).

While plant defence signalling pathways against biotrophs and necrotrophs are typically reported as distinct and antagonistic, there are many examples of synergism which challenge this. For example, it has been shown that SAR and other forms of induced resistance are dependent on both SA and JA, and that both JA and ET are involved in defence against biotrophs (Leon-Reyes *et al.*, 2009, Thaler *et al.*, 2004, Truman *et al.*, 2007). The relationship between infection lifestyle and type of defence response activated is therefore likely to be less clear cut than previously described.

1.4.3 Key genes involved in plant defence signalling

Many genes involved in plant defence signalling and regulation of cross-talk between distinct signalling pathways have been identified both in *Arabidopsis* and other plant species. Some of those which are pertinent to this thesis and related studies include:

NPR1 (*Non-expresser of PR genes 1*) is a central regulator of SA signalling during plant defence. Mutants in this gene show decreased expression of *PR* (*Pathogenesis Related*) genes and increased susceptibility to a range of pathogens including *Pseudomonas syringae* (Cao *et al.*, 1994, 1997). The gene is constitutively expressed but only activated upon induction of SA signalling, when it is reduced from an oligomeric to a monomeric form and translocated to the nucleus, where it induces expression of *PR* genes via activation of WRKY domain containing and TGA(CG) binding transcription factors (Kinkema *et al.*, 2000, Mou *et al.*, 2003, Tada *et al.*, 2008, Dong, 2004). NPR1 also acts as a negative regulator of JA signalling (Beckers and Spoel, 2006). Mutants lacking functional NPR1 show increased levels of JA and expression of JA responsive genes such as *PDF1.2*. NPR1 is believed to down-regulate JA signalling through a cytosolic function which is unrelated to its SA signalling function in the nucleus (Spoel *et al.*, 2003). Two NPR1-like proteins, **NPR3** and **NPR4**, have recently identified as SA receptors which modulate NPR1 activity (Fu *et al.*, 2012, Yan and Dong, 2014).

PAD4 (phytoalexin deficient 4) and **EDS1** (enhanced disease susceptibility 1) both encode intracellularly located lipase like proteins and are thought to be involved in both basal and *R* gene mediated defence responses. EDS1 is required for *PAD4* expression, and is also involved in inducing HR. EDS1 is therefore thought to act early in SA mediated defence signalling, while PAD4 is induced later during the signal transduction cascade (Feys *et al.*, 2001).

EDS5 is required for SA accumulation following infection, and is also up-regulated by SA treatment, indicating a positive feedback loop (Glazebrook *et al.*, 2003, Nawrath *et al.*, 2002). *EDS5* has recently been found to encode a transporter protein

responsible for the movement of SA from the chloroplast to the cytosol (Serrano *et al.*, 2013).

RAR1 and **SGT1** and **HSP90** have an integrated role in activation of both *R* gene mediated and basal defence responses to biotrophic pathogens (Kadota *et al.*, 2010, Tor *et al.*, 2002, Azevedo *et al.*, 2006, Azevedo *et al.*, 2002). However, silencing of this system in the tobacco plant *Nicotiana benthamiana* has been shown to increase resistance to the necrotrophic fungal pathogen *Botrytis cinerea* (El Oirdi and Bouarab, 2007).

Genes required for JA mediated defence signalling include **JAR1** (*jasmonate resistant 1*) and **COI1** (*coronatine insensitive 1*) (Loreti *et al.*, 2008). The ethylene receptors **ETR1 (EIN1)** and **EIN2** are required for response to ET and subsequent defence activation (Alonso *et al.*, 1999, Cancel and Larsen, 2002). The transcription factor **ERF1** (ethylene response factor 1) acts as a convergence point for ET and JA signalling, requiring activation of both hormones for transcriptional activation and subsequent expression of defence related genes (Lorenzo *et al.*, 2003).

1.4.4 The role of other plant hormones in defence

Other phyto-hormones with a role in defence include Absciscic Acid (ABA), auxin, and Gibberellic Acid (GA) (Robert-Seilaniantz *et al.*, 2011). ABA, which is known for its involvement in abiotic stress responses, has been found to both positively and negatively regulate plant defence, depending on the pathogen type and the stage at which a defence response is mounted (Ton *et al.*, 2009). As reviewed by Kazan and Manners (2009), auxin is thought to affect plant defence via antagonism of SA signalling and synergism with JA signalling, thus having a negative impact on defence against biotrophs while aiding immunity to necrotrophs. GA may also have

either a positive or negative role on plant defence, mediated by its down-regulation of DELLAS – plant growth suppressors which increase resistance to infection by necrotrophs by promoting JA signalling, while increasing susceptibility to biotrophs (Navarro *et al.*, 2008).

1.4.5 Exploitation of host defence responses by pathogens

Successful defence against pathogens with different lifestyles requires careful and timely regulation of distinct defence signalling pathways, with some pathways being up-regulated while others are down-regulated. Some pathogens exploit or perturb plant defence signalling in order to promote colonisation. For example, the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) produces coronatine, a jasmonate mimicking toxin which reduces SA mediated defence. This mimicry is blocked in coronatine insensitive (*coi1*) mutants, which show enhanced resistance to *Pst* associated with increased SA dependent defence responses. However, *coi1* mutants have enhanced susceptibility to the necrotrophic pathogen *Alternaria brassicicola* (Xie *et al.*, 1998, Kloeck *et al.*, 2001, Penninckx *et al.*, 1998). Thus, *Pst* hijacks the plant defence response to necrotrophic pathogens in order to facilitate its own infection.

The necrotrophic fungal pathogen *B. cinerea* elicits HR ahead of infection and subsequently colonises the dead tissue (Govrin *et al.*, 2006, Govrin and Levine, 2000). Another necrotrophic pathogen, *Sclerotinia sclerotiorum*, secretes oxalic acid which promotes ROS production and PCD in the host at high concentrations (Kim *et al.*, 2008). Interestingly, low concentrations of oxalic acid secreted during initial colonisation act to suppress PCD, allowing establishment of infection (Williams *et al.*, 2011).

In addition to toxins active against a broad range of host plants, some necrotrophic pathogens also appear to induce disease on specific hosts by secreting host specific toxins (HSTs) which aid disease progression in the presence of a single plant *susceptibility* (*S*) gene (Friesen *et al.*, 2008). This is analogous to the gene-for-gene interaction whereby effectors from biotrophs are recognised by plant *R* genes.

For example, the HST ToxA produced by the wheat pathogens *P. nodorum* and *Pyrenophora tritici repentis* induces PCD in the host, mediated by the wheat susceptibility gene *Tsn1*. *Tsn1* encodes an NB-LRR protein reminiscent of an *R* gene product (Faris *et al.*, 2010, Oliver and Solomon, 2010, Vleeshouwers and Oliver, 2014, Friesen *et al.*, 2006). Similarly, *Cochliobolus victoriae* produces victorin, which induces PCD in both oats and Arabidopsis, aiding infection. In Arabidopsis this is dependent on LOV1, an NB-LRR protein (Lorang *et al.*, 2007). The equivalent *S* gene in oats triggers plant defence-like responses and is thought to share identity with a rust resistance gene. This suggests that HSTs from necrotrophs may hijack *R* gene mediated plant defence responses evolved against biotrophs.

1.4.6 The interaction between *Fusarium* and wheat during FEB infection

The molecular basis of the interaction between cereal infecting *Fusaria* and their hosts is poorly understood, with few characterised effector proteins and no evidence of a gene-for-gene interaction mediating either susceptibility or resistance; the currently identified sources of resistance in wheat are QTL based, as previously described.

***Fusarium* virulence mechanisms**

Transcriptome analyses have identified a number of candidate secreted effector proteins from *F. graminearum* but many of these have yet to be functionally

evaluated *in planta* (Brown *et al.*, 2012). One characterised *F. graminearum* secreted effector is the secreted lipase Fgl1, which is thought to be responsible for the generation of polyunsaturated free fatty acids (FFAs) which suppress callose biosynthesis. Callose deposition is associated with decreased colonisation by $\Delta fgl1$ mutants (Blümke *et al.*, 2014, Voigt *et al.*, 2005).

The trichothecene mycotoxin deoxynivalenol (molecular weight, 296.32), though not a protein, may arguably be classed as an effector molecule since it is required for symptomless colonisation of wheat, and its biosynthesis genes are highly expressed during the initial symptomless colonisation phase (Brown *et al.*, 2011, Brown *et al.*, 2012, Cuzick *et al.*, 2008b, Jansen *et al.*, 2005). Studies in Arabidopsis leaves suggest that low levels of DON production may aid early symptomless growth of *F. graminearum* by inhibiting PCD (Diamond *et al.*, 2013), while higher concentrations induce ROS production (Desmond *et al.*, 2008). This is similar to the observed role of oxalic acid in the pathogenicity of *S. sclerotiorum* as previously described.

However, DON biosynthesis is not required for infection of maize cobs, or Arabidopsis flowers and siliques (Bormann *et al.*, 2014, Cuzick *et al.*, 2008b). This may indicate that DON toxicity is host, and also tissue, specific. *F. graminearum* also secretes cell wall degrading enzymes (CWDEs), such as xylanases and polygalacturonases (PGs) which are thought to aid infection by breaking down cell wall components (Kikot *et al.*, 2009, Tomassini *et al.*, 2009).

Transcriptional analyses of wheat defence responses to FEB

Several studies have examined the effect of FEB infection on gene transcription in wheat, in order to elucidate the defence signalling responses induced by infection, and differences in transcription profiles between resistant and susceptible cultivars.

Ding *et al.* (2011) found that SA signalling was initially induced by infection 6 hours after infection in the resistant cultivar Wangshuibai, followed by ET and then JA signalling after 12 hours. SA signalling was delayed and JA/ET signalling reduced in a susceptible mutant. Gottwald *et al.* (2012) similarly found that JA and ET signalling related genes were up-regulated during infection of the resistant cultivar Dream compared to susceptible cultivar Lynx, and that the expression pattern of Dream was similar to that of the resistant spring wheat Sumai 3. Yang *et al.* (2013) identified three wheat orthologues of the *Arabidopsis* defence regulator *NPR1*, and found that two of them were up-regulated in a resistant compared to a susceptible cultivar during infection. Resistant and susceptible cultivars also show quantitative differences in expression of *PR* genes – for example the resistant cultivar Sumai 3 shows increased expression of chitinases, which target the fungal cell wall, compared to susceptible mutants (Li *et al.*, 2001). Pritsch *et al.* (2000) found that these *PR* genes were expressed in distal tissues following *Fusarium* inoculation, indicating that a defence response is mounted ahead of the infection front. Collectively, these transcript analyses demonstrate that the timing and magnitude of diverse defence responses is important for resistance to FEB, and that resistance pathways typically associated with both biotrophs and necrotrophs are recruited for defence against FEB.

These transcriptome analyses provide substantial insights into the induction of defence signalling during FEB disease in resistant compared with susceptible cultivars. However, the functionality of many of the identified genes remains to be tested, and wheat's large, hexaploid genome makes transgenic and / or mutagenesis approaches to analyse gene function difficult. Furthermore, it is difficult to estimate the importance of some differentially expressed genes which have no known function

or close homologues in other species (Bernardo *et al.*, 2007). The complete sequencing, alignment and annotation of the wheat genome are only now nearing completion, owing to its size and complexity (The International Wheat Genome Sequencing Consortium, 2014, Lai *et al.*, 2012, Brechley *et al.*, 2012).

1.5 Arabidopsis as a model for FEB infection

In contrast to wheat, the model plant *Arabidopsis thaliana* has a small, diploid, fully sequenced genome, which extends to a large number of different ecotypes (Cao *et al.*, 2011b, Ossowski *et al.*, 2008a, Arabidopsis Genome Initiative, 2000). This is supported by large collections of genetic mutants along with a wealth of online resources such as annotated genome browsers, gene expression data, and pathway information (Hruz *et al.*, 2008, Kanehisa *et al.*, 2014, Lamesch *et al.*, 2011, Alonso *et al.*, 2003, Schmid *et al.*, 2005) (www.plantcyc.org). Much of this is accessible via the Arabidopsis Information Resource (TAIR), with the recently launched Arabidopsis Information Portal further facilitating access to community resources (Baerenfaller *et al.*, 2012).

Arabidopsis has therefore been widely adopted to study the genetic basis of plant-pathogen interaction outcomes as previously outlined, results from which then have the potential to be translated into important crops (Piquerez *et al.*, 2014, Dangl *et al.*, 2013). This has extended to its use as a model for studying infection by FEB causing isolates of *Fusarium*:

Urban *et al.* (2002) demonstrated that both *F. culmorum* and *F. graminearum* infect the floral and silique tissue of *Arabidopsis*, but that this infection does not spread to the main stem or leaf tissue. DON mycotoxin is also produced during infection. Floral infection of *Arabidopsis* by FEB causing *Fusarium* species was therefore put forward

as a suitable model system for the study of plant defence signalling against the causal agents of FEB, without the need for complex genetic studies in wheat (Fig. 1.2).

Since the publication of this original study, several research groups have used the pathosystem to investigate the role of various defence associated genes and signalling pathways in determining the outcome of the *Fusarium-Arabidopsis* interaction, as well as the variation in susceptibility among *Arabidopsis* ecotypes. While some studies have made use of the floral infection system and disease scoring method originally published by Urban *et al.* (2002), others have used leaf and seedling systems to study the *Fusarium-Arabidopsis* interaction.



Figure 1.2: Wheat ear experimentally infected with *Fusarium* (a), compared to spray inoculated *Arabidopsis* floral tissue (b) and droplet inoculated siliques (c). All images show infection at 10 dpi.

1.5.1 Variation in *Fusarium* susceptibility between *Arabidopsis* ecotypes

Urban *et al.* (2002) screened 236 *Arabidopsis* ecotypes and did not find that any were extremely resistant or susceptible to *Fusarium* floral infection. However, it was found that inoculation of ecotype Landsberg *erecta* 0 (*Ler*-0) results in consistently severe floral infection with low variability between plants and experimental replicates, while infection of ecotype Columbia-0 (*Col*-0) is highly variable. This difference was attributed to the compact floral morphology of *Ler*-0 facilitating fungal spread, namely in that developing siliques which were open flowers at the time of inoculation remain in close proximity to the apical inflorescence. This is in contrast with the more relaxed, spread out floral morphology of *Col*-0, where developing siliques stretch away from the apical inflorescence, preventing spread of fungal hyphae. The effect of the *erecta* mutation on floral susceptibility to *F. culmorum* is explored further in Chapter 7.

Chen *et al.* (2006) developed a detached leaf assay involving wounding and the application of exogenous DON mycotoxin to induce consistent *F. graminearum* infection in *Arabidopsis* rosette leaves. Using this method, these researchers found differences in susceptibility between *Arabidopsis* ecotypes; Bay-0, Kas-1 and *Ler*-0 were all found to be more susceptible to *F. graminearum* compared to four distinct Columbia lines. This indicated that the differences in susceptibility to *Fusarium* between *Col*-0 and *Ler*-0 were not purely the result of *erecta* acting on the floral morphology. Indeed, Chen and colleagues performed QTL analysis on a *Col*-0 x *Ler*-0 mapping population and found that *Fusarium* susceptibility was associated with a major QTL on Chromosome 4, not linked to the *erecta* mutation.

It is worth noting however that this study relied on significant manipulation of the *Arabidopsis*-*Fusarium* interaction via wounding and exogenous DON

application, and that detachment of the leaves might also impact on the plant defence response. It is therefore unclear how well findings from this study might relate to FEB susceptibility in wheat floral tissue.

1.5.2 Arabidopsis genes screened for altered Fusarium susceptibility

Several studies have used knock-out mutants and/or over-expression lines to study the role of specific defence-related genes on the interaction between wheat-infecting *Fusarium* strains and *Arabidopsis* floral and/or leaf tissue, as summarised in Table 1.1 and detailed below (Cuzick *et al.*, 2009, Cuzick *et al.*, 2008a, Makandar *et al.*, 2010, Makandar *et al.*, 2006, Savitch *et al.*, 2007, Van Hemelrijck *et al.*, 2006).

The *Arabidopsis* mutant *esa1* (*enhanced susceptibility to Alternaria 1*) carries a mutation in an unmapped gene, which renders it susceptible to several necrotrophic pathogens. The mutation does not alter susceptibility to biotrophic pathogens such as the oomycete *Hyaloperonospora arabidopsidis*. Further analysis showed that *esa1* is attenuated in ROS-induced production of the antimicrobial phytoalexin camalexin, along with JA and ET induced defence responses (Tierens *et al.*, 2002). Van Hemelrijck *et al* (2006) screened the *esa1* mutant for susceptibility to several pathogens of the genus *Fusarium*, including the FEB causing species *F. graminearum* and *F. culmorum*. The floral tissue of the *esa1* mutant was found to be significantly more susceptible to *F. culmorum* than wild-type plants. A similar but non-significant trend was observed for *F. graminearum*. This finding indicated that *ESA1* mediated defence against necrotrophs is also involved in defence against cereal infecting *F. culmorum*.

The *Arabidopsis* gene *GLK1* (*Golden Like Kinase 1*) is a transcriptional activator involved in chlorophyll biosynthesis (Waters *et al.*, 2009, Waters *et al.*, 2008).

Savitch *et al.* (2007) found that over-expression of *GLK1* resulted in up-regulation of a number of defence related genes including antimicrobial peptides, but down-regulation of PR1, used as a marker SA mediated defence signalling. Arabidopsis plants overexpressing *GLK1* also supported less *F. graminearum* growth in leaves infiltrated with *F. graminearum* spores using a needleless syringe. Transgenic Arabidopsis plants expressing the bacterial SA hydroxylase NahG, which breaks down SA, showed increased *F. graminearum* infection levels.

The most extensive studies on responses of Arabidopsis mutants to wheat infecting *Fusarium* have so far been reported by Cuzick *et al.* (2008-2009) and Makandar *et al.* (2010). The former used the floral spray assay devised by Urban *et al.* (2002) and *F. culmorum* inoculum, while the latter used a combination of the floral spray assay and leaf syringe infiltration, both with *F. graminearum*. These studies particularly focussed on the effect of mutation of genes in the SA, JA and ET signalling pathways on *Fusarium* susceptibility.

Both Cuzick *et al.* (2008a) and Makandar *et al.* (2010) found that mutation of the central signalling regulator *NPR1* resulted in increased *Fusarium* susceptibility. Makandar *et al.* (2010) attributed this to the requirement of a functional SA signalling pathway for defence against *Fusarium*, due to the susceptibility profiles observed in a number of other SA signalling mutants and transgenic lines (see table 1.1), including the SA induction mutant *sid2-2*. By contrast, Cuzick *et al.* (2008a) did not observe a significant difference in *F. culmorum* floral infection levels between *sid2-2* and wild type plants, casting doubt over the role of SA signalling in resistance to *F. culmorum*. Similarly, later studies found no effect of mutation of *EDS1* or *EDS5* on *F. culmorum* floral

susceptibility. However, *eds12*, which is attenuated in SA mediated systemic resistance, shows enhanced susceptibility to *F. culmorum* (Cuzick *et al.*, 2009, and unpublished data). The phenotypes of the Arabidopsis *enhanced disease susceptibility (eds)* mutants are further explored in Chapters 3 and 4.

Constitutive expression of Arabidopsis *NPR1* in wheat has previously been shown to reduce FEB infection, and this was later shown to be associated with SA accumulation (Makandar *et al.*, 2006, Makandar *et al.*, 2011). However, the potential of these findings for reducing FEB infection in the field is hampered by the increased Fusarium seedling blight seen in the *NPR1* transgenic plants (Gao *et al.*, 2013).

Contrasting conclusions have also been drawn from different studies on the roles of JA and ET signalling in defence against Fusarium. Cuzick *et al* (2008a) found that the JA signalling mutants *coi1* and *jar1* were more resistant to *F. culmorum* floral infection, but attributed this to alterations in floral morphology such as increased stem elongation and decreased fertility affecting nutrient availability to the fungus. Only one of four ET signalling genes studied (*EIN2*) appeared to contribute to *F. culmorum* floral resistance, rendering the role of ET signalling inconclusive.

However, Makandar *et al.* (2010) put forward evidence for a role of JA signalling in *F. graminearum* susceptibility, based on decreased leaf and floral infection of JA signalling mutants *jar1* and *opr3* (Table 1.1). As described by Cuzick *et al* (2008), *jar1* has altered floral morphology, and *opr3* is male sterile (Sanders *et al.*, 2000). The evidence from this study of the role of these genes in leaf susceptibility is therefore more reliable than the floral data. Interestingly, the *jar1 npr1* double mutant was found by Makandar *et al.* (2010) to be more

susceptible to floral and leaf infection by *F. graminearum* than both wild-type plants and the *npr1* single mutant. Indeed, the disease scores for the double mutant were higher than the maximum value allowed for by the original scoring system devised by Urban *et al.* (2002), indicating that the authors had to amend the scoring protocol to represent the severity of disease in the double mutant. This would suggest that JAR1, though initially contributing to susceptibility, may play a role in resistance at some later stage in infection, or that JA mediated defence may help to block infection in the absence of *NPR1* mediated defence responses. Evidence for the former hypothesis was supported by the finding that application of methyl jasmonate (MeJA) early during leaf infection enhanced susceptibility, while later application enhanced resistance. This correlates with the findings by Ding *et al.* (2011) that JA signalling is induced in resistant wheat plants, but not susceptible plants, 12 hours after infection.

Chen *et al.* (2009) reported that ET signalling mutants are more resistant to *F. graminearum* infection, using the previously described DON-amended detached leaf assay. However, only the *ein2* mutant showed significantly less disease on detached flowers, correlating with the findings of Cuzick *et al.* (2008). Ethylene signalling was also found to contribute to disease susceptibility of detached wheat leaves and ears. It was postulated that ET signalling contributes to DON induced host cell death facilitating infection. However, these results conflict with findings by Gottwald *et al.* (2012) on the role of ET signalling in Fusarium resistant wheat lines. Analysis of the function of ET signalling genes in FEB resistance in wheat is ongoing, using an RNA silencing approach (Scofield *et al.*, unpublished).

Cuzick *et al* (2008a) also reported that the *Pseudomonas* susceptible mutant *eds11* (*enhanced disease susceptibility 11*) is highly susceptible to *F. culmorum* floral infection. As discussed further in Chapters 3 and 5, the genomic location and function of *EDS11* are unknown, and it does not appear to be required for SA or JA mediated defence responses (Glazebrook *et al.*, 1996). Its role in defence signalling is therefore unclear.

It was also reported that mutation of *SGT1b*, which is involved in oxidative burst mediated basal and effector triggered defences, results in reduced susceptibility to *F. culmorum* floral infection (Cuzick *et al.*, 2009). The reason for this remains unclear. It was previously indicated that abolition of PCD by silencing of *SGT1* was responsible for enhanced resistance to necrotrophs in tobacco (El Oirdi and Bouarab, 2007), however Cuzick *et al.* (2009) found no differences in ROS accumulation or PCD between *sgt1b* mutant and wild type plants.

These findings, when taken together, present a complex and at times contradictory picture of the relative roles of various defence signalling pathways in defence against *Fusarium* in *Arabidopsis*. The general model appears to be that SA mediated defence signalling reduces infection, while JA and ET mediated signalling increases infection, at least for *F. graminearum* on *Arabidopsis* leaves. However, roles for both JA and ET in resistance have also been elucidated. It is not clear to what extent the success of defence responses are dependent on the fungal species, since no direct comparisons have been made between *F. culmorum* and *F. graminearum* under the same laboratory conditions. Furthermore, findings from *Arabidopsis* leaf assays may not always be translatable to FEB disease of wheat. While floral infection may be more of a

representative model, the effects of many defence signalling mutants on plant development and floral morphology compound results.

Table 1.1: Arabidopsis mutants with altered defence responses to *F. graminearum* (Fg) and *F. culmorum* (Fc) strains. Key: (-) indicates less disease than wild-type, (+) indicates more disease than wild-type, (wt) indicates equivalent disease to wild-type. (++) indicates increased disease in *jar1 npr1* double mutant compared to *npr1* mutant alone. (*) indicates expression of a transgene. OE signifies over-expression of a transgene.

GENOTYPE	GENE FUNCTION	PATHOGEN	INOCULATION	ASSESSMENT	DISEASE	REFERENCE
<i>coi1</i>	JA signalling	Fc 98/11	floral spray	disease score	-	(Cuzick <i>et al.</i> , 2008a)
		Fg Z-3639	leaf infiltration	% leaf infected	-	(Makandar <i>et al.</i> , 2010)
		Fg DAOM233423	seeding inoculation	cotyledon infection	-	(Schreiber <i>et al.</i> , 2011)
<i>cpr5</i>	SA and JA down-regulation	Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	-	(Makandar <i>et al.</i> , 2010)
<i>eds11</i>	unknown	Fc 98/11	floral spray	disease score, DON	+	(Cuzick <i>et al.</i> , 2008a)
<i>ein2</i>	ET signalling	Fc 98/11	floral spray	disease score, DON	+	(Cuzick <i>et al.</i> , 2008a)
<i>ERF1 OE</i>	JA/ET signalling	Fc 98/11	floral spray	disease score, DON	wt	(Cuzick <i>et al.</i> , 2008a)
<i>esa1</i>	unknown	Fc 180420	floral spray	disease score	+	(Van Hemelrijck <i>et al.</i> , 2006)
<i>eto1</i>	ET regulation	Fc 98/11	floral spray	disease score, DON	wt	(Cuzick <i>et al.</i> , 2008a)
<i>etr1</i>	ET signalling	Fc 98/11	floral spray	disease score, DON	wt	(Cuzick <i>et al.</i> , 2008a)
<i>GLK1 OE</i>	chloroplast development	Fg Z-3639	leaf infiltration	imaging	-	(Savitch <i>et al.</i> , 2007)
		Fg DAOM233423	seeding inoculation	cotyledon infection	-	(Schreiber <i>et al.</i> , 2011)
<i>jar1</i>	JA signalling	Fc 98/11	floral spray	disease score	-	(Cuzick <i>et al.</i> , 2008a)

GENOTYPE	GENE FUNCTION	PATHOGEN	INOCULATION	ASSESSMENT	DISEASE	REFERENCE
		Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	-	(Makandar <i>et al.</i> , 2010)
		Fg DAOM233423	seedling inoculation	cotyledon infection	-	(Schreiber <i>et al.</i> , 2011)
<i>jar1 npr1</i>	See single mutants	Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	++	(Makandar <i>et al.</i> , 2010)
<i>opr3</i>	JA signalling	Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	-	(Makandar <i>et al.</i> , 2010)
<i>NahG*</i>	SA degradation	Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	+	(Makandar <i>et al.</i> , 2010, Savitch <i>et al.</i> , 2007)
<i>npr1</i>	central regulator	Fc 98/11	floral spray	disease score, DON	+	(Cuzick <i>et al.</i> , 2008a)
		Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	+	(Makandar <i>et al.</i> , 2010)
<i>NPR1 OE</i>	central regulator	Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	-	(Makandar <i>et al.</i> , 2010)
<i>sgt1b</i>	<i>R</i> gene mediated defence	Fc 98/11	floral spray	disease score, DON	-	(Cuzick <i>et al.</i> , 2009)
<i>sid2</i>	SA synthesis	Fc 98/11	floral spray	disease score	wt	(Cuzick <i>et al.</i> , 2008a)
		Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	+	(Makandar <i>et al.</i> , 2010)
<i>ssi2</i>	SA down-regulation	Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	-	(Makandar <i>et al.</i> , 2010)
<i>wrky18</i>	NPR1 mediated defence	Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	+	(Makandar <i>et al.</i> , 2010)
<i>WRKY18 OE</i>	NPR1 mediated defence	Fg Z-3639	leaf infiltration, floral spray	% leaf infected, disease score	-	(Makandar <i>et al.</i> , 2010)

1.5.3 Transgenic and chemical approaches

The Arabidopsis-Fusarium pathosystem has also been used to test a number of transgenic and chemical approaches to controlling FEB in wheat. Schreiber *et al.* (2011) used a high throughput cotyledon infection assay to screen for chemicals which inhibit *F. graminearum* growth *in planta*. Two chemicals, sulfamethoxazole and gramine, inhibited infection of Arabidopsis seedlings and also FEB disease and DON accumulation in wheat ears, though neither showed *in vitro* antifungal activity. This study also upheld the previous findings that *GLK1* overexpression and mutation of *JAR1* and *COI1* reduce foliar disease (Table 1.1).

Ferrari *et al.* (2012) demonstrated that constitutive expression of polygalacturonase-inhibiting proteins (PGIPs) in Arabidopsis resulted in inhibition of Fusarium PG activity (involved in cell wall degradation) and enhanced floral resistance to *F. graminearum*. Transgenic wheat plants expressing the bean PGIP *PvPGIP2* showed reduced FEB symptoms, indicating the translatability of this study. However, accumulation of DON mycotoxin was not compared between wild-type and transgenic lines; successful FEB control in wheat requires not just reduction in symptoms but also in mycotoxin production.

Conversely, Shin *et al.* (2012) created transgenic Arabidopsis plants expressing a barley UDP-glucosyltransferase, which detoxifies DON. These plants were able to grow on plates supplemented with high levels of DON, where wild-type plants died. However, the resistance of these transgenic lines to Fusarium infection was not assessed. It is therefore unclear whether increased DON tolerance correlates with enhanced resistance to infection.

Several studies have also shown a role of secreted antimicrobial defensin-like compounds in Arabidopsis resistance to Fusarium using transgenic approaches. Expression of the *Medicago trunculata* defensin targeted to either the vacuole, endoplasmic reticulum or extracellular space decreased silique infection by *F. graminearum* following point wound inoculations (see Chapter 2) and also resulted in lower DON accumulation (Kaur *et al.*, 2012). This defensin also inhibited *in vitro* Fusarium growth. Targeting of the *M. trunculata* defensin to the extracellular space also reduced growth of the obligate biotrophic oomycete pathogen *Hyaloperonospora arabidopsidis*.

The insect defensin thanatin from the spiny soldier bug *Podisus maculiventris* was also found to inhibit *F. graminearum in vitro* growth. Furthermore, Arabidopsis plants transgenically expressing thanatin showed less *F. graminearum* leaf infection, as well as reduced infection by the necrotrophic fungus *B. cinerea* (Koch *et al.*, 2012).

Thionins have been found to accumulate in the cell walls of wheat spikes following infection with *F. culmorum* (Kang and Buchenauer, 2003). Asano *et al.* (2013) found that the Arabidopsis thionin Thi2.4 inhibited *in vitro* Fusarium growth and that constitutive expression in Arabidopsis reduced leaf and floral infection by *F. graminearum*. This was thought to be mediated by its interaction with fungal fruiting body lectin (FFBL), a virulence factor from *F. graminearum*.

These studies provide proof of concept for the use of transgenic plants expressing small antimicrobial peptides in control of FEB. However, further studies in wheat are needed, particularly with relation to thanatin and thionin.

1.5.4 Host Induced Gene Silencing of Fusarium genes

One final exciting use of the Arabidopsis-Fusarium pathosystem has come to light during the development of this thesis. In addition to the expression of antimicrobial compounds or alteration of defence signalling pathways, the Arabidopsis-Fusarium pathosystem has also been used to demonstrate a potential role of host induced gene silencing (HIGS) in disease control. *F. graminearum* contains three paralogues of the sterol 14 α -demethylase encoding gene *CYP51*, which are important for growth and virulence on wheat and Arabidopsis and are the target site for azole fungicides (Fan *et al.*, 2013). Expression of double stranded (ds) RNA complementary to the *F. graminearum* *CYP51* genes in Arabidopsis and barley resulted in their silencing in the fungus, inhibiting fungal growth and blocking infection of both Arabidopsis and barley (Koch *et al.*, 2013). This is one of the first examples of host induced gene silencing of fungi, which may provide a novel and effective mechanism for disease control (Bailey, 2014).

1.6 Metabolomics, an emerging research tool in plant biology

Metabolomics – the analysis of all the metabolites in a given biological sample – is becoming an increasingly important tool for the study of plant metabolic processes in the post genomics era (Ward *et al.*, 2003; 2007). Several distinct analytical techniques including ¹H Nuclear Magnetic Resonance (NMR) spectroscopy, Electrospray Ionisation (ESI) mass spectroscopy, and gas and liquid chromatography have been used to study the plant metabolome with a number of applications, as reviewed by Schauer and Fernie (2006), Hall (2006), Saito and Matsuda (2010), Sumner (2010). These applications include the assessment of factors contributing to food taste and nutrition, and the search for novel pharmaceutical compounds. Metabolomic analyses are also

used to assess the effects of genetic mutations and to screen for unintended impacts of genetic modification (Ren *et al.*, 2009, Baker *et al.*, 2006, Hofmann, 2011, Shepherd *et al.*, 2006).

Metabolic signatures may also be useful in predicting plant growth rates and other agronomically desirable traits, with potential applications for metabolic marker assisted breeding (Meyer *et al.*, 2007, Fernie and Schauer, 2009, Lisec *et al.*, 2008). The effect of abiotic stresses on plant metabolism, and the role of plant metabolites in modulating stress responses, has also been studied using metabolomics approaches (Ward *et al.*, 2011, Nikiforova *et al.*, 2006).

1.6.1 Metabolomics and plant-pathogen interactions

Most pertinent to this thesis is the use of metabolomics to advance understanding of plant pathogen interactions. The plant immune response relies heavily on both primary and secondary metabolites, making metabolomics an important tool for dissecting defence responses, as recently reviewed by Leiss *et al.* (2011), Rojas *et al.* (2014) and Heuberger *et al.* (2014). Plants must carefully balance distinct primary metabolic pathways such as those involved in photosynthesis, respiration carbon and nitrogen partitioning against pathways required for inducible defences to pathogens, without compromising essential functions. By contrast, pathogens have been shown to modulate host metabolism in order to aid infection (Truman *et al.*, 2006, Ward *et al.*, 2010). An overview of some of the plant metabolites which may be involved in defence responses, from Heuberger *et al.* (2014), is shown in **Figure 1.3**.

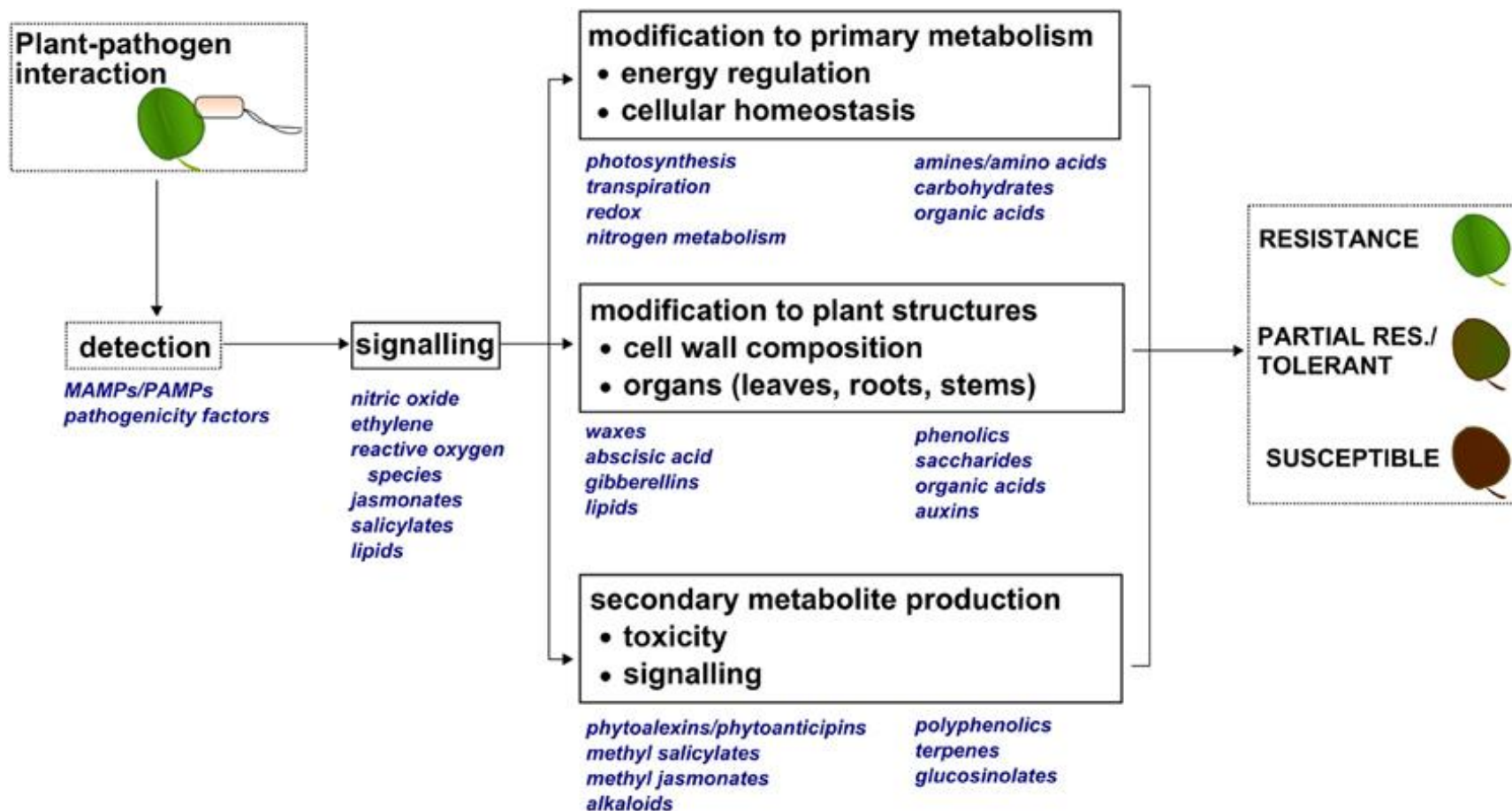


Figure 1.3: Modulation of metabolism during plant responses to pathogen invasion, from Heuberger *et al* (2014). Metabolites are involved in pathogen detection and defence signalling, along with modification to primary metabolism, physical defence responses and production of antimicrobial secondary metabolites.

Several studies have analysed changes in the plant metabolome during pathogen infection. For example, gas chromatography – mass spectroscopy (GC-MS) analysis of *Medicago trunculata* cell cultures treated with pathogen elicitors such as yeast extract and MeJA found alterations in levels of the amino acids glycine, threonine and serine (Broeckling *et al.*, 2005), while analysis using liquid chromatography – mass spectroscopy (LC-MS) found alterations in phenylpropanoid and isoflavonoid biosynthesis and phytoalexin accumulation (Farag *et al.*, 2008). Meanwhile NMR spectroscopy has been used to study the response of *Nicotiana tabacum* to infection by tomato mosaic virus (TMV) and of *Catharanthus roseus* to phytoplasma infection (Choi *et al.*, 2006, Choi *et al.*, 2004).

Further to the role of metabolomics in understanding plant defence responses, metabolomics can also be used to identify metabolic biomarkers of plant disease, which can be used for diagnostic purposes. For example, Hantao *et al.* (2013) used GC-MS to identify biomarkers of Eucalyptus infection by the necrotrophic fungal pathogen *Teratosphaeria nubilosa*, while Aksenov *et al.* (2014) used GC-MS to identify biomarkers of bacterial citrus greening disease (Huanglongbing), a major threat to the citrus industry.

1.6.2 Use of metabolomics to study FEB

Several studies have used metabolomics approaches to identify compounds and pathways which may influence the severity of FEB infection of wheat, maize and barley:

Two separate comparative analyses of the metabolomes of FEB susceptible and resistant barley lines have independently identified several resistance related (RR) compounds including flavonoids, fatty acids and phenylpropanoid

derived compounds such as *p*-coumaric acid, which is a hydroxycinnamic acid. *p*-coumaric acid was also found to inhibit fungal growth *in vitro* (Bollina *et al.*, 2010, Bollina *et al.*, 2011, Kumaraswamy *et al.*, 2011). The latter study also found that jasmonic acid, linolenic acid and a detoxified form of DON (DON-3-O-glucoside) accumulated to a greater extent in resistant lines, and suggested that these compounds could be used as biomarkers for resistance during breeding. Several of these compounds were also independently put forward as RR metabolites following a metabolome analysis of FEB resistant double haploid barley (Chamarthi *et al.*, 2014).

Flavonoids and phenylpropanoid derived metabolites such as cinnamic acid and hydroxycinnamic acid have also been identified as RR metabolites in wheat during comparative metabolome analysis of resistant and susceptible lines (Gunnaiah *et al.*, 2012, Paranidharan *et al.*, 2008, Hamzehzarghani *et al.*, 2005)

A study of maize resistance to FEB also found that *p*-coumaric acid was induced during infection, although the presence of this compound alone was not thought to delay infection (Cao *et al.*, 2011a).

Flax, an important biofuel and fibre crop, is also susceptible to FEB causing *Fusarium* species. It has been shown that transgenic flax with increased production of glycosylated flavonoids has enhanced resistance to *F. culmorum* seedling infection (Lorenc-Kukula *et al.*, 2009).

These studies demonstrate the usefulness of metabolomics for assessing the basis of resistance traits in *Fusarium* hosts, and highlight flavonoids and phenylpropanoids, particularly hydroxycinnamic acids, as resistance related metabolites which could be useful biomarkers for resistance during crop breeding.

1.7 Project Objectives

The main focus of this project was to investigate the factors determining resistance and susceptibility in Arabidopsis floral and silique tissue to the FEB causing pathogens *F. culmorum* and *F. graminearum*. Specific aims of the project were:

1. To analyse the metabolomic fingerprints of the Arabidopsis *enhanced disease susceptibility (eds)* mutants along with other mutants previously screened for *F. culmorum* susceptibility, in both the absence and presence of *F. culmorum* infection. It is hypothesised that mutants susceptible to *F. culmorum* will have metabolic traits in common which distinguish them from wild-type and non-susceptible mutant plants (Chapters 3 and 4).
2. To identify the genomic location of the gene harbouring the *eds11* mutation, using a mapping-by-sequencing approach (Chapter 5).
3. To identify additional mutations which alter floral and/or silique susceptibility to *F. culmorum* (and *F. graminearum*) by screening mutants with altered defence to other biotrophic or hemi-biotrophic pathogens (Chapters 6 and 7).

1.8 Project scope

1.8.1 Use of floral and silique inoculation methods

Several studies have reported infection of Arabidopsis leaves by *F. graminearum* using a variety of experimental infection methods on attached and detached leaves (Skadsen and Hohn, 2004, Chen *et al.*, 2006, Makandar *et al.*, 2010, Makandar *et al.*, 2006, Savitch *et al.*, 2007, Schreiber *et al.*, 2011). However, attempts to induce *Fusarium* infection in healthy Arabidopsis leaves under the conditions reported in this thesis were not successful (Figure 1.4).

This thesis therefore focusses on *Fusarium* floral, silique and pedicel infection

of Arabidopsis, although differences in the surface colonisation of senescent rosette leaves following spray inoculations are also reported.

1.8.2 Focus on *F. culmorum*

While some of the studies reported in this thesis involve *Fusarium graminearum*, this research project predominantly focuses on the interaction between Arabidopsis and *F. culmorum*. There are several reasons for this. Firstly, *F. culmorum* has historically been considered the primary causal agent of FEB in the UK and its optimum growth and infection conditions are more suited to the UK climate (Parry *et al.*, 1995, Doohan *et al.*, 2003). Secondly, previous research on the Arabidopsis-FEB interaction which has fed into this research project has focussed on resistance to *F. culmorum* (Cuzick *et al.*, 2008a, Cuzick *et al.*, 2009). Finally, while *F. culmorum* is an important cause of FEB disease in the UK, *F. graminearum* is considered globally to be the predominant causal agent, and many research groups are therefore focussing on the interaction between *F. graminearum* and its host plants. Focussing on *F. culmorum* as a causal agent therefore avoids duplication of research efforts and optimises research output.

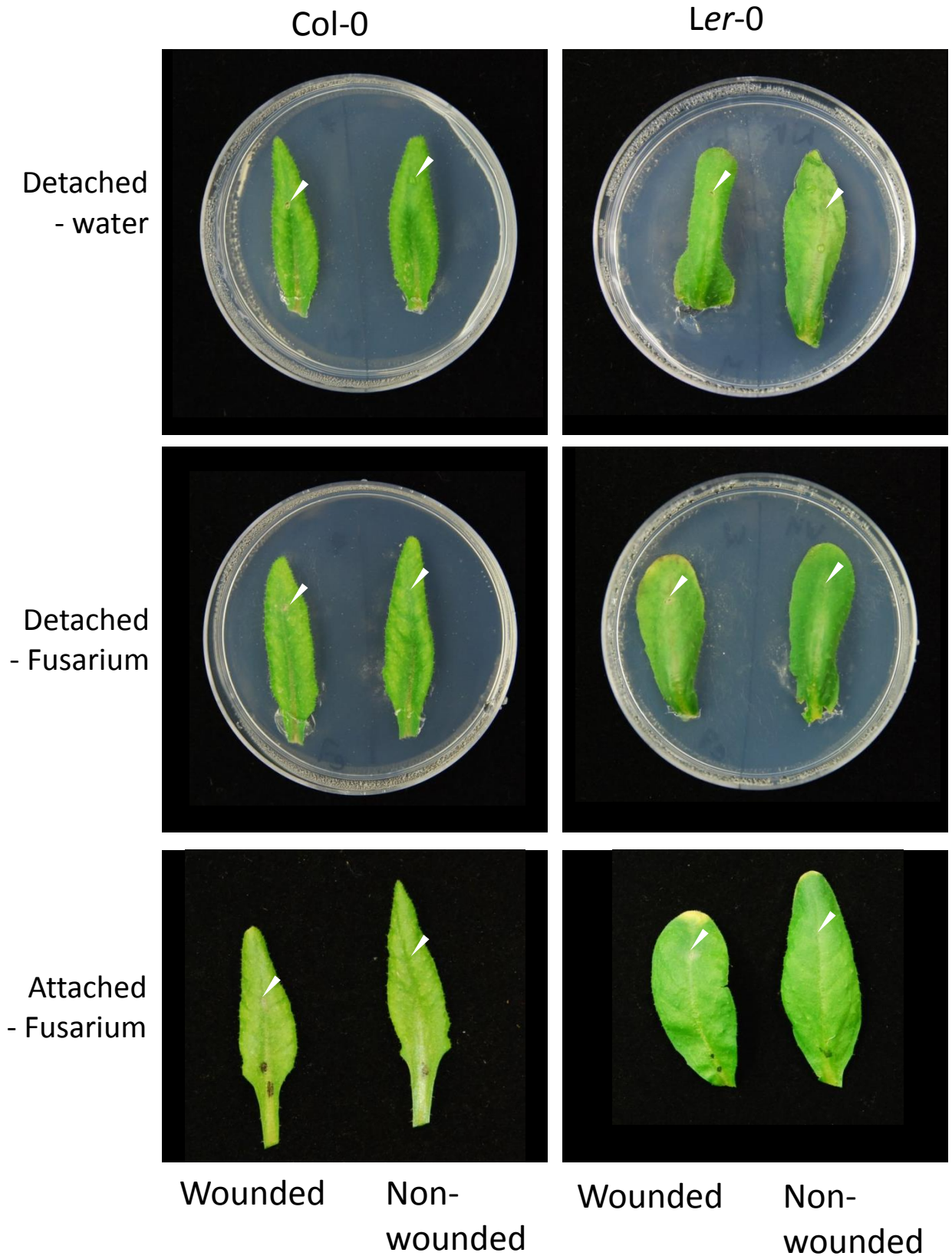


Figure 1.4: Arabidopsis leaves 5 days after droplet inoculation with $\sim 10^5$ *F. graminearum* spores, onto the abaxial surface of wounded or unwounded, attached or detached leaves of Arabidopsis ecotypes Col-0 and Ler-0. White arrow indicates location of spore/water droplet.

2 General Materials and Methods

2.1 Arabidopsis growth and propagation

Arabidopsis plants for *Fusarium* assays and metabolomics analysis were grown in Levingtons F2+S compost (Ipswich, UK) in a Fitotron® 'walk in' plant growth chamber (www.fitotron.co.uk), with a 16 h light / 8 h dark cycle at temperatures of 20°C (light) and 17°C (dark), with 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ fluorescent illumination, at 70% humidity. Seeds were sown onto compost in 40 well flats (each well 4cm² wide and 5cm deep) placed on capillary matting in plastic trays. These were then covered with kitchen foil and transferred to 5°C for four days to allow for seed imbibition and stratification prior to transfer to the growth chamber. A list of seed stocks and their sources is given in Appendix 1.

2.2 Fusarium growth, maintenance and storage

F. culmorum strain 98/11 and *F. graminearum* strain PH-11 were obtained and propagated as previously described (Cuzick *et al.*, 2008b, Urban *et al.*, 2002): Conidia were transferred from frozen stocks stored at -80°C onto synthetic nutrient poor agar plates (SNA -0.1% KH₂PO₄, 0.1% KNO₃, 0.1% MgSO₄·7H₂O, 0.05% KCl, 0.02% glucose, 0.02% saccharose, 2% Agar) for 8-11 days, and then transferred onto potato dextrose agar (PDA, Sigma Aldrich UK) plates for 48h to encourage high levels of conidial production. Conidia were then suspended in sterile distilled water and filtered through sterile Miracloth (Calbiochem®, Watford, UK), and stored at -80°C prior to plant inoculations. Concentration of stock suspensions was calculated using a haemocytometer (Hausser Bright-Line, Horsham, PA, USA). . Studies using *F. graminearum* strain PH-1 were conducted under PHSI license 101948/198285/2.

2.3 Arabidopsis-Fusarium infection assays

Unless otherwise stated, all Arabidopsis-Fusarium inoculations were done approximately 14 hours into the light period of the light:dark cycle. Inoculated plants were kept in Perspex boxes measuring 50 x 50 x 100cm at 100% humidity for the duration of the experiment, with darkness maintained for the first 20 hours of the experiment by covering the box with a black pond liner. Each box had a capacity of up to 80 plants divided across 4 plastic trays (Figure 2.1a). Plants were randomised within each tray. At least 5 treated and 2 control plants were used per genotype in each experiment.

2.3.1 Spray inoculation

Inoculations were done as described in Urban *et al.* (2002):

Plants with 2-3 open flowers but no siliques (Growth stage 6, Boyes *et al.*, 2001) were spray inoculated with *Fusarium* conidia using a Humbrol airbrush powerpack and spray gun (Humbrol, Margate, UK). Stored conidial stocks were defrosted and diluted in sterile water to a concentration of 10^6 conidia ml⁻¹ unless otherwise stated. Each plant received approximately 0.5ml of suspension (Figure 2.1b). Control plants were treated with sterile water.

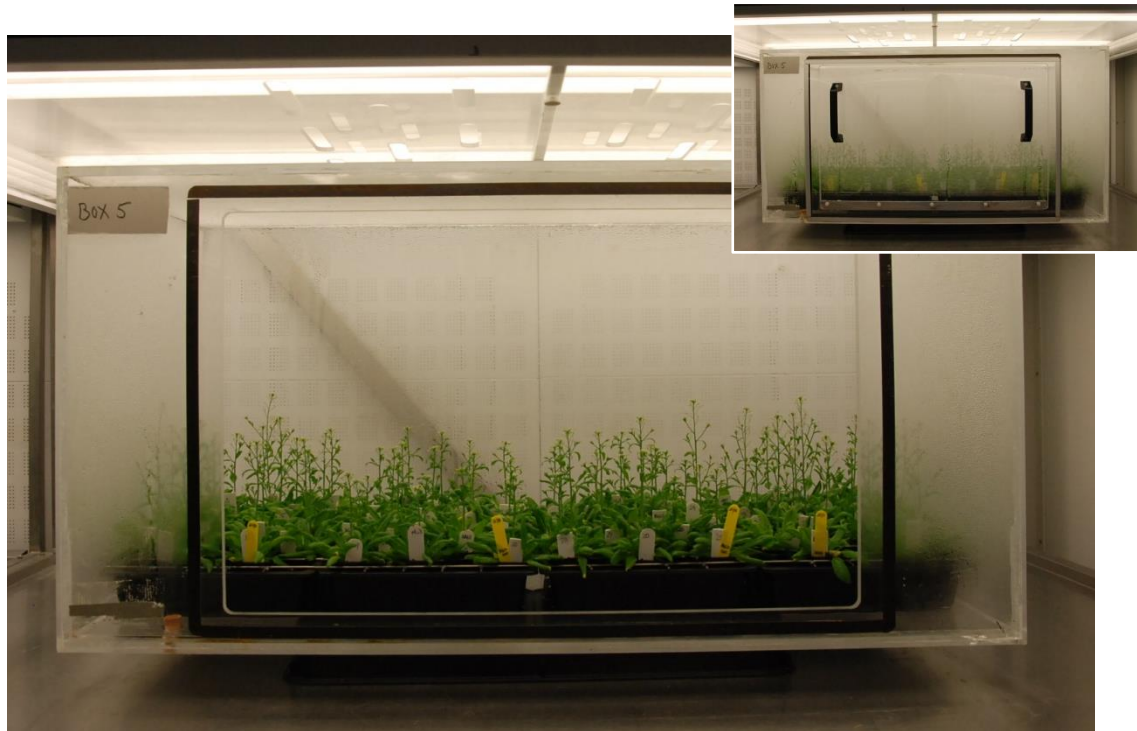
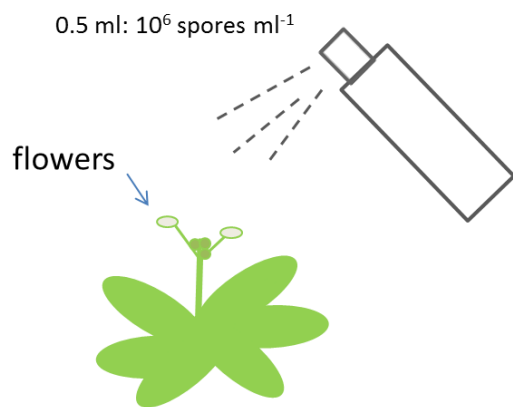
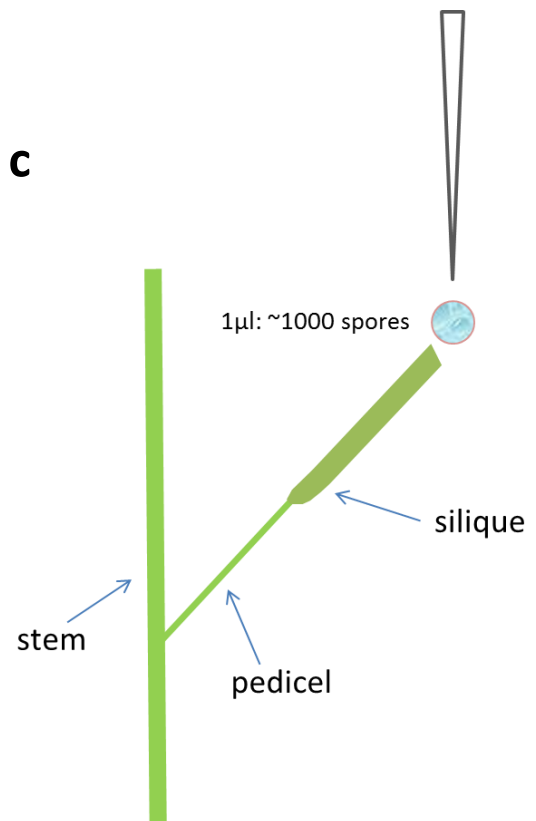
a**b****c**

Figure 2.1: Inoculation of *Arabidopsis thaliana* floral tissue with *Fusarium* conidia. Perspex inoculation boxes used for all inoculations are shown in (a). Inoculations were done either by spraying flowering plants with *Fusarium* conidia (b) or by droplet inoculation of conidia onto the wounded tips of siliques (c).

2.3.2 Assessment of disease progression after spray inoculation

Disease progression was typically assessed at 7, 11 and 14 dpi for Ler-0 genotypes and at 14 and 21 days for other genotypes, but this varied between experiments. Visible infection symptoms on the flowers and developing siliques were assessed using the Fusarium-Arabidopsis Disease (FAD) scoring system described in Urban *et al.* (2002) but with some modifications (Table 2.1). In addition, in some experiments the number of non-diseased green siliques and infected/uninfected rosette leaves were also counted.

Table 2.1: Scoring of *Fusarium* disease in Arabidopsis floral and silique tissue, adapted from Urban *et al.*, 2002. Plants were given separate scores for floral and silique infection from 0 (no disease) to 7 (constriction of the main stem). The intermediate scores of 2 and 4 (F), and 2, 4 and 6 (S) were reserved for when all the tissue arising from the inoculated inflorescence exhibited the disease phenotype described for the preceding score.

Organ	Score	Description of disease phenotypes
Flower (F)	0	No disease
	1	Aerial mycelium visible on flower
	3	Drying of flowers
	5	Stem constriction within flower head
	7	Main stem constriction
Silique (S)	0	No disease
	1	Aerial mycelium on silique surface
	3	Drying of silique surface
	5	Pedicel constriction or loss of siliques by disease travel within pedicel
	7	Main stem constriction

2.3.3 Single silique wound point inoculation

For single silique wound point inoculations, plants were selected with 3-6 immature siliques, depending on the experiment. Approximately 1mm of silique

tissue was removed from the tip of each silique and immediately afterwards a 1 µl droplet of *Fusarium* conidial suspension at a concentration of 10^6 conidia ml⁻¹ was placed on the cut tip (Figure 2.1c).

2.3.4 Assessment of disease progression after single silique wound point inoculation

Following single silique *Fusarium* inoculation, the silique is rapidly colonised and infection progresses along the length of the pedicel, arresting at the pedicel-main stem junction. Infection is characterised by tissue necrosis visible under white light and as green fluorescence under UV light with a GFP2 or Violet filter, viewed using a Leica 205FA stereomicroscope (see imaging, below). There is no evidence of symptomless fungal growth beyond the visible infection front in *Arabidopsis* pedicel tissue.

Green fluorescence under UV light was therefore used to determine the extent of fungal growth along each infected pedicel, and measured along with the total length of the pedicel using the ruler function in the Leica imaging software (Fig. 2.2).

UV light with a Violet filter was used to identify the presence of blue-green auto-fluorescence at the stem-pedicel junction, characteristic of accumulation of the plant defence compounds scopolin and scopoletin (see Chapter 4 and 7).

2.4 *Pseudomonas* growth, maintenance and storage

Pseudomonas syringae pv. *maculicola* strain ES4326 was provided by the Ausubel laboratory (MIT, Boston, USA). For long term storage, 15% glycerol stocks were made and kept at -80°C. Stocks were then cultured overnight in liquid LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7) at 28°C.

2.5 Infection of Arabidopsis with *Pseudomonas* bacteria

Plants for *Pseudomonas syringae* assays were grown in a Vindon™ upright growth chamber (www.vindon.co.uk) with a 12 h light / 12 h dark cycle. Bacteria were centrifuged and re-suspended in 10mM MgSO₄ at a concentration of approximately 10⁶ cfu/ml (OD₆₀₀=0.0002) equating to 10³ cfu/cm² leaf tissue, unless otherwise stated. Plants were inoculated with the bacterial suspension by needleless syringe infiltration onto the abaxial leaf surface. Plants were inoculated at 4 weeks of age unless otherwise stated. Two leaves were inoculated per plant, with at least 6 plants inoculated per genotype.

2.6 DNA extraction, PCR amplification and sequencing

2.6.1 DNA extraction

Total DNA from Arabidopsis plants was extracted using the DNeasy Plant Mini Kit (Quiagen, see manufacturer's instructions). DNA from fungal spores was extracted using the phenol chloroform method described for extraction from archived air samples in Rogers *et al.* (2009) with the modification of using ammonium acetate and isopropanol in place of sodium acetate and ethanol for DNA precipitation.

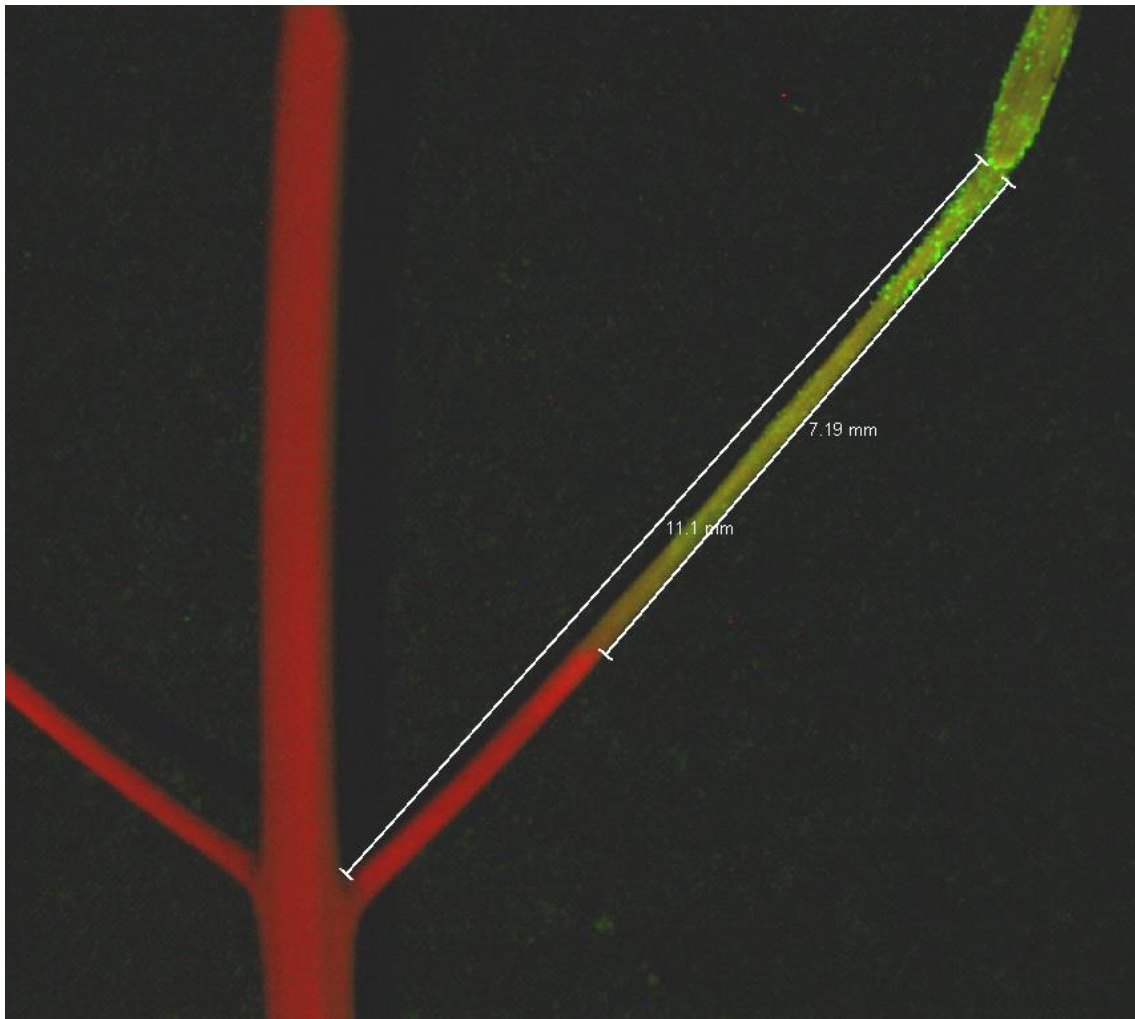


Figure 2.2: Assessment of fungal growth through Arabidopsis pedicel tissue. Infected tissue was viewed using a Leica 205 FA stereomicroscope under UV light with a GFP2 filter. The length of pedicel infected by the fungus and the total length of the pedicel were measured using the ruler tool within the LAS-AF6000 software (Leica microsystems, Milton Keynes, UK), giving absolute and relative levels of fungal infection within each pedicel.

2.6.2 Polymerase Chain Reaction (PCR) analyses

PCR was done using either REDTaq® ReadyMix™ PCR Reaction Mix, or Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs, Ipswich, MA, USA) where sequencing of the product was required (see manufacturers' instructions). Reactions were done using a G-STORM thermal cycler (Somerton, Somerset, UK).

2.6.3 Purification and sequencing of PCR products

Amplified DNA was purified using the QIAquick PCR Purification Kit (Quiagen, Manchester, UK, see manufacturer's instructions) and submitted to Eurofins MWG Operon (Ebersberg, Germany) for sequencing along with primers, in accordance with their submission guidelines. Geneious™ software (Auckland, NZ) was used for viewing and alignment of sequenced amplicons.

2.6.4 Gel Electrophoresis

Arabidopsis genomic DNA and DNA amplified from PCR reactions was visualised on agarose gels, typically subjected to 80v for 50 minutes. Gels were made using 1 x TBE (tris borate EDTA) and 1% or 0.8% agarose (Fisher Scientific, UK) for amplicons >1kb and whole genomic DNA, respectively.

2.6.5 Primer design

Primers were designed using Primer3 software (Wageningen, Netherlands) within the Geneious programme and synthesised by Sigma Aldrich UK.

2.7 Imaging

2.7.1 Stereomicroscopy

A Leica 205FA stereomicroscope and accompanying LAS-AF6000 software (Leica microsystems, Milton Keynes, UK) were used for microscopic analysis

and image capture, using either white light or UV light with a Violet filter (Excitation 405-445, Emission 460) or a GFP2 filter (Excitation: 460-500, Emission: 510nm). Scale bars were automatically generated by the LAS-AF6000 software.

2.7.2 Photography

A Nikon D80 digital camera with a Sigma DC MACRO HSM 17-70mm lens was used for image capture. Plants were photographed on blue or black velvet under growth room lighting conditions using the close-up camera mode without the use of flash. Cropping, resizing and annotation of photographs and generation of figures was done using Microsoft PowerPoint software

2.8 Statistical Analysis

The statistical tests used for analysis of each dataset are outlined in the respective figure legends. Tables of means, standard errors and least significant differences (LSDs) can be found in Appendix 5. An effect of genotype or treatment was considered significant at $p < 0.05$, unless otherwise stated. All statistical analysis was done using Genstat v16 (Payne *et al.*, 2011). Commonly used statistical analyses are detailed below.

2.8.1 Analysis of Arabidopsis-Fusarium spray inoculation data

Arabidopsis – Fusarium disease susceptibility data generated using the FAD scoring system following spray inoculation (described in Table 2.1), along with numbers of infected leaves or uninfected siliques, were subjected to regression analysis fitted to a Generalized Linear Model with assumed Poisson distribution using the log link function. For mutant experiments with multiple time points, the effects of genotype and time, and the interactions between genotype and time, were examined for each measured variable. Where a significant effect of

genotype or treatment was found ($p < 0.05$), genotype/treatment means and least significant differences (LSDs) between genotypes or treatments were calculated at a 5% confidence level and means for all genotypes compared to the control genotype, and all treatments (such as amino acid treatment) compared to the control treatment, to identify significant differences. In the absence of an interaction between genotype and time, one mean per genotype was predicted by amalgamating data from all time points, with corresponding LSDs. Where a significant interaction between genotype and time was observed, means and LSDs between genotypes were calculated for each time point assessed.

2.8.2 Analysis of Arabidopsis-Fusarium silique inoculation data

For single silique point wound inoculations, one-way analysis of variance (ANOVA) was used to compare fungal growth (in mm) along the silique between genotypes. LSDs ($p < 0.05$) from the control genotype were calculated.

2.8.3 Analysis of susceptibility to *P. olsonii* floral infection and *F. culmorum* infection of the stem-pedicel junction

Disease probability data presented in figure 4.2 were statistically analysed using a Generalized Linear Model with assumed Binomial distribution and using the logit link function. LSDs ($p < 0.05$) were then calculated for predicted mean probabilities for each genotype compared to wild type plants. For pedicel infection data, the percentage of infected pedicel-stem junctions (/6) was calculated for each plant.

3 Assessment of metabolome differences between *Arabidopsis* genotypes with altered defence responses

3.1 Introduction

Metabolomics is a powerful tool for understanding plant metabolic processes and the possible role(s) of uncharacterised genes, as outlined in Chapter 1.

This chapter examines deviations from the wild type *Arabidopsis* metabolome in a collection of defence related mutants, along with transgenic insertion lines known to affect pathogen recognition and defence signalling. This study was done in collaboration with the Rothamsted Metabolomics Facility (MeT-RO).

A selection of 13 mutants/overexpression lines previously tested for altered *F. culmorum* floral susceptibility were selected for metabolomic ‘triple fingerprinting’ using a combination of proton nuclear magnetic resonance (¹H NMR) spectroscopy and electron spray ionisation (ESI) mass spectroscopy in positive and negative ion modes (Ward *et al.*, 2003, Ward *et al.*, 2007). The metabolic complement of whole, healthy wild type and mutant/transgenic plants at first flower stage was assessed. The mutants selected show a range of responses to *F. culmorum* and other phyto-pathogens (Table 3.1):

The *enhanced disease susceptibility* mutants *eds4*, *eds5*, *eds7*, *eds8*, *eds10*, *eds11*, *eds12* and *eds13* all show increased susceptibility to virulent *Pseudomonas syringae* pv. *tomato* (*Pst*) and *P. syringae* pv. *maculicola* (*Psm*) but show varying responses to other pathogens including the mildew *Erysiphe ornontii* and the bacterial wilt *Xanthomonas campestris* (Table 3.1). In addition, *eds7*, *eds11* and *eds12*, along with the SA signalling mutant *npr1* (isolated in the same mutant screen) all show increased susceptibility to floral infection by *F. culmorum* (Cuzick *et al.*, 2008 and unpublished). Only one of the *EDS* genes

(*eds5*) has been mapped to a genomic location, and the role of the remaining genes in plant defence signalling is poorly understood.

RAR1 and *SGT1b* have been shown to have a coupled role in mounting a hypersensitive response (HR) mediated by resistance (*R*) gene recognition in both monocotyledonous and dicotyledonous plants (Azevedo *et al.*, 2006, Azevedo *et al.*, 2002). However, mutation of *SGT1b*, but not *RAR1*, results in decreased susceptibility to *F. culmorum* in Arabidopsis floral tissue (Cuzick *et al.*, 2009). It is unclear why the *sgt1b* mutant would be more resistant rather than less resistant to attack by *Fusarium*, and why the *rar1* mutant does not show the same phenotype.

The *Cladosporium fulvum* effector ECP6 has previously been shown to mask recognition of the fungal pathogen associated molecular pattern (PAMP) chitin (de Jonge *et al.*, 2010). Constitutive expression of the *C. fulvum* ECP6 gene in Arabidopsis ecotype Col-0 increases susceptibility to the vascular wilt pathogen *Verticillium daliae* (Thomma *et al.*, pers. comm.) and was shown to suppress resistance to *F. culmorum* floral infection in preliminary studies (Hammond-Kosack *et al.*, unpublished). However this reduced resistance phenotype was not observed in follow-up studies (see Chapter 7).

The role of ethylene (ET) signalling in defence against *Fusarium* is not clearly understood in wheat or Arabidopsis, with evidence that it may promote or suppress defence, or be uninvolved (Chen *et al.*, 2006; Cuzick *et al.*, 2008; Chen *et al.*, 2009; Ding *et al.*, 2011; Scofield *et al.*, unpublished). Therefore the transgenic Arabidopsis line constitutively expressing *ETHYLENE RESPONSE FACTOR 1* (*ERF1*), which has previously shown wild type levels of defence against *F. culmorum* (Table 3.1), was also included in the study.

The aim of this chapter was to generate a unique metabolomic fingerprint for each of the 13 mutant/transgenic lines of interest. It was hypothesised that mutants with similar susceptibility profiles (Table 3.1) might share similar metabolic perturbations, which could increase understanding of how these genes and their associated metabolic processes affect plant resistance. In addition, any alterations in defined metabolic pathways in the unmapped *eds* mutants might help to elucidate the function and perhaps identity of the genes and pathways in which the unmapped causal mutations lie.

Table 3.1: Defence response phenotypes of mutants and overexpression lines investigated in this chapter. wt = wild type phenotype, S = increased susceptibility, R = increased resistance, SAR+ = wild type systemic acquired resistance, SAR- = reduced systemic acquire resistance. ISR+ = wild type induced systemic resistance, ISR- = reduced induced systemic resistance. Information collated from Glazebrook *et al.* (1996), Volko *et al.* (1998), Rogers and Ausubel (1997), Ton *et al.* (2002), Cuzick *et al.* (2008a) and Cuzick *et al.* (unpublished)

Genotypes	Pathogens / treatments					
	<i>Pseudomonas syringae</i> leaves	<i>Erysiphe orontii</i> leaves	<i>Xanthomonas campestris</i> leaves	<i>Fusarium culmorum</i> floral	systemic acquired resistance	induced systemic resistance
Col-0	wt	wt		wt	SAR+	ISR+
Col-0 <i>npr1-1</i>	S	S	S	S	SAR-	ISR+
Col-0 <i>eds4</i>	S			wt	SAR+	ISR-
Col-0 <i>eds5-2 / sid1</i>	S	S	S	wt	SAR-	ISR+
Col-0 <i>eds7</i>	S			S	SAR+	ISR+
Col-0 <i>eds8</i>	S			wt	SAR+	ISR-
Col-0 <i>eds10-1</i>	S	S	S	wt	SAR+	ISR-
Col-0 <i>eds11-1</i>	S	R	wt	S	SAR+	ISR+
Col-0 <i>eds12-1</i>	S	wt	wt	S	SAR-	ISR+
Col-0 <i>eds13-1</i>	S	S	S	wt	SAR+	ISR+
Col-0 <i>ERF1</i>	S			wt		
Col-0 <i>ECP6</i>				S/wt		
Ler-0	wt			wt		
Ler-0 <i>sgt1b</i>	wt			R		
Ler-0 <i>rar1</i>	wt			wt		

3.2 Materials and Methods

3.2.1 Tissue harvesting and metabolomic analysis

Arabidopsis genotypes Col-0, *eds4*, *eds5*, *eds7*, *eds8*, *eds10*, *eds11*, *eds12*, *eds13*, *ERF1*, *Ler-0*, *rar1*, *sgt1b*, *npr1* and *ECP6* were subjected to metabolomic analysis (see Appendix 1.1 for seed stock origins).

Plants were grown until first flower stage (Growth stage 6.00, Boyes *et al.* 2001). This is the growth stage at which *Fusarium* floral inoculations are done. This growth stage and tissue type also precisely matches the main Arabidopsis mutant screen currently underway at MeT-RO (Metabolomics at Rothamsted) as part of large scale analysis of Arabidopsis mutants generated via forward or reverse genetics (HiMET consortium project). This experimental design therefore allowed for the possibility of direct comparison between the unmapped *eds* mutants and mutants included in the HiMET screen.

All aerial tissue from each plant was harvested directly into liquid nitrogen, between 14 and 15 hours into the light period. Samples were then stored at -80°C prior to freeze drying. Three experimental replicates were done, with 6 plants harvested from each genotype.

Plant tissue from each experimental replicate was then pooled, freeze-dried, ground using a pestle and mortar and then submitted to MeT-RO (Metabolomics at Rothamsted) for analysis using methods previously described (Ward *et al.*, 2011, Ward *et al.*, 2003). Briefly, freeze dried samples were split into three technical replicates and extracted using polar solvent mixture of 80:20 D₂O:C₃OD, and then analysed using ¹H NMR and ESI-Mass spectroscopy. SIMCA-P 11 software (Umetrics, Umea, Sweden) was used for multivariate analysis. All data were mean centre scaled.

Principle component analysis, hierarchical clustering, statistical analysis and construction of heat maps were done by Dr Jane Ward, MeT-RO. Manipulation and interpretation of information provided in heat map form was done by the author.

3.2.2 Amplification and sequencing of *FAH1*, *NPR1*, *NPR3* and *NPR4*

To verify background mutations in the various genetic stocks and/or the presence of various wild-type sequences of key defence genes, several

diagnostic PCR analyses were done. A 381bp region of the Arabidopsis *FAH1* gene surrounding the *fah1-2* mutation (located at the 292nd nucleotide of the ORF) was amplified from genotypes Col-0, *eds4*, *eds5*, *eds7* and *eds8* using the primers in Table 3.2.

Overlapping sequences of the Arabidopsis *NPR1*, *NPR3*, and *NPR4* gene and promoter regions that were approximately 600bp each in length were amplified from *eds11* using five forward and reverse primer sets per gene (Table 3.2).

Table 3.2: Primers used for PCR and sequencing in this chapter.

Gene	Forward Primer	Reverse Primer
<i>FAH1</i>	5'-TCAGCTTCATCACACGGCGGC-3'	5'ACGAACTGAAGCCCATGACTCAGC-3'
<i>NPR1</i>	5'-TCTCACCACCACTCTCGTTG-3'	5'-CTGCGCATTGAGAACTCCT-3'
	5'- TTCGGTTGTGACTGTTTTGG -3'	5'- AATGAAGAGCACACGCATCA -3'
	5'- TCGAATGTACATAAGGCACTTGA -3'	5'- TTGAAAAAGACGTTGAGCA -3'
	5'- ACGCTGCTCGATCTTGAAAA -3'	5'- GGGACGAATTTCTTAATTCCA -3'
	5'- GCGGAGAAGACGACACTGCTGA -3'	5'- CAGGATGCAAAACGAAGAGCG -3'
<i>NPR3</i>	5'-TGGTTCTGGGTTTGGTTGAT-3'	5'-AATGGCAGGTCGACAACAAT-3'
	5'-TTTCGAAAAGTGAAGAACCAAA-3'	5'-TCTCCGAAATCTTGGGACTG-3'
	5'-TTGACTCAGCTTCTTGATCAGTG-3'	5'-AGAACCATGGGGTTCTTCCT-3'
	5'-CGCCAATGCATCTGAGTTTA-3'	5'-TGCATTGTGAAACGTAATGA-3'
	5'-TGGGTAAACGGAAGCTTGA-3'	5'-TTTTGGGTAAATCAGCACTCC-3'
<i>NPR4</i>	5'-AAGCAAAGCAAAAAGGAAAGG-3'	5'-ATGAAACGCCTTATGTGCAA-3'
	5'-TGGGAAGTATCTCCGACCTG-3'	5'-GGAGACTCACTAGGCCGAAA-3'
	5'-GAATGGGAACAAGTGGGTGT-3'	5'-TTGAGAGAGTGGCGAGATCA-3'
	5'-TCTGAATCCAATGCCTTGAGTA-3'	5'-TGAGAAGAAACCTAAGTATCAAATGAA-3'
	5'-AATCAATGGCCGGTTTACAA-3'	5'-GGATCTTTTCTTCAGGGCTTG-3'

3.3 Results

3.3.1 Metabolomic analysis of healthy *Arabidopsis* plants reveals differences in primary metabolite composition between genotypes

Healthy plants at first flower stage were subjected to metabolomic analysis in order to assess differences in primary metabolism between wild type and mutant plants in the absence of pathogen challenge.

Samples were submitted to MeT-RO for triple fingerprinting analysis (^1H NMR spectroscopy and ESI mass spectroscopy in both positive and negative ion modes). Data from ^1H NMR reads are presented and assessed in this chapter. Metabolite data for each genotype were subjected to principal component analysis (PCA, Fig. 3.1). A heat map was generated by MeT-RO showing relative abundance of the metabolites identified in each genotype with hierarchical clustering of the metabolites and genotypes shown on the heat map axes (Fig. 3.2). Note that wild type *Ler-0* and the *Ler-0* background mutants *rar1* and *sgt1b* were omitted from the PCA due to having metabolic fingerprints which are very distinct from genotypes in the Col-0 background, in order to visualise differences between Col-0 background genotypes more clearly.

3.3.2 Principal Component and Hierarchical Clustering Analysis reveal the relationship between *Arabidopsis* mutant and wild type metabolic fingerprints.

The Principal Component Analysis (Fig. 3.1) reveals *eds4* and *eds8* to be clear outliers with fingerprints distinct from both wild type Col-0 and the other genotypes assessed. Hierarchical Cluster Analysis and heat map generation (Fig. 3.2) place *eds8* in a cluster with the 3 *Ler-0* genotypes assessed, despite the mutant being in the Col-0 background. The *eds4* mutant clusters separately to all the other mutants assessed, denoting its distinct metabolic fingerprint.

Mutants *eds11* and *npr1-1* also segregate from the other mutants due to higher

PC1 values, and cluster together. Hot and cool metabolite regions relating to *eds11* and *npr1-1* mutants are broadly the inverse of those observed for *eds4* and *eds8* (Fig. 3.2). Statistically significant differences (Tukey Kramer, $p < 0.05$) in metabolite abundance between mutant and wild type plants are shown in Figure 3.3.

The transgenic line overexpressing *ECP6* co-clusters with wild type Col-0, revealing limited differences in metabolite synthesis between these two genotypes: The insertion of *ECP6* alters plant cell perception of the fungal PAMP chitin and so alterations in metabolite synthesis in the absence of chitin might be expected to be minimal. Likewise, the SA signalling mutants *eds5/sid1* and *eds12* also cluster together closely with Col-0 and *ECP6*. Differences in metabolite synthesis between these mutants and wild type may also only become apparent upon pathogen inoculation and /or treatment with defence inducing compounds such as SA.

The 3 *Ler-0* genotypes, *Ler-0*, *rar1* and *sgt1b*, are all more similar to one another than to the Col-0 genotypes, with the exception of *eds8* (see above). This complements previous findings that *Ler-0* and Col-0 have very distinct metabolic fingerprints (Ward *et al.*, 2003). Mutants *rar1* and *sgt1b* cluster more closely with one another than with wild type *Ler-0*, possibly alluding to the known paired role of these genes in *R* gene mediated defence signalling (Azevedo *et al.*, 2002).

3.3.3 Wild type and mutant plants differ in their abundance of 29 known and 26 unknown metabolites

Known metabolites identified with different levels of abundance between genotypes were the amino acids leucine, isoleucine, asparagine, serine, valine, proline, alanine, glutamine, threonine, aspartate and glutamate, the sugars

maltose, raffinose, stachyose, glucose, fructose, sucrose, galactose and galactinol, along with choline, glycine betaine, malate, fumarate, sinopoyl malate, γ -aminobutyric acid (GABA) and the flavonoids 3-p- α -coumaroyl glucosyl rhamnoside (KGR) and kaempferol 3,7-dirhamnoside (KRR).

An additional 26 unknown compounds were also present in different abundances across the selected genotypes, most of which occurred in the carbohydrate and aliphatic regions of the spectrum (Ward *et al.*, 2003). Many of these show similar relative abundances and distribution patterns across genotypes to known metabolites, indicating that these may be precursors or compound derivatives (Fig. 3.2). However, there is a large cluster containing 10 unknown metabolites at the centre of the HCA map. This splits into two smaller clusters, the first of which contains 4 unknown metabolites along with leucine and isoleucine, indicating that the unknown compounds may be associated with the synthesis of these amino acids. The other cluster contains 5 unknown compounds which do not appear to be closely associated with any known metabolites, making their role in metabolism difficult to ascertain. One unknown compound (position-4.485) does not cluster with any other known or unknown compounds and displays a unique pattern of relative abundance between genotypes.

A map of primary metabolism showing the different abundance of known metabolites between wild type plants and the *eds* mutant suite is shown in Figure 3.4. These mutants have been selected for further scrutiny because, excepting *eds5* and *npr1*, their genomic location and gene function currently remain unknown.

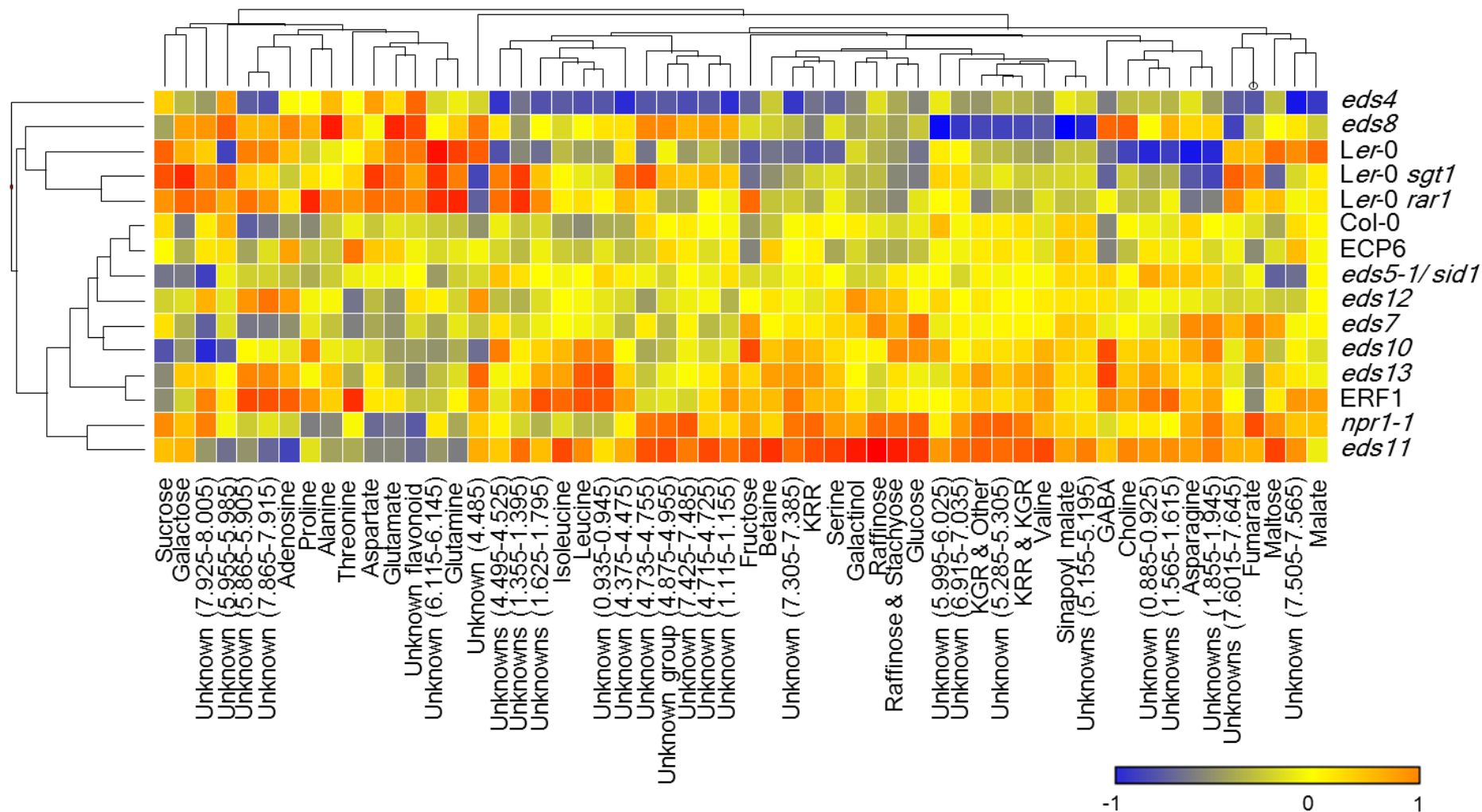


Figure 3.2: Heat map representation generated by Jane Ward, MeT-RO of discriminatory metabolites between Arabidopsis wild type and defence related mutant and transgenic plants. Comparisons were generated via HCA using the complete linkage method with similarity based on Euclidian distance. Metabolite levels are based on characteristic chemical shift ranges from $^1\text{H-NMR}$ intensities. For each metabolite, data are mean centred and normalised to unit variance.

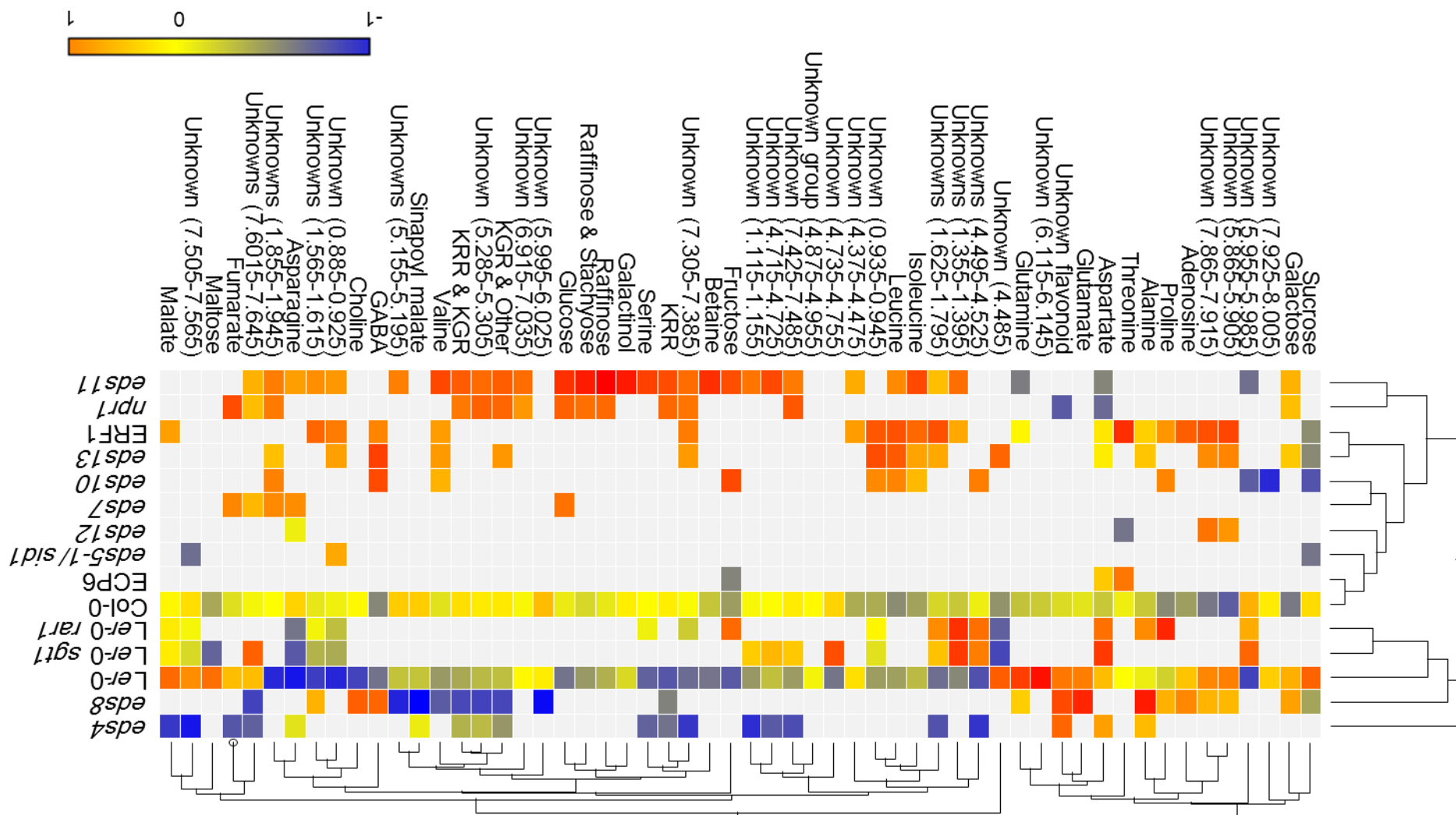


Figure 3.3: Heat map representation of statistically significant discriminatory metabolites between mutant/transgenic and wild type lines. All genotypes compared to Col-0, with the exception of *sgt1* and *rar1* which are compared to Ler-0. Statistical analysis done by Jane Ward, MeT-Ro using Tukey-Kramer test. $p < 0.05$. Heat map adapted for statistical significance by H. Brewer.

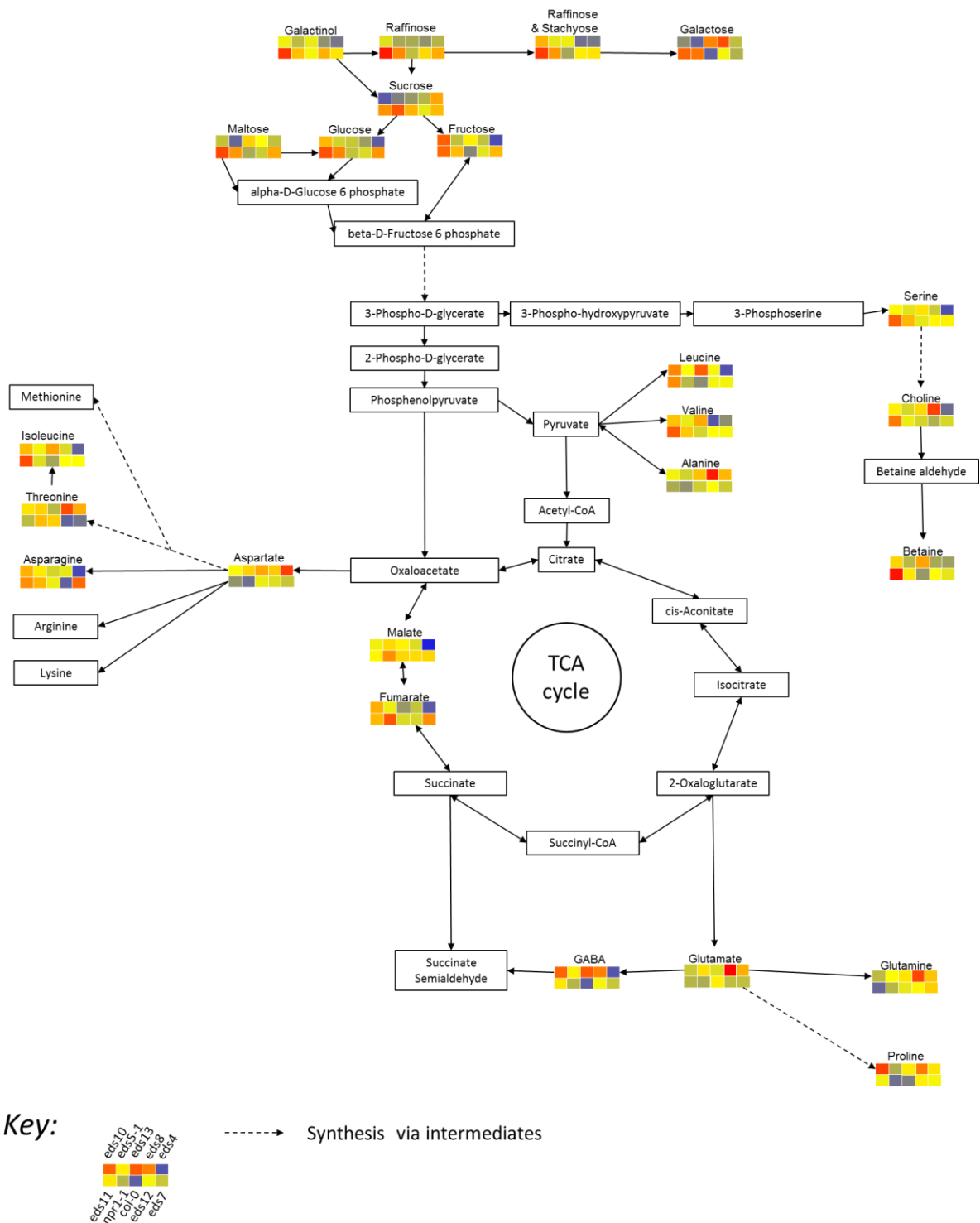


Figure 3.4: Simplified map of primary metabolism in *Arabidopsis thaliana*, showing the relationships between the metabolites identified in this analysis and variation in relative abundance of these metabolites between the genotypes analysed, according to data generated from ^1H NMR. Metabolites without abundance information were either not identified in the analysis, or their abundance did not vary significantly between genotypes. Pathway information was obtained using the KEGG database.

3.3.4 Sinapoyl malate biosynthesis is unaltered in mutants generated in the *fah1-2* background

The mutants *eds4*, *eds5*, *eds7* and *eds8* were generated during an EMS mutagenesis screen of plants in the *ferulic acid hydroxylase 1-2* (*fah1-2*) background, which cannot synthesise the sinapic acid ester sinapoyl malate (Chapple *et al.*, 1992). However, this study revealed that only *eds4* and *eds8* had reduced levels of sinapoyl malate, and this reduction is only statistically significant for *eds8* (Tukey-Kramer, $p < 0.05$). Mutants *eds5* and *eds7* had levels equivalent to those found in wild type Col-0 (Fig. 3.2). The gene fragment containing the *fah1-2* mutation was therefore amplified from wild type and *eds* mutant plants and sequenced in order to assess whether the mutation was still present, revealing that only *eds8* still contains the *fah1-2* mutation. The mutation has most likely been lost from the other *eds* mutants during a backcrossing event, but not from *eds8*. This may be due to a lower number of backcrosses for *eds8*, or genetic linkage between the *eds8* and *fah1-2* mutations.

3.3.5 Mutants *eds11* and *npr1* have similar metabolic fingerprints, which are distinct from wild type Col-0 and other mutant plants

The mutants *npr1* and *eds11* are both highly susceptible to leaf tissue infection by virulent strains of the bacteria *Pseudomonas syringae*, and to floral infection by *Fusarium culmorum* (Volko *et al.*, 1998, Cuzick *et al.*, 2008a). Both mutants are also late flowering under the growth conditions used for this study (Fig. 3.5). These mutants were found to have distinct metabolic fingerprints from the other genotypes assessed, but were very similar to one another; both had significantly elevated levels of the sugars galactose, raffinose, and glucose, and the flavonoids KRR and KGR along with three associated unknown compounds

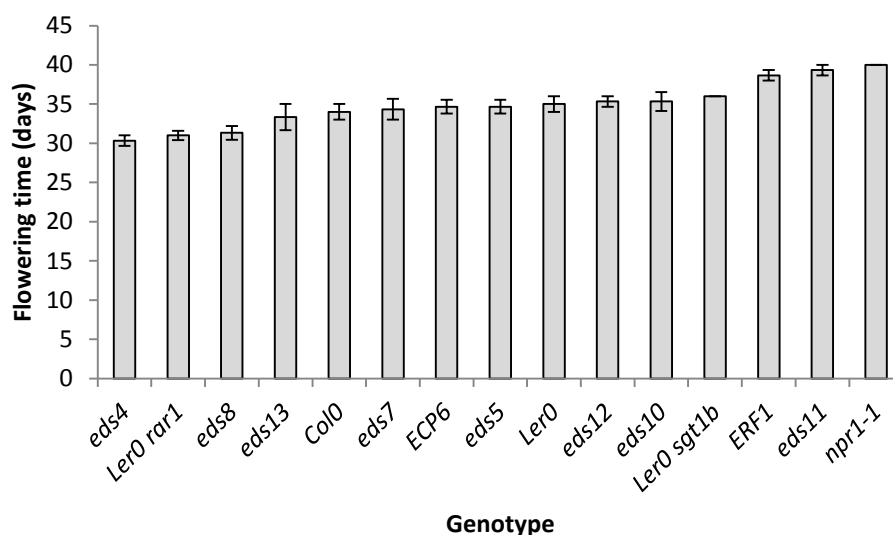


Figure 3.5: Flowering time of plants used for metabolomic analysis, based on time from sowing to harvest at first flower stage. Data are means of three experimental replicates. Bar = standard error.

(7.305-7.385, 6.915-7.035 and 5.285-5.305). Collectively, this data indicates that both mutants have altered primary sugar metabolism and flavonoid biosynthesis compared to wild type Col-0. Both mutants also had elevated levels of two unknown compounds (4.875-4.995 and 7.425-7.485) which clustered separately to any known metabolites, and two unknown compounds (1.855-1.945 and 7.6015-7.645) which associated with asparagine and fumarate, respectively. It is of note that these two unknown compounds are also elevated in the *Fusarium* susceptible mutant *eds7*, indicating that these may play a role in this susceptibility. The *eds11* mutant also had significantly elevated levels of eight additional unknown metabolites, many of which cluster independently of known metabolites, along with leucine, isoleucine, valine, serine, betaine and galactinol. Many of these were also elevated in some *npr1* samples but this was not statistically significant. The *eds11* mutant has previously been found not to be an allele of *npr1* (Volko *et al.*, 1998) and sequencing of the *NPR1* gene and promoter region in *eds11* confirmed this.

Additionally, sequencing of the related genes *NPR3* and *NPR4* (Zhang *et al.*, 2006, Moreau *et al.*, 2012, Fu *et al.*, 2012) from *eds11* confirmed that *eds11* is wild type for these genes. However, the close relationship between the metabolic fingerprints of the two mutants suggests that EDS11 and NPR1 may be involved in a common signalling pathway.

3.3.6 Mutations in *EDS8* and *EDS4* cause broad spectrum changes in primary metabolism

The *eds8* and *eds4* mutants are both characterised by small rosettes and early flowering (Figure 3.5), along with increased susceptibility to *Pseudomonas syringae*, and a reduction in Induced Systemic Resistance (ISR) mediated by Jasmonic Acid (JA) signalling (Table 3.1). Both these mutants have metabolic fingerprints which are distinct from wild type plants (Figures 3.1 – 3.3): Both have significantly reduced levels of the major flavonoids KRR and KGR, along with an associated unknown metabolite (5.205-5.285). In addition, *eds8* has elevated levels of glutamate and its derivatives GABA, proline and glutamine, along with adenosine (and two associated unknown compounds), and alanine and galactose. The *eds4* mutant has decreased levels of the TCA (tricarboxylic acid) cycle components fumarate and malate, and two associated unknowns (7.505-7.565), along with the amino acids asparagine, alanine and serine, and 5 unknown metabolites which cluster together.

3.3.7 Mutations in the *R* gene mediated defence signalling genes *RAR1* and *SGT1b* cause similar alterations in primary metabolism

Both *rar1* and *sgt1b* showed decreased malate levels, but elevated levels of asparagine and aspartate compared to wild type Ler-0 plants. These mutants also shared altered levels of eight unknown compounds, and decreases in a further three unknown compounds. These shared alterations may reflect the

coupled role of SGT1b and RAR1 in plant defence signalling (Azevedo *et al.*, 2002). Further investigation into the identity of the unknown compounds is required.

3.3.8 *ERF1*, *eds10* and *eds13* show common increases in metabolite abundance compared to wild type Col-0

The Arabidopsis line overexpressing *ETHYLENE RESPONSE FACTOR 1*, and mutants in *EDS10*, which is thought to be involved in ISR mediated by JA signalling, and *EDS13*, a gene of unknown function, are all more susceptible to the virulent bacterial pathogen *P. syringae* but the connection between them in terms of metabolite synthesis and signalling are unknown. This study reveals that all 3 mutants produce elevated levels of GABA, valine, leucine and isoleucine, and 2 associated unknown compounds (0.935-0.945 and 1.625-1.795), but have reduced levels of sucrose. This indicates that overexpression of *ERF1* disrupts primary metabolism in a similar manner to mutations in *EDS10* and *EDS13*.

3.3.9 Mutation of *EDS7* and *EDS12* results in a small number of significant metabolome alterations from wild type plants

The floral tissue of both *eds7* and *eds12* is more susceptible to infection by *F. culmorum*. While neither of these mutants have metabolic fingerprints which differ extensively from wild type plants, both are altered in their relative abundance of a small number of compounds:

Asparagine and threonine were both reduced in *eds12*, while levels of two unknown compounds (5.865-5.905 and 7.865-7.915), which were linked to adenosine by HCA, were elevated. Mean levels of adenosine were also found to be increased compared to wild type plants but this finding was not statistically significant (Tukey-Kramer, $p \geq 0.05$).

Alterations in the *eds7* metabolome corresponded to a subset of the alterations observed in *eds11* and/or *npr1*; plants had elevated levels of glucose, along with fumarate and asparagine and their associated unknown compounds (7.6015-7.645 and 1.855-1.945, respectively).

3.4 Discussion

This study has revealed differences between Arabidopsis defence related mutant and wild type plants in terms of their metabolic fingerprint. Each of the 13 mutant/transgenic lines investigated deviates from wild type in its abundance of at least one primary metabolite.

3.4.1 Metabolism and plant defence

The spectroscopic analysis of the Arabidopsis *eds* mutants, along with other defence related mutants, reveals alterations in primary metabolic processes compared to wild type plants even in the absence of pathogen challenge. This indicates that their compromised defence response may be due to altered levels of primary metabolites before, and at the time of, pathogen attack, which may facilitate infection and /or prevent induction of a defence response involving key defence related secondary metabolites. In the case of *eds11* and *npr1*, alterations in primary sugar metabolism could be aiding pathogen attack, as seen in other studies with the bacterial pathogen *Pseudomonas syringae* (Ward *et al.*, 2010). However, the finding that the *Fusarium* susceptible mutants *npr1* and *eds11* have elevated flavonoids is unexpected, considering the elucidated role of flavonoids in FEB resistance in barley (Kumaraswamy *et al.*, 2011, Bollina *et al.*, 2010, Bollina *et al.*, 2011).

Several mutants also show altered primary amino acid abundance, indicating perturbations in amino acid biosynthesis. Alterations in amino acid biosynthetic pathways, namely aspartate metabolism and threonine biosynthesis, have

recently been shown to impact plant defence to a range of pathogens (van Damme *et al.*, 2009, Huibers *et al.*, 2013, Zeier, 2013, Stuttmann *et al.*, 2011). This is explored further in Chapter 6.

3.4.2 Requirement for identification of unknown compounds

Almost half (26) of the compounds found to differ between genotypes in this study are ‘unknowns’ meaning that their chemical structures have not been identified. While some of these were linked to known metabolites based on relative abundance patterns across genotypes, others showed unique distribution. This makes it difficult to link these metabolites to known pathways and processes and understand how they may be involved in plant defence, and how they related to the specific genotype of the line in which they are altered.

For example, mutants *rar1* and *sgt1b*, which have a coupled role in *R* gene mediated defence responses, also show changes in metabolite production in common with one another. However, the majority of these changes occur in compounds whose identity is not yet known, impeding further investigation into the roles of these metabolic changes in altering the defence response.

Characterisation of the unknown metabolites identified in this chapter is therefore crucial to further understanding of the genotypes of interest.

Unfortunately their chemical characterisation requires a great deal of further analysis which is beyond the scope of this project (Ward *et al.*, 2011, Nakabayashi and Saito, 2013).

3.4.3 Effects of non-target SNPs

The mutants investigated in this chapter were generated by ethyl methanesulfonate (EMS) mutagenesis, which typically introduces hundreds of single nucleotide polymorphisms (SNPs) into the plant genome, only one of which is typically responsible for the phenotype under selection (Ashelford *et*

al., 2011, Jander *et al.*, 2003). In the case of the *eds* mutants this phenotype is reduced resistance to *Pseudomonas* infection. However, while only one SNP may be responsible for the defence phenotype, other SNPs may be contributing to the metabolomic fingerprint. It cannot therefore be assumed that every metabolomic deviation between these mutants and the wild type is directly linked to their susceptibility phenotype and underlying causal mutation.

3.4.4 Effects of using growth stage based analysis

Growth stage (first flower opening), rather than absolute age, was used to select comparable plants between genotypes. There were a number of reasons for this, as detailed in the methods section:

Flower opening is the growth stage at which plants are inoculated with *Fusarium* spores during the spray inoculation assay detailed in chapter 2. It was therefore of interest to assess how the base-line metabolome of *Fusarium* susceptible genotypes differs from that of resistant genotypes at the time of inoculation. Furthermore, there is evidence that large-scale metabolomic changes occur throughout the rosette as the plant transitions from vegetative to reproductive growth (Ward *et al.*, unpublished). It would therefore not be appropriate to select plants based on age when only some genotypes were flowering.

However, it is interesting to note that the genotypes with the most divergent metabolomes from wild type Col-0 are *eds4* and *eds8* (early flowering) and *eds11* and *npr1-1* (late flowering), and that some metabolites show contrasting abundances between the early and late flowering genotypes. This occurs namely in the major flavonoids KRR and KGR, which are elevated in the late flowering genotypes and reduced in the early flowering genotypes, along with

an additional unidentified flavonoid which shows the reversed abundance pattern. There are similar trends in other compounds which are not statistically significant in all four genotypes. This raises the question of whether these deviations are due to differences in rosette age, or the causal genotype, or a combination of both; it is not clear whether flowering time is directly linked to the mutation of interest in these genotypes.

3.4.5 Conclusions

This chapter shows that many of the mutant/transgenic *Arabidopsis* lines which have been tested for altered resistance to *F. culmorum* have metabolic fingerprints that are significantly divergent from wild type plants, in the absence of pathogen challenge. In addition, there are shared deviations in *eds7*, *eds11* and *eds12*, all of which have elevated floral *F. culmorum* susceptibility.

In the next chapter, the susceptibility of these lines to *Fusarium* pedicel infection following silique inoculation is examined, along with changes in the metabolome induced by this infection.

4 The effect of *Fusarium culmorum* infection on the plant metabolome

4.1 Introduction

Metabolism is a central element of plant pathogen interactions, as discussed in Chapters 1 and 2. Synthesis of primary and secondary metabolites is essential for many plant defence responses, and pathogens in turn may re-engineer plant metabolism to aid invasion and nutrient acquisition.

Previously, metabolomic analysis of *F. culmorum* infected *Arabidopsis* pedicel and upper stem tissue eleven days after silique inoculation revealed eight secondary metabolites induced by *Fusarium* infection which were absent from mock inoculated samples (Figure 4.1: , Baker, Hammond-Kosack *et al.*, unpublished). These included two glucosinolates, and camalexin, which are well characterised in their role in plant defence against fungal pathogens (Bednarek *et al.*, 2009, Sanchez-Vallet *et al.*, 2010) and two coumarins; scopoletin and its glucoside scopolin, thought to be responsible for plant auto-fluorescence in infected samples (Figure 4.1, inset). These coumarins are known to be involved in *Arabidopsis* root defence against the related fungal pathogen *Fusarium oxysporum* (Kai *et al.*, 2006) as well as defence against *Sclerotinia* head rot in sunflowers (Prats *et al.*, 2006). Coumarins have also been implicated in resistance to FEB in barley following metabolomic analysis of resistant cultivars (Bollina *et al.*, 2010, Kumaraswamy *et al.*, 2011).

In chapter 3, the metabolic fingerprints of thirteen *Arabidopsis* defence related mutants were assessed, under normal growth conditions. The aim of this chapter is to generate metabolic fingerprints for these mutants following *F. culmorum* infection, compared to mock inoculated plants. These mutants had previously been screened for altered floral susceptibility following spray

inoculations. The intention was therefore to study metabolic changes following both floral spray inoculation, and silique point wound inoculation. However, infection following spray inoculations was not sufficiently severe and consistent to permit comparisons between genotypes. Therefore, only silique point wound inoculation was used in this study. It was hypothesised that some of the mutants investigated might show alterations in production of the previously described secondary metabolites which accumulate in wild type plants following *F. culmorum* inoculation.

The susceptibility of the mutants of interest to infection of the pedicel-stem junction following silique inoculation was also assessed, as was the susceptibility of these mutants to floral infection by an opportunistic fungal contaminant.

Plant growth, inoculations, disease assessments, harvesting and grinding, and interpretation of processed data, were done by the author. Metabolic fingerprinting and associated analysis was done by Dr Jane Ward and colleagues in the Rothamsted Metabolomics facility (Met-RO).

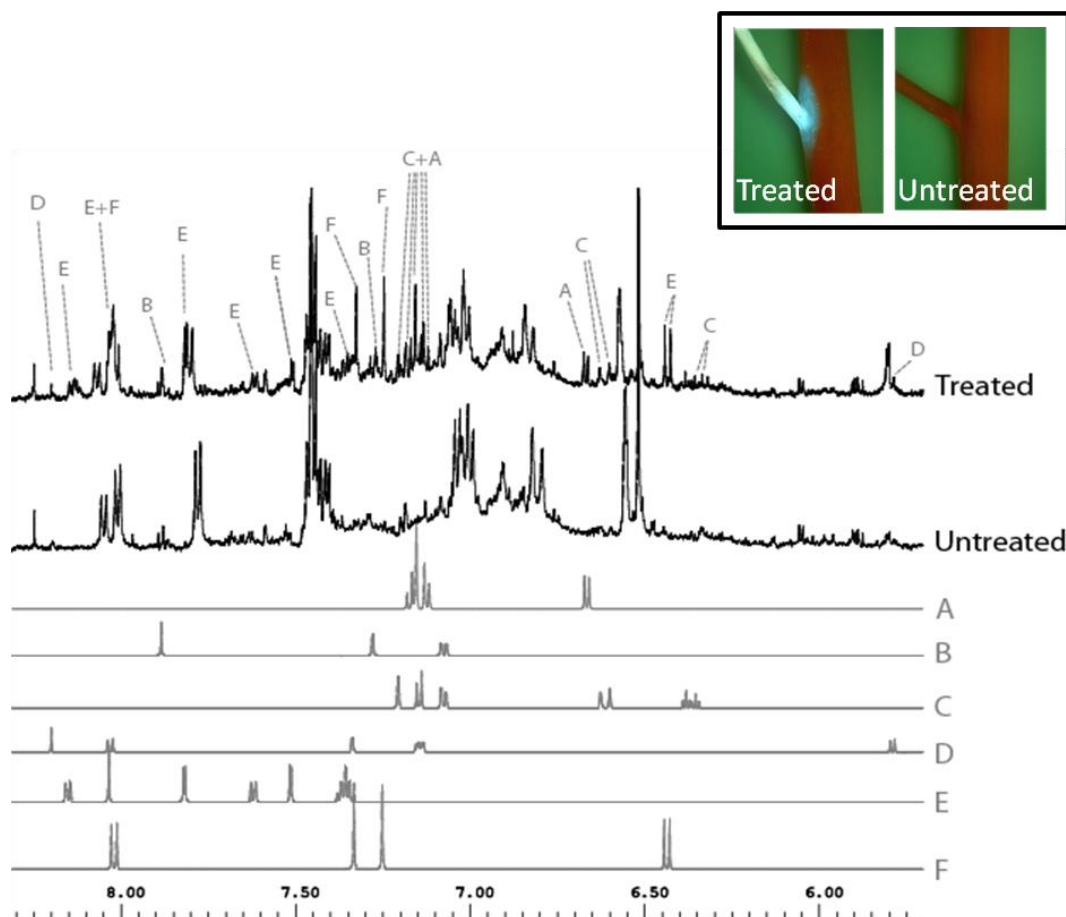


Figure 4.1: NMR spectra revealing compounds induced in *Arabidopsis* pedicel/upper stem tissue by infection of siliques with *F. culmorum*. Upper: Aromatic regions from NMR spectra of crude extracts of treated and untreated samples harvested at 14 dpi (shown inset- UV light with violet filter). Lower: Aromatic regions from edited (to remove noise and impurities) NMR spectra of A: 4-methoxyglucobrassicin, B: 6-O-β-D-glucopyranosyl indole-3-carboxylic acid, C: coniferyl β-D-glucopyranoside, D: 6-O-β-D-glucopyranosylindole-3-carboxylic acid β-D-glucopyranosyl ester, E: camalexin, F: scopolin. Source: MeT-RO at Rothamsted Research (Bakker *et al.*, unpublished).

4.2 Materials and Methods

4.2.1 Plant Growth and Inoculations

Plants were grown and inoculated by single silique wound point inoculation as described in Chapter 2; Plants with 6 immature siliques were chosen for inoculation. Inoculations were done 14-16 hours into the light period. Plants were grown and inoculated in four independent experiments. Ten mock and ten *F. culmorum* inoculations were done for each genotype in each experiment.

4.2.2 Harvesting and analysis

Infected pedicel and adjoining stem tissue was harvested at 14 dpi, directly into liquid nitrogen as described in Chapter 3. Plant tissue of each genotype and treatment was pooled, each pool containing tissue from two independent experiments, giving rise to two pooled biological samples per treatment/genotype combination. Samples were freeze dried and ground and submitted to Metabolomics at Rothamsted (Met-RO) for metabolic fingerprinting as described in Chapter 3. Each sample was split into three technical replicates for analysis. Data presented here is generated from ¹H-NMR spectroscopic analysis.

4.2.3 Contaminant analysis

The growth room fungal contaminant isolated during this study was cultured on SNA, as described in Chapter 2 for *Fusarium* cultures.

The ITS region of the rRNA of the fungal contaminant was amplified using the universal primers ITS1 (5' - TCCGTAGGTGAACCTGCGG -3') and ITS4 (5'- TCCTCCGCTTATTGATATG -3') (White *et al.*, 1990).

4.3 Results*

4.3.1 Susceptibility to the contaminant fungus *Penicillium olsonii* differs between genotypes

Plants kept at high humidity for extended periods following silique inoculation with either *F. culmorum* conidia or sterile water often succumbed to infection of the apical inflorescence by an unidentified fungal contaminant. These apically restricted infections were first visible after 10 days in the inoculation chamber and, due to the elongation of the floral apex post –silique inoculation, were then at least 7 to 10 cm above the point of the original silique inoculation. Isolation of the contaminant and sequencing of the ITS region revealed the species to be *Penicillium olsonii* (NCBI Taxonomy ID 99116). This ascomycete fungus is the causal agent of blue mould disease on a variety of dicotyledonous species and a known growth room contaminant and opportunistic pathogen of *Arabidopsis* floral and leaf tissue (Wagner *et al.*, 2000). Variation in contamination levels between genotypes was formally assessed by recording the number of plants per genotype that had become contaminated 14 days after mock or *F. culmorum* silique inoculation (Figure 4.2a). No effect of treatment was observed, indicating that distal infection with *Fusarium* neither inhibits nor facilitates *P. olsonii* floral infection. However, there was a significant effect of genotype on infection rates (d.f. = 14, 81, $p < 0.001$). Genotypes *eds4*, *eds7*, *eds12* and *npr1* all showed higher contamination levels across four experimental replicates than wild type Col-0, regardless of treatment (70.8%, 68.0%, 92.6%, 90.0% and 36.1% respectively). Higher susceptibility was also seen in *rar1* compared to wild type Ler-0 ($p = < 0.05$). All other mutants showed

* Prepared samples for metabolome analysis were submitted by the author to MeT-RO on 24th October 2012. Analysed data were returned to the author on 28th November 2014. This thesis was submitted on 19th December 2014. As such, reporting and interpretation of results from the metabolome analysis described in this chapter is not as comprehensive as intended.

wild type levels of contamination. Overall contamination levels decreased with each replicate, but remained high in the significantly affected genotypes. No effect of this floral contaminant on the metabolite composition of upper stem and pedicel tissue was seen (Jane Ward, *pers. comm.*).

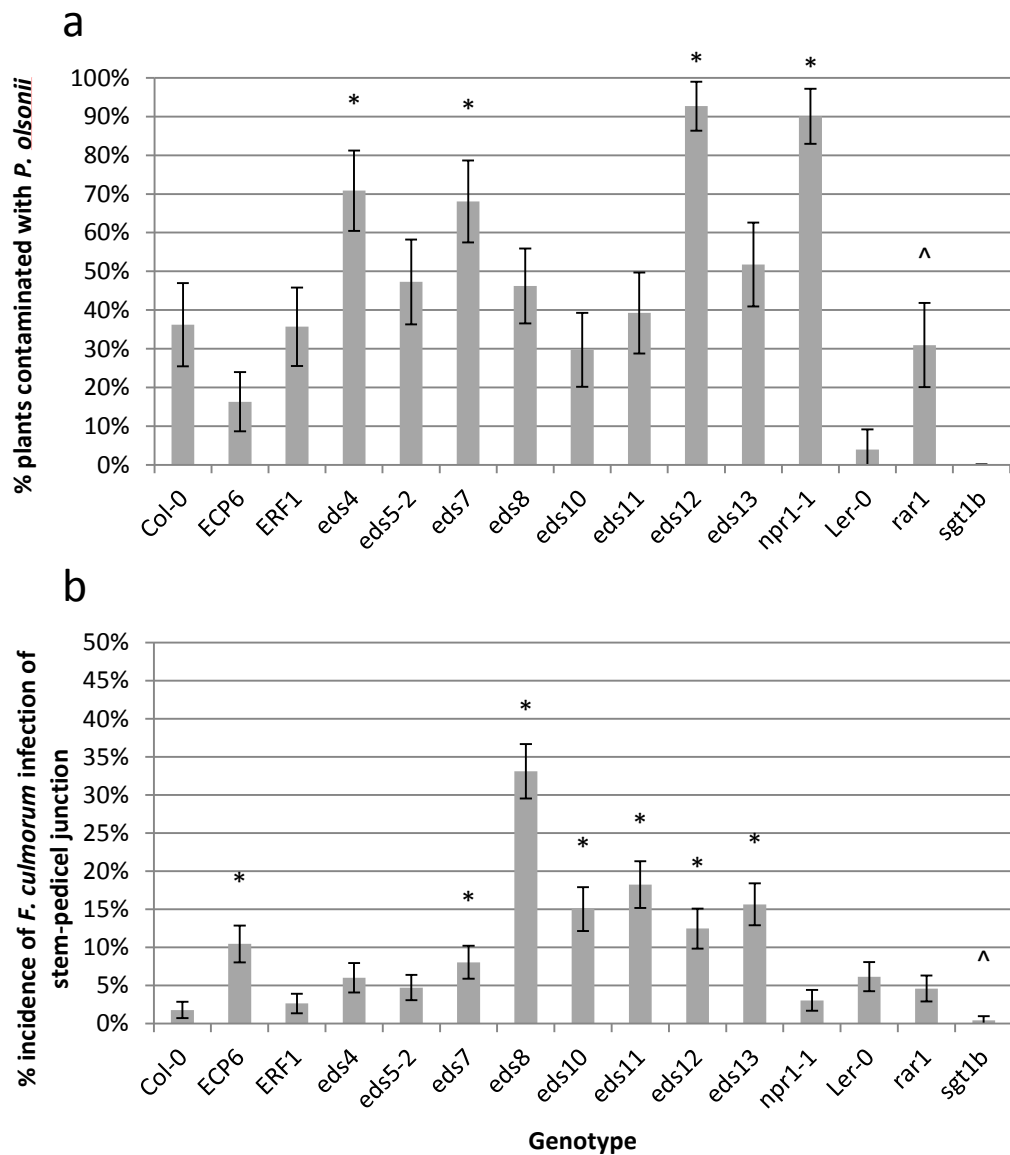


Figure 4.2: Susceptibility of investigated Arabidopsis genotypes to a) floral infection by *P. olsonii* and b) infection of the stem-pedicle junction by *F. culmorum*. The percentage of plants of each genotype florally infected by the opportunistic blue mould pathogen *P. olsonii* was recorded at 14 dpi (a). The incidence of infection of the stem-pedicle junction in each genotype was recorded at 14 dpi (b). *significantly different from Col-0, ^significantly different from Ler-0 (regression followed by calculation of LSDs, $p < 0.05$). Data is pooled from 4 independent experiments.

4.3.2 Rate of infection of the pedicel-stem junction differs between genotypes

Previous studies had shown that silique inoculation with *F. culmorum* results in complete infection of the silique, pedicel and pedicel-upper stem junction by 14 dpi, with progression of infection symptoms arresting at the junction (Baker *et al.*, unpublished, Cuzick *et al.*, 2008a). However, in this study, few inoculation events resulted in complete pedicel colonisation by 14 dpi. The incidence of infection at the junction was formally assessed and compared between genotypes across four independent experiments (n=40).

A significant effect of genotype on incidence of disease at the stem pedicel junction was observed (Figure 4.2b, d.f. = 14, 550, $p < 0.001$). The incidence of visible disease symptoms at the pedicel-stem junction was significantly higher in the transgenic line expressing *C. fulvum* ECP6 (10.4%), *eds7* (8.0%), *eds8* (33.1%), *eds10* (15.0%), *eds11* (18.2%), *eds12* (12%) and *eds13* (15.6%) compared to wild type Col-0 (1.7%), but not in *ERF1*, *eds4*, *eds5* or *npr1*. Infection incidence in the *R* gene mediated signalling mutant *sgt1b* was significantly lower than in wild type Ler-0 (0.4% vs. 6.1%) while mutation in its signalling partner *RAR1* did not significantly alter disease levels. These results were significant across four experimental replicates ($p = < 0.05$). However, it is noteworthy that with the exception of *eds8*, the rate of infection at the junction did not exceed 20% in any genotype.

4.3.3 The metabolic profile of uninfected pedicel/upper stem tissue differs from that of whole flowering plants

In chapter 3, the metabolic fingerprint of the 15 genotypes of interest was investigated in whole plants at first flower stage. In that analysis, genotypes *eds11* and *npr1* were found to have similar metabolic fingerprints that were also

distinct from those of other genotypes in the Col-0 (Figure 3.2). Similarly, principal component analysis (PCA) and analysis of individual metabolite abundances indicated that *eds4* and *eds8* were similar to one another but distinct from other genotypes.

As shown in Figure 4.3, PCA did not indicate strong similarities in pedicel/upper stem metabolite composition analysed in this study between *eds11* and *npr1*. Large differences were also seen in *npr1* between experimental replicates for PC1, which represents 39% of the variation between samples. Genotypes *eds4* and *eds8* segregated from other genotypes and clustered together (Figure 4.3).

4.3.4 A collection of 32 known metabolites was detected in the upper stem/pedicel tissue

The known metabolites detected in this analysis were the amino acids asparagine, arginine, aspartate, GABA, glutamate, glutamine, isoleucine, leucine, phenylalanine, alanine, proline, threonine, and valine, the amino acid derivatives glycine betaine and proline betaine, the major flavonoids KRR and KGR, the sugars glucose, maltose, trehalose, sucrose, fructose and raffinose, the TCA cycle components citrate, fumarate, acetate and malate, along with choline, choline-O-sulphate, inositol and a uracil-like compound. All of these metabolites showed changes in abundance following *F. culmorum* infection of the pedicel, in some or all of the genotypes examined. Interestingly, none of the defence related compounds previously identified by ¹H-NMR to be induced by *F. culmorum* infection of the stem-pedicel junction were detected in this study. Several unknown metabolites were also detected but these have been omitted from subsequent analysis.

Differences in abundance of metabolites between genotypes in uninfected tissue are presented in heat map form in Figure 4.4a (Col-0 genotypes) and

4.5b (Ler-0 genotypes), following hierarchical cluster analysis (HCA). Note that colour denotes relative abundance compared to wild type plants, rather than absolute abundance or abundance relative to the mean as presented in Chapter 3.2. While detailed analysis of differences between metabolites for each genotype in uninfected pedicel/upper stem is beyond the scope of this study, it is interesting to note that *npr1*, which showed increased levels of KRR and KGR compared to wild type when whole flowering plants were assessed, has reduced levels of these compounds in the tissues assessed here. However, these compounds were elevated in the pedicel/stem tissue of *eds11*, consistent with findings for whole plants. Primary sugars were also elevated in both genotypes in both whole plants and pedicel/stem junctions. The flavonoids KRR and KGR were also reduced in genotypes *eds8* and *eds4*, consistent with findings for whole plants in Chapter 3.

In addition, *sgt1b*, which has reduced susceptibility to *F. culmorum* infection in both floral and pedicel tissue, shows reduced levels of several metabolites in comparison to both wild-type Ler-0 and the related mutant *rar1*.

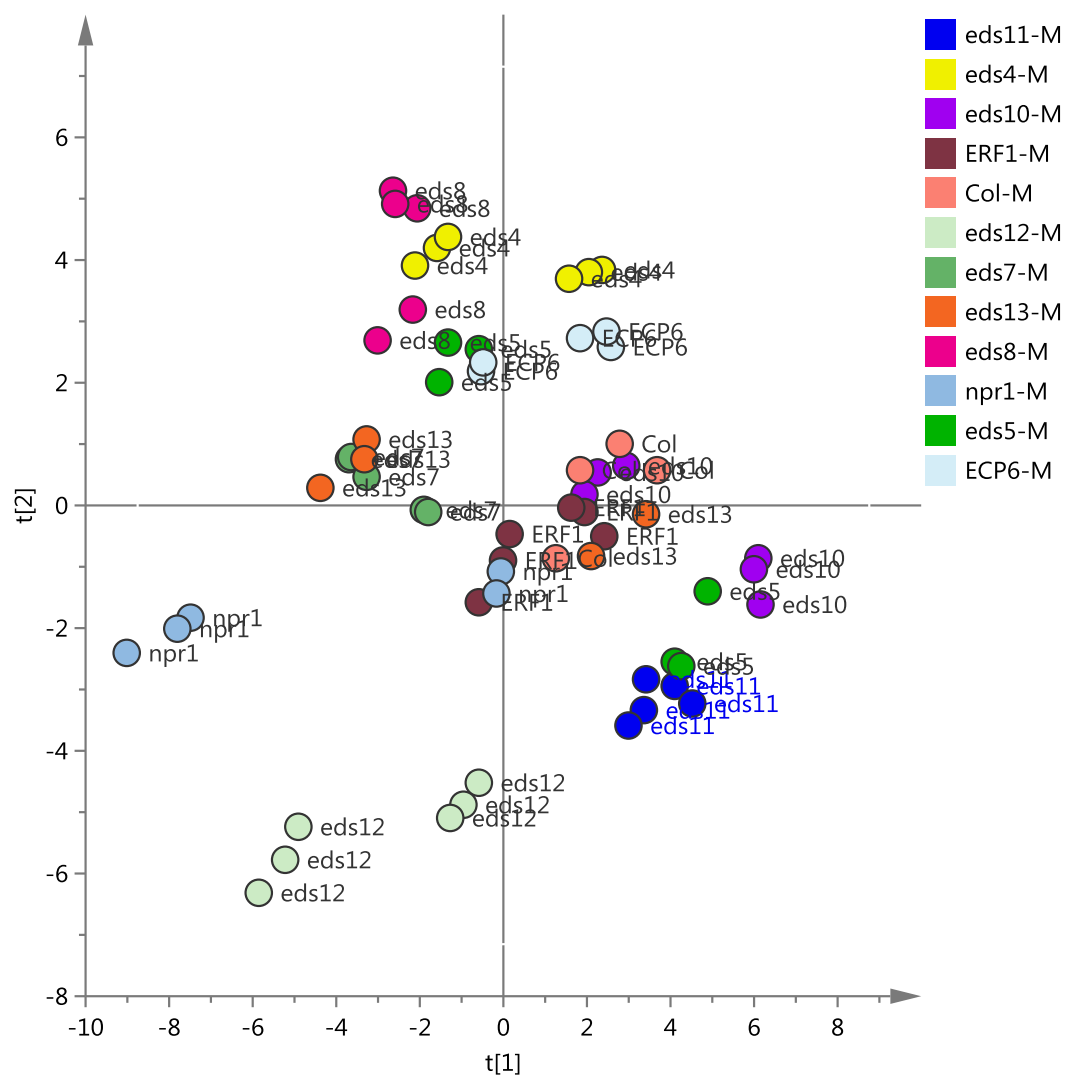


Figure 4.3: Principal component analysis (PCA) of the metabolic composition of 13 genotypes in the Arabidopsis Col-0 background, following ^1H -NMR spectroscopic analysis of upper stem/pedicle junctions of uninfected plants. PCA constructed from ^1H -NMR data using extracted regions of known characteristic regions. Models constructed using unit variance scaling. $t[1]$ = PC1 = 39%, $t[2]$ = PC2 = 25%. Figure generated by Jane Ward, MeT-RO

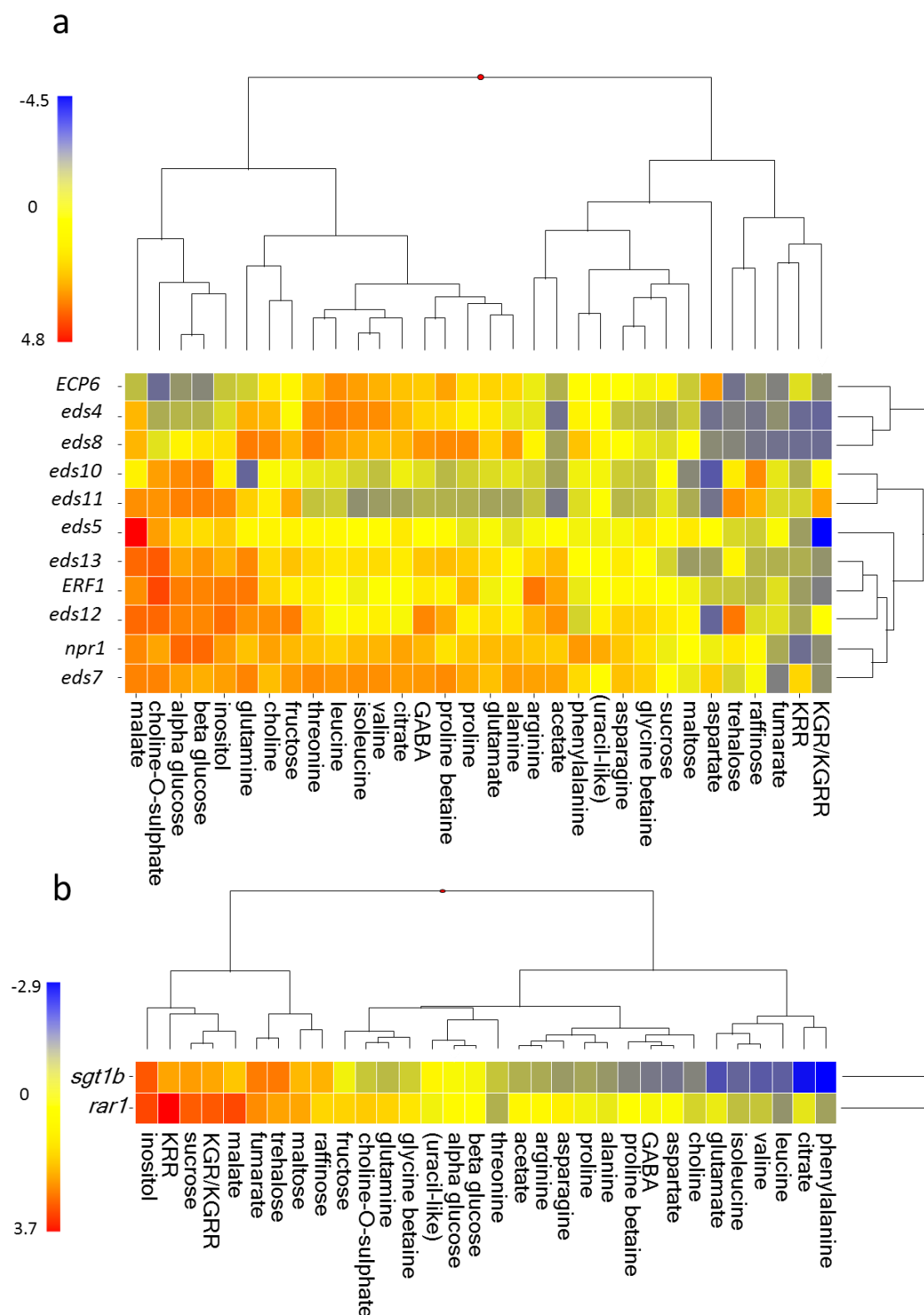


Figure 4.4: Differences in metabolite abundances between genotypes.

Hierarchical cluster analysis generated from discriminatory metabolite profiles of uninfected pedicel/upper stem tissue for each mutant compared to wild-type Col-0 (a) or Ler-0 (b). Data generated from pairwise PCA analysis of mock-treated samples of each mutant. HCA analysis has been generated by the complete linkage method using Euclidian distance as the similarity measure. Red = elevated relative to wild-type. Blue = reduced relative to wild-type. Heat maps and HCA generated by Jane Ward, MeT-RO

4.3.5 The effect of *F. culmorum* infection on the metabolic profile of pedicel/upper stem tissue

The metabolic profiles of pedicel/upper stem junction samples analysed in this chapter were expected to segregate based on treatment (mock versus *Fusarium* infected) and also genotype. Differences between experimental replicates were also expected. As shown in Figure 4.5, PCA revealed that samples of ecotype Col-0 segregated predominantly into treated and untreated groups based on PC1, which accounts for 45% of the variation between samples. Genotype differences were denoted by PC2 (21%). It is interesting to note that the metabolome of infected *eds4* pedicels was comparable with that of mock inoculated tissue from some other genotypes, namely *npr1*, *eds5* and *ECP6*. While the profiles of most genotype/treatment combinations were comparable between experimental replicates, some, such as *eds13*, showed higher levels of variation between replicates. By contrast, for genotypes in the *Ler-0* background, differences between treatments were reflected in PC2 (25%), with differences between experimental replicates and genotypes accounting for PC1 (51%) (Figure 4.6).

Individual PCA plots representing differences in metabolic profile between mock and *Fusarium* infected samples in each replicate for each genotype are shown in Appendix 6. In all of these analyses, differences between mock and *Fusarium* infected samples were represented by PC1, with variation between experimental replicates reflected in PC2.

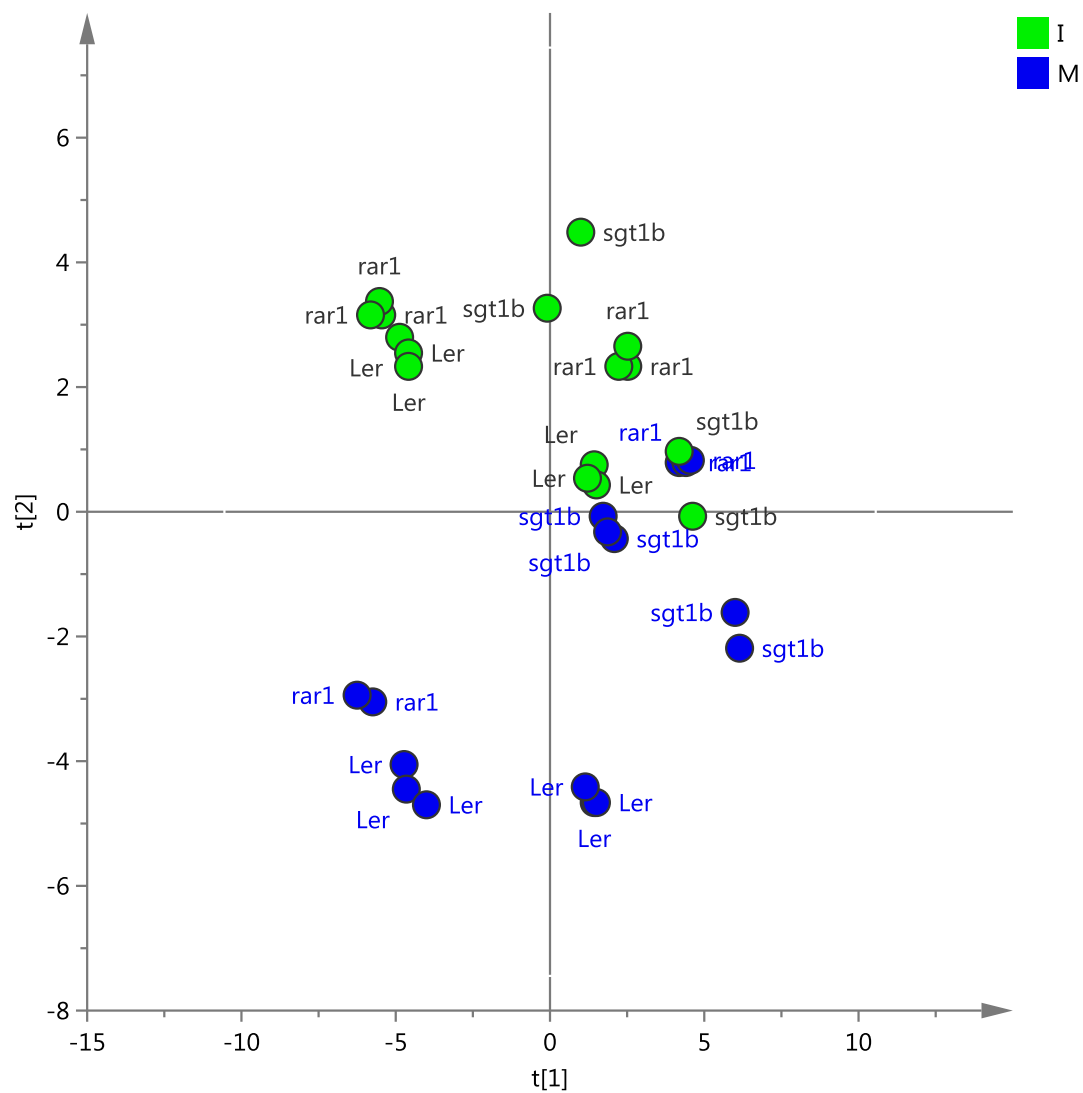


Figure 4.6: Effect of *F. culmorum* on metabolome profile of genotypes in the Ler-0 background. PCA constructed from $^1\text{H-NMR}$ data using extracted regions of known characteristic regions. Models constructed using unit variance scaling. M = mock, I = infected. $t[1]$ = PC1 = 51%, $t[2]$ = PC2 = 25%. Figure generated by Jane Ward, MeT-RO

4.3.6 Shifts in individual metabolite abundances caused by *F. culmorum* infection

The metabolites outlined in section 4.3.3 and Figure 4.4 showed changes in abundance following infection with *F. culmorum*. Fusarium-induced changes in abundance of each metabolite across the genotypes assessed are shown in Figure 4.7 and Figure 4.8. The majority of compounds were elevated in *F. culmorum* infected samples rather than reduced.

Compounds which were elevated in *F. culmorum* infected samples, irrespective of genotype, included the sugars trehalose and fructose, the amino acids alanine and asparagine, and inositol, glycine betaine and a uracil-like compound. Only fumarate and phenylalanine were reduced following infection in all genotypes, with the level of reduction differing between genotypes.

Proline betaine, proline, choline, aspartate, arginine, glutamate, GABA, acetate, and the sugars raffinose and maltose were all elevated in most but not all of the genotypes. It is particularly interesting to note that choline, proline, proline betaine, GABA, glutamate, arginine, acetate, asparagine and inositol all accumulated to a lesser degree in *Ler-0* genotypes than *Col-0*. GABA, glutamate and proline (betaine) are all synthesised via the TCA cycle component 2-oxaloglutarate, while asparagine and arginine both derive from aspartate, indicating that these pathways are altered by *F. culmorum* infection to different degrees between ecotypes.

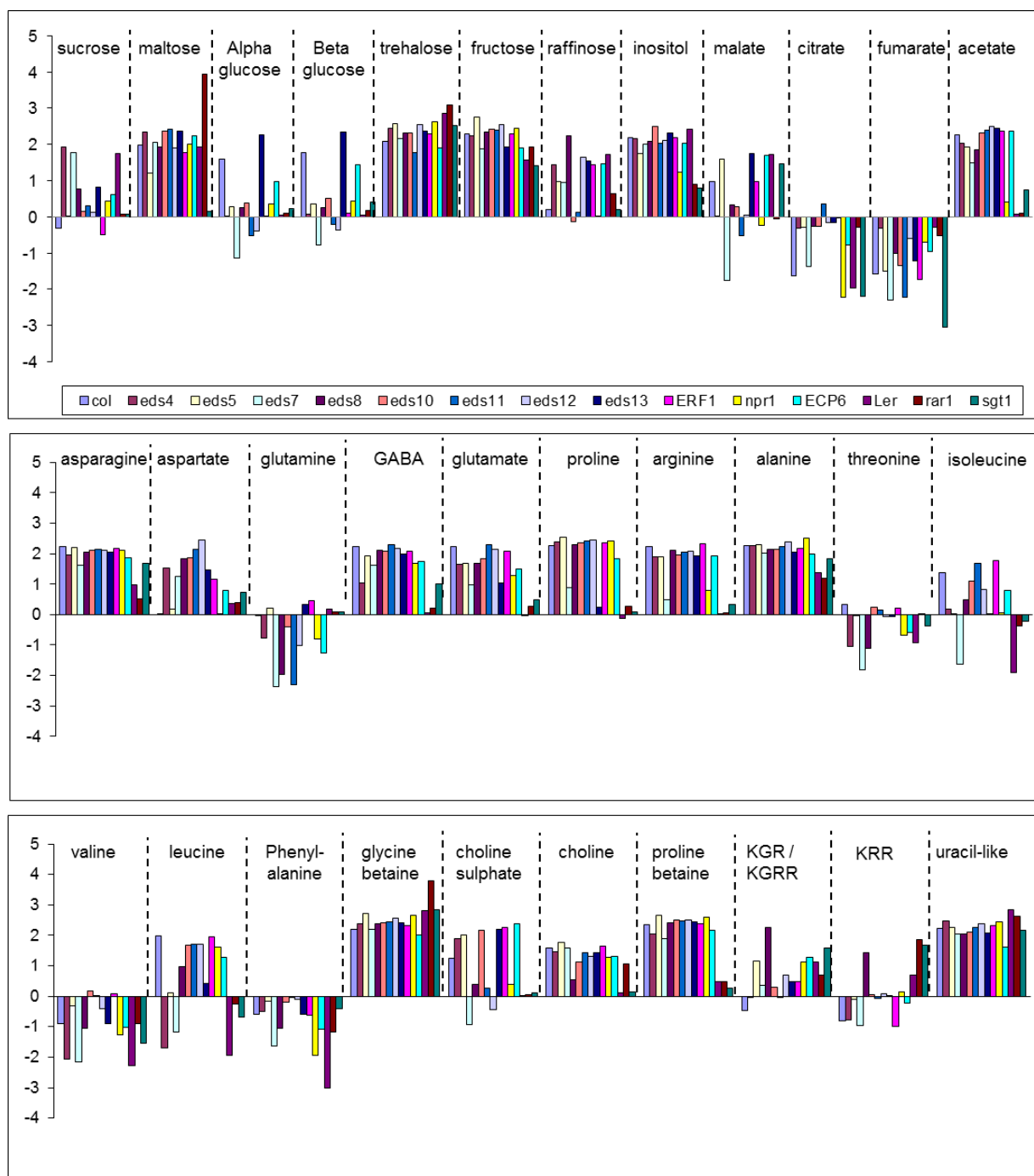


Figure 4.7: Changes in abundance of compounds due to *F. culmorum* infection of pedicel tissue, compared to mock infected samples. Bars show abundance for each genotype relative to mock inoculated plants. Data generated from pairwise PCA of each mutant using unit-variance scaling. Figure generated by Jane Ward, MeT-RO.

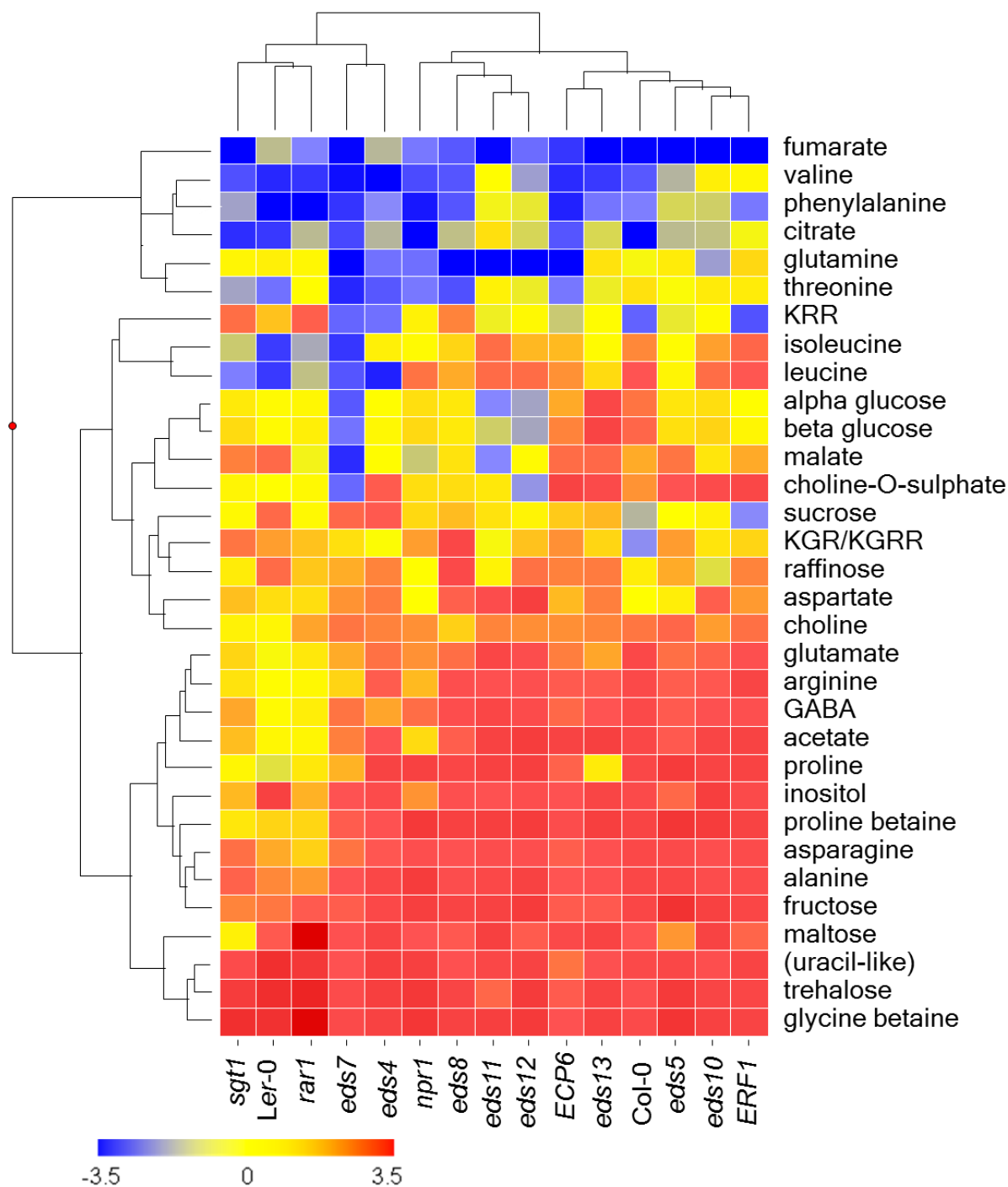


Figure 4.8: Hierarchical cluster analysis generated from discriminatory profiles (for each mutant) of metabolites induced or reduced by *F. culmorum* infection. Data generated from pairwise PCA analysis of each mutant. HCA analysis has been generated by the complete linkage method using Euclidian distance as the similarity measure. Red = induced by infection relative to mock treatment; Blue reduced by infection relative to mock treatment. Analysis and figure provided by Jane Ward, MeT-RO. Note- The genotypes *sgt1b* and *rar1* are in the Ler-0 background.

4.3.7 Genotype-dependent changes in metabolite composition following *F. culmorum* infection

For the remaining compounds, the direction (increase or decrease) and magnitude of change in abundance was dependent on genotype. These compounds were sucrose, glucose, malate, citrate, glutamine, isoleucine, threonine, leucine, choline-O-sulphate and the flavonoids KRR and KGR. In Figure 4.9, heat map data from Figure 4.8 is re-aligned to the susceptibility data in Figure 4.2b, in order to identify possible correlations between alterations in metabolite abundance and susceptibility levels.

For example, while citrate levels were reduced by *F. culmorum* infection in all genotypes, this reduction is less pronounced in the more susceptible genotypes. By contrast, the reduction in glutamine is more pronounced in the majority of susceptible genotypes compared to those which are less susceptible. The exceptions to this are *npr1* and *eds4*. While these genotypes did not show higher levels of infection at the pedicel-stem junction than wild type Col-0, *npr1* is highly susceptible to *F. culmorum* floral infection. Elevation of aspartate was also generally more pronounced in susceptible genotypes. Similarly, the reduction in the flavonoids KRR and KGR observed in Col-0 was not detected in the *F. culmorum* susceptible genotypes, although it was also absent in other genotypes.

Glucose was found to increase in response to *F. culmorum* infection in Col-0, but decreased in the mutants *eds7*, *eds11* and *eds12*. These mutants all show enhanced susceptibility to *F. culmorum* in both floral and pedicel tissue. Several other compounds were reduced following infection of *eds7* but not Col-0, namely glutamine, threonine, isoleucine, leucine, malate, and choline-O-

sulphate. However, many of these compounds were initially more abundant in untreated eds7 pedicel/upper stem tissues compared to Col-0 (Figure 4.4a).

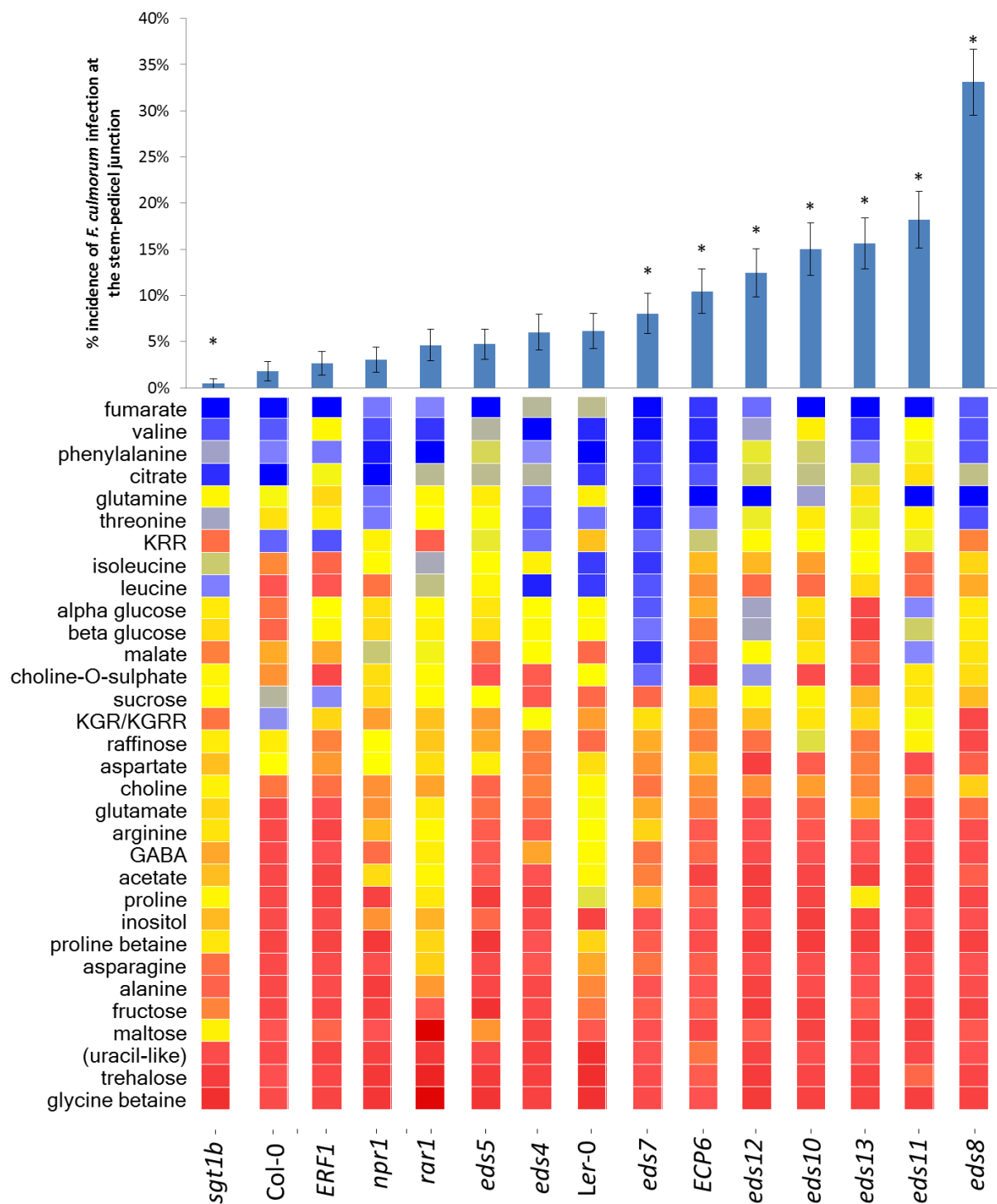


Figure 4.9: Analysis of relationship between incidence of infection at the stem-pedicel junction and alterations in metabolite abundance, following *F. culmorum* infection of the stem pedicel junction of 15 Arabidopsis genotypes. Red = induced by infection relative to mock treatment; Blue reduced by infection relative to mock treatment. (Adapted from Figure 4.8). Note- The genotypes *sgt1b* and *rar1* are in the Ler-0 background.

*=infection levels significantly different from wild type plants ($p < 0.05$). Bar = standard error.

4.4 Discussion

In this chapter, metabolome changes in the pedicel/upper stem tissue of a collection of *Arabidopsis* defence related mutants was analysed in response to infection by *F. culmorum*. The incidence of infection at the stem-pedicel junction was also examined between genotypes. In addition, the susceptibility of different genotypes to the opportunistic contaminant *P. olsonii* was examined.

4.4.1 Mutants with wild type *F. culmorum* floral susceptibility phenotypes have increased susceptibility to pedicel infection

Several of the mutants investigated displayed an increased incidence of *F. culmorum* infection at the stem-pedicel junction compared to wild type plants. Some of these, *eds7*, *eds11*, *eds12*, have already been identified as more susceptible to *F. culmorum* floral infection. However the remaining genotypes, *eds8*, *eds10* and *eds13*, previously showed wild type levels of floral infection, as outlined in Chapter 3. These differential infection outcomes may indicate differences in the requirements for the individual *EDS* genes for defence in floral and silique/pedicel tissue. It is interesting to note that both *eds8* and *eds10* have been associated with reduced JA signalling, generally associated with defence against necrotrophs (Ton *et al.*, 2002). It may therefore be that while defence against floral infection is mediated by the SA signalling pathway, defence against pedicel/stem infection requires aspects of the JA signalling pathway. The lack of susceptibility in *npr1* to stem invasion by *F. culmorum* is interesting. On the one hand it could indicate that SA signalling via NPR1 may be less important for defence against *F. culmorum* in *Arabidopsis* upper stem tissue than it is in *Arabidopsis* floral tissue. However, if JA signalling limits pedicel infection then mutation of *NPR1* might be expected to reduce infection rates, since NPR1 negatively regulates JA signalling (Spoel *et al.*, 2003).

Brown *et al.* (2010) demonstrated an extensive intracellular symptomless growth phase of the *F. graminearum* hyphal front in wheat ears followed by an intercellular phase with extensive host necrosis and visible disease symptoms. A similar infection process has been observed in *F. culmorum* (van de Meene, *et al.*, unpublished). However, no comparable symptomless phase has been observed during infection of *Arabidopsis* silique and pedicel tissue, and necrosis appears concurrent with hyphal invasion. This would suggest that *F. culmorum* induces cell death during infection of *Arabidopsis* pedicel/upper stem tissue, and infection can proceed more rapidly in mutants with reduced JA signalling. However, detailed analysis of the lifestyle of *Fusarium* hyphae within *Arabidopsis* pedicel tissue is required to confirm this, as a brief symptomless phase may exist.

4.4.2 Susceptibility to *P. olsonii* differs between genotypes

Contamination of a growth room with an opportunistic plant pathogen is rarely desirable, but can be useful for further phenotypic characterisation of mutant genotypes, highlighting those which are more susceptible to opportunistic attack. In this study, the opportunistic fungal pathogen *P. olsonii* (blue mould) was found to infect the floral tissue of *eds4*, *eds7*, *eds12* and *npr1* plants more readily than wild type plants. Three of these mutants, *eds7*, *eds12* and *npr1*, were already known to be more susceptible to floral infection by *F. culmorum* (Chapter 3, Table 3.1). While *npr1* shows broad spectrum susceptibility to a large number of virulent biotrophic and hemibiotrophic pathogens, susceptibility in *eds7* and *eds12* has so far been limited to virulent *P. syringae* and *F. culmorum*, along with the mildew *Erysiphe orontii* in the case of *eds12*, but not *eds7* (Glazebrook *et al.*, 1996, Rogers and Ausubel, 1997, Volko *et al.*, 1998). These mutants are also able to express SA induced *PR* genes upon pathogen

challenge and mount some level of systemic acquired resistance (SAR). This indicates that defence responses against *P. olsonii* and *F. culmorum* may be mediated by a common signalling pathway, which is more specific than, and independent of, broad spectrum SA mediated defence.

4.4.3 Differences in metabolite profiles between whole plants and pedicel/upper stem tissue

In Chapter 3, the metabolome of whole, healthy plants was analysed at first flower stage, to match an analysis done by MeT-RO of the SLAT Arabidopsis lines generated by the Sainsbury Laboratory (Tissier *et al.*, 1999) . In this study, genotypes were clustered based on similar metabolic profiles. For example, mutants *eds11* and *npr1*, which are more susceptible to *F. culmorum* floral infection than wild type plants, clustered together, and were more distinct from wild type plants than other genotypes. In this chapter, which analysed the upper stem/pedicel tissue, not all of the patterns observed for whole plants were upheld. This indicates the potential importance on focusing specifically on the tissues of interest when conducting metabolome analyses. These results also clearly indicate that the 'naïve' non-induced host metabolome encountered by potentially invading *Fusarium* hyphae is very different between these two biological niches. A healthy attached leaf is considered to be non-host tissue, whilst a healthy attached pedicel is readily colonised.

As previously outlined, comparison between healthy and infected inflorescences was not carried out due to low and variable floral infection rates following spray inoculation. However, it would be interesting to examine differences in metabolic composition of inflorescences between wild type and *F. culmorum* susceptible mutants in the absence of *F. culmorum* infection.

4.4.4 Absence of antimicrobial secondary metabolites identified in this study

Previous analysis had indicated induction of several defence associated antimicrobial compounds following *F. culmorum* infection of the pedicel-stem junction in wild type plants (Figure 4.1). These included glucosinolates, and the coumarins scopolin and scopoletin. However, in this study, these compounds were not detected. This is most likely to be due to the low incidence of infection reaching the stem-pedicel junction; infection was still limited to the pedicel in the majority of plants used for this analysis at 14 dpi. This does suggest that these compounds may play a role in preventing spread of infection into the main stem, since they are not detected in the pedicel. However, it also suggests these eight compounds are not induced ahead of infection, with their induction only occurring once disease is present at the junction. Alternatively, it is possible that the fungal hyphae are able to entirely metabolise these eight compounds in the pedicel tissue, but this degradation does not occur in stem tissue. It would be interesting to assess whether there are any transcriptional changes in pedicel or stem tissue ahead of the infection front, such as those seen in wheat (Pritsch *et al.*, 2000).

Collectively, these results indicate that the accumulation of known defence associated antimicrobial compounds, at levels detectable by ¹H-NMR, is not taking place in advance of *F. culmorum* infection of the pedicel. This suggests that differences in susceptibility of the 15 genotypes explored in this study are due to other factors. However, analysis of samples using ESI mass spectroscopy is ongoing, which may permit detection of some of these compounds.

The reason for the slow progression of disease development in this study is not clear, but it is likely due to differences in growth room conditions, such as light quality, between studies. It is noteworthy that the original study was done when the growth rooms were < 6 months old, whereas this study was done 3 years later.

4.4.5 *F. culmorum* infection results in elevation levels of several metabolites

Of the 32 known metabolites identified in this study, 17 were elevated in all or most of the genotypes assessed in infected compared to uninfected tissue.

These included many components of primary metabolism, namely sugars and amino acids. It is not clear however whether this elevation is due to increased production by the plant, or production by the invading fungus. Increases in carbohydrates in infected plants due to alterations in respiratory function and source-sink relationships have been widely reported for a number of pathogens, as have alterations in amino acid abundances (Heuberger *et al.*, 2014, Berger *et al.*, 2007). The infected siliques are supporting seed development. Therefore the pedicel tissue would be a conduit for many of the metabolites destined for accumulation in the sink tissue. The presence of the *Fusarium* hyphae is highly likely to alter sink strength and the ability of the pedicel to transport metabolites to the various sink tissues in the silique.

Of particular interest is the finding that several metabolites show differential accumulation levels between Col-0 and Ler-0 genotypes. Compounds which are induced to a greater extent in Col-0 compared to Ler-0 include GABA and derivatives glutamate, proline and proline betaine. This indicates differences in metabolic responses to pathogens between these *Arabidopsis* ecotypes, which warrant further investigation.

4.4.6 Possible association between metabolite alterations and *F. culmorum* susceptibility in pedicel tissue

No metabolites were identified which showed a clear cut difference in accumulation between genotypes with enhanced *F. culmorum* susceptibility and those with wild types infection levels. However, some trends were observed. For example, reduction in citrate level was less pronounced in *F. culmorum* susceptible mutants, while glutamine and glucose were reduced in these mutants but not wild type plants. However, it is difficult to determine whether alterations in abundance of these amino acids are a result of plant or fungal metabolism. It is also impossible to determine what effect each metabolite might have on the outcome of the interaction between Arabidopsis and *F. culmorum*. Furthermore, these alterations in abundance are compared to uninfected plants for each genotype, rather than absolute abundances being compared between genotypes. Metabolites which increase in abundance during infection in a given mutant might still have a lower final abundance than in wild type plants.

The presented data for both incidence of *F. culmorum* junction infection and shifts in metabolite abundance are also combined from four biological replicates (*F. culmorum* infection) which were split into two pooled biological samples (metabolome data). The differences between samples for each genotype were greater between infected and uninfected samples than between biological replicates. However, better understanding of the data might be achieved by analysing induced shifts in metabolite abundances in the two pooled biological replicates individually, and linking these to the disease levels for these samples.

Furthermore, while there were statistically significant differences in infection incidence at the stem-pedicel junction, infection incidence did not exceed ~20% in any genotype and was largely restricted to a portion of the pedicel. The tissue

harvested for this study therefore contained a substantial amount of visibly non-infected tissue, since the entire pedicel and adjoining stem tissue was harvested regardless of infection levels. This may have affected metabolome analysis, preventing detection of subtle shifts in metabolite abundance which might have differed between genotypes, due to dilution of the sample with non-infected tissue.

4.4.7 Conclusions

This study has revealed alterations in abundance of 32 known metabolites in *Arabidopsis* pedicel/upper stem tissue as a result of *F. culmorum* infection in wild type and defence related mutant plants. While many of these compounds were induced in all genotypes, some showed discriminatory levels of induction (or reduction) between wild type and mutant plants, and also between ecotypes. It is not clear to what extent these differences in induction are linked to the susceptibility profiles of these mutants. The data presented here will require further analysis in order to identify additional patterns and correlations. In addition, the importance of these metabolites in other plant-pathogen interactions and responses to abiotic stresses should be further evaluated.

5 Assessment of the genetic basis of the *eds11* susceptibility phenotype using a mapping by sequencing approach

5.1 Introduction

Forward genetic screens have been widely used to create and identify collections of *Arabidopsis* mutants with altered defence responses to a range of plant pathogens. These screens are typically done by generating a mutagenised population using a chemical mutagen such as ethyl methanesulfonate (EMS), which introduces large numbers of single nucleotide polymorphisms (SNPs) into the genome. These mutant populations are then screened for altered resistance to a chosen pathogen. Mutant collections generated from these screens include the *powdery mildew resistant* (*pmr*) mutants (Vogel and Somerville, 2000), the *downy mildew resistant* (*dmr*) mutants (van Damme *et al.*, 2005, see chapter 6) and the *enhanced disease susceptibility* (*eds*) mutants to *Pseudomonas* bacterial infection (Glazebrook *et al.*, 1996, Rogers and Ausubel, 1997).

Following isolation of a mutant of interest, it is theoretically possible to map the causal mutation to a genomic location, revealing the disrupted gene responsible for the mutant phenotype. This has traditionally been done by outcrossing the mutant to another *Arabidopsis* accession and then selecting plants with the mutant phenotype from the F₂ population. DNA is then extracted from this mutant pool and analysed using genetic markers which differentiate between the two accessions used, such as CAPS (cleaved amplified polymorphic sequence) markers which contain restriction sites in only one accession (Neff *et al.*, 1998, Konieczny and Ausubel, 1993, Cao *et al.*, 1997). Assuming that the mutant phenotype is recessively inherited, markers close to the mutation of

interest will largely represent the accession in which the mutant was generated, while unlinked markers will equally represent both the parent and the outcross accession, since they are not under selection.

This marker based approach has been invaluable in the map based cloning of a huge number of Arabidopsis mutations and corresponding genes. However, its drawbacks include being time consuming and labour intensive, and the requirement for a high density of molecular markers surrounding the causal mutation. There is also a requirement for large numbers of mutant F₂ progeny, which can be challenging to obtain if the mutant phenotype is subtle. In addition, the requirement to outcross to another accession makes this approach unsuitable where a mutant phenotype is reliant on a specific genetic background, or where the mutant has been selected in a screen for suppressors of another mutation, which is only present in the parent ecotype (Ashelford *et al.*, 2011, Hartwig *et al.*, 2012).

Next generation re-sequencing of mutant genomes is now being widely used to rapidly identify candidate mutations and corresponding genes, in combination with or in place of traditional molecular marker based mapping. This is thanks to significant advances in analysis of whole genome sequencing (WGS) data and the ever reducing cost of re-sequencing projects, making mapping by sequencing a cost effective and efficient alternative to traditional marker based mapping.

One mapping by sequencing approach makes use of the SHOREmap pipeline: Whole genomic DNA from bulked mutant outcrossed F₂ plants is sequenced using the Illumina sequencing by synthesis (SBS) platform, which generates high quantities of short reads with ~50 fold coverage of the genome. These are

then aligned to the reference genome using the SHORE (short read alignment) pipeline. SHOREmap is an extension of this pipeline, whereby candidate genes are called based on the identification of SNPs in regions of the genome which have been predominantly inherited from the accession in which the mutant was generated (Ossowski *et al.*, 2008b, Schneeberger *et al.*, 2009). This follows the same principles as marker based mapping, in that the region of the genome surrounding the causal mutation will be inherited in the F₂ mutant population purely from the mutant accession, while unlinked regions may be heterozygous or homozygous for the outcrossed accession. An equivalent pipeline was used by Austin *et al.* (2011) to isolate a candidate mutation from a pool of 80 F₂ - plants from a cross between mutant Col-0 and wild type Ler-0 accessions.

These mapping by sequencing pipelines still have the limitation of requiring the mutant to be outcrossed to another accession, and mutant plants selected from a hybrid F₂ population. As previously described this can be difficult for mutations with subtle or quantitative phenotypes which may be hard to select for in the F₂ population due to natural variation in the phenotypic trait of interest between accessions. Pathogen susceptibility is a good example of such a phenotype. Several mapping by sequencing projects for recessive mutations have therefore used the alternative strategy of re-sequencing single or pooled F₂ individuals following a backcross to the wild type parental accession, both in *Arabidopsis* and other model species. EMS mutagenesis typically introduces hundreds to thousands of SNPs into the genome, but only those causing or linked to the mutant phenotype will be consistently present in the mutant genome following backcrossing. Candidate mutations can therefore theoretically be identified in a single F₂ backcrossed individual by identifying a region or regions of the genome which still have a high density of EMS derived mutations following

multiple backcrosses (Zuryn *et al.*, 2010, Ashelford *et al.*, 2011) (Figure 5.1a), or from a mutant pool of backcrossed F₂s by analysing the proportion of SNPs supported by the sequencing reads at each locus after re-sequencing the pooled DNA: Assuming that only mutant individuals have been selected, the SNP responsible for the mutant phenotype will occur in 100% of the reads (frequency = 1), while SNPs unrelated to the causal mutation are expected to have a frequency of around 50% (Fig 5.1b). The SHOREmap pipeline has recently been updated to incorporate a *backcross* function, which facilitates the identification of candidate SNPs from backcrossed populations (Hartwig *et al.*, 2012). The re-sequencing of the parental accession(s) is recommended for any backcross based mapping by sequencing strategy, in order to rule out variation between the parent and the reference genome.

James *et al.* (2013) have recently published a 'User guide to mapping by sequencing' in which *in silico* modelling was used to estimate the number of candidate mutations generated by different mapping by sequencing approaches by altering parameters such as of number of backcrosses, sequencing depth and size of mutant F₂ pool. James *et al.* (2013) predict that 50 fold sequencing coverage of ~50 F₂ plants following one backcross (BC₁F₂) would yield ~25 candidate SNPs, however any incorporation of the wild type allele into the pooled F₂s would increase the number of candidates significantly.

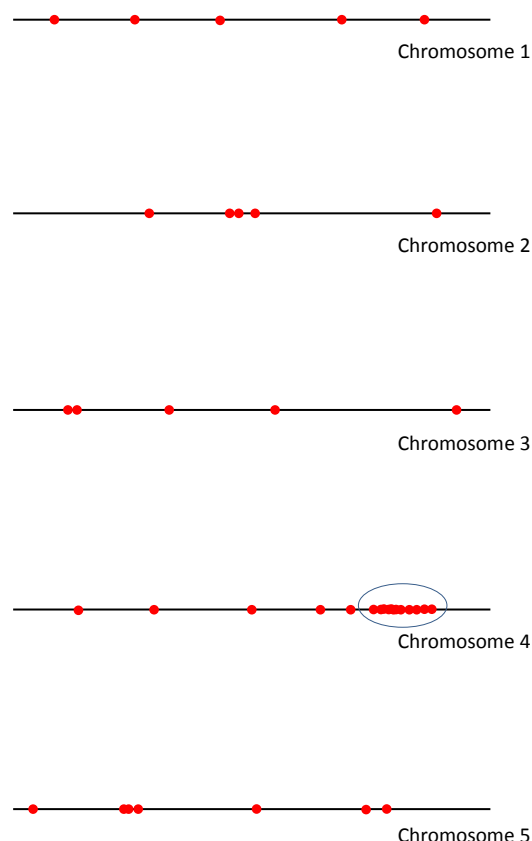
The *eds11* (*enhanced disease susceptibility 11*) mutant was isolated from a population of EMS mutagenised plants from the *BGL2:GUS* reporter line in accession Col-0, based on enhanced susceptibility to the virulent bacterial pathogen *Pseudomonas syringae* pv. *maculicola* strain ES4326. The phenotype is thought to be caused by a single recessive mutation (Glazebrook *et al.*,

1996). Mutant plants are also more susceptible to *F. culmorum* (Cuzick *et al.*, 2008a) but not to the bacterial wilt pathogen *Xanthomonas campestris* or the powdery mildew *Erysiphe orontii*. The *eds11* mutant was shown to be unaffected in expression of *PR* genes including *BGL2*, and can successfully mount Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR) responses (Volko *et al.*, 1998, Ton *et al.*, 2002). Despite its isolation nearly a decade ago and its effect on resistance to both *Pseudomonas syringae* and *Fusarium*, the causal mutation in *eds11* has not been identified and the corresponding gene has not been cloned or its genetic location determined.

In this chapter, mapping of the *EDS11* locus responsible for altered susceptibility to *F. culmorum* was attempted by re-sequencing a population of 58 *F. culmorum* susceptible F₂ plants generated from a backcross to the Col-0 *BGL2:GUS* line. The approach used was similar to that of Hartwig *et al.* (2012), making use of Illumina sequencing followed by the SHOREmap *backcross* pipeline (Fig. 5.2). The mapping strategy and preliminary results are presented and future work is discussed.

Crosses, screening of F₂s, DNA extraction and analysis of SHOREmap *backcross* output were done by the author. Library assembly, Illumina sequencing, read alignment and preliminary SNP calling using SHOREmap *backcross* were done by Konrad Paszkiewicz and colleagues within the University of Exeter Sequencing Service, UK.

A) Identification of candidate SNPs from a single F_2 individual following multiple backcrosses, based on SNP density across the genome.



B) Identification of candidate SNPs from a pool of F_2 individuals with the mutant phenotype following one or more backcrosses, based on the proportion of sequence reads supporting each SNP

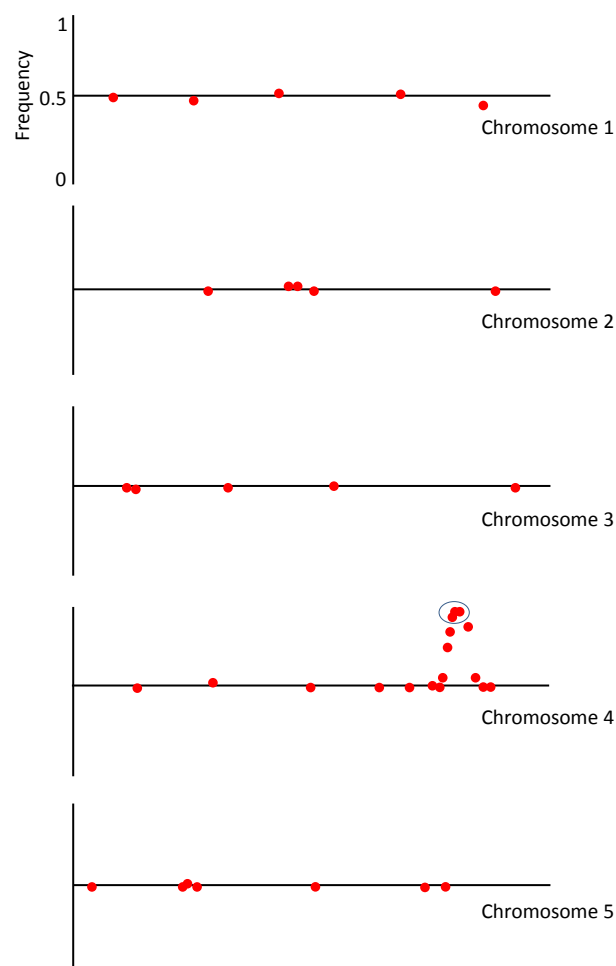


Figure 5.1: Schematic of contrasting approaches to identification of candidate EMS induced mutations in backcrossed individuals and pools. Black horizontal lines = chromosomes. Red dots = SNPs. Blue ellipse = region containing candidate causal SNPs. A) Re-sequencing of a single individual following multiple backcrosses to the wild type parent and identification of genomic regions with high density of SNPs, due to selection through multiple backcrosses. B) Re-sequencing of a pool of F_2 individuals with the mutant phenotype, following one or more backcrosses to the wild type parent. Since whole genome sequencing generates multiple reads for each genomic region, the proportion of reads supporting the SNP at each locus can be ascertained. SNPs unrelated to the selected phenotype will be represented in ~50% of the reads (frequency = 0.5) while the causal SNP will occur in 100% of the reads (frequency = 1).

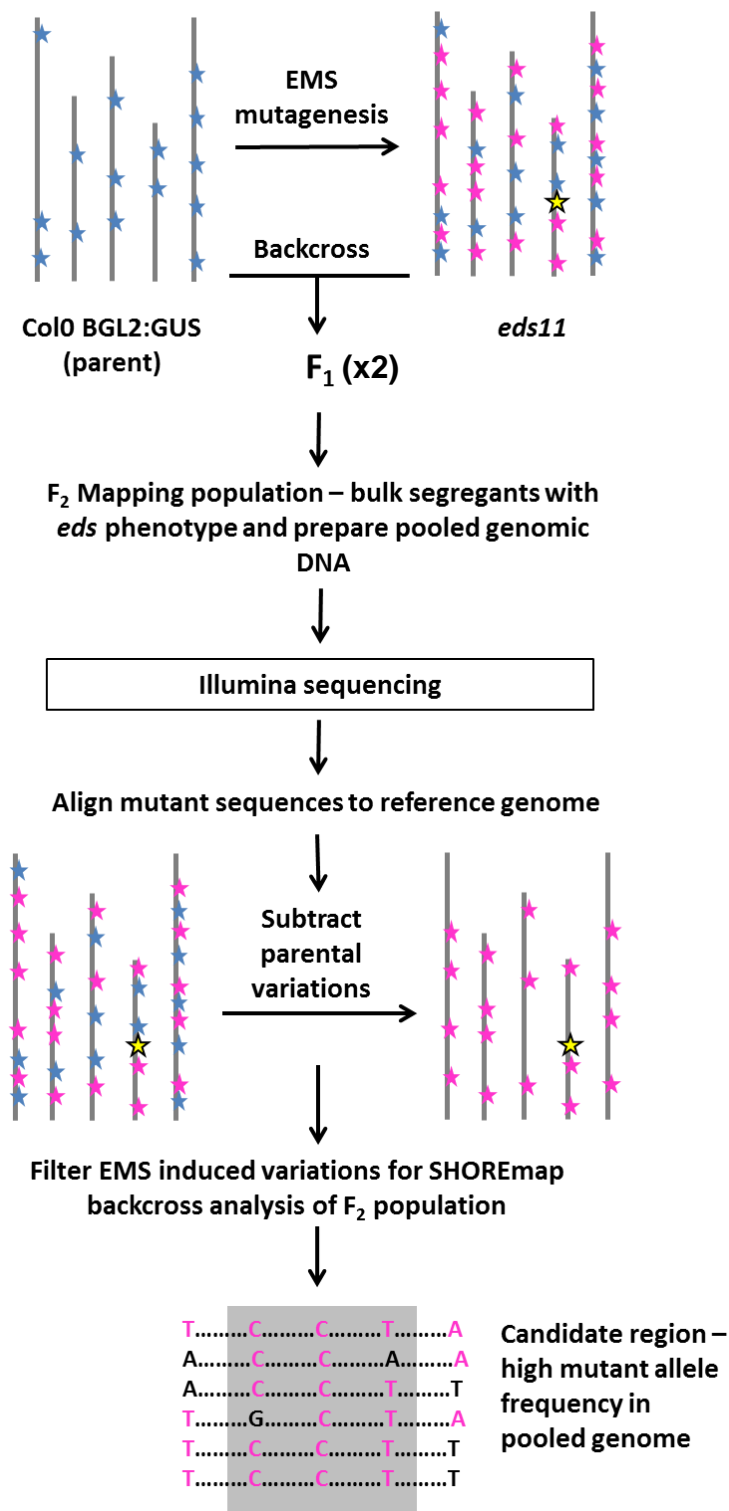


Figure 5.2: Schematic of the mapping by sequencing approach used in this chapter, adapted from Hartwig *et al.* (2013). Blue stars = polymorphisms between parental line and reference genome. Pink stars = EMS derived mutations. Yellow star = causal mutation. The *eds11* mutant was generated by EMS mutagenesis of the reporter line Col-0 *BGL2:GUS* by Glazebrook *et al.* (1996). The *eds11* mutant is backcrossed to the parental *BGL2:GUS* line and resulting F_1 plants selfed to give rise to recombinant F_2 populations. Plants from two arising F_2 populations are screened for the *eds* phenotype (enhanced susceptibility to *F. culmorum*) and susceptible plants selected for re-sequencing using the Illumina platform, along with the parental line *BGL2:GUS*. The SHOREmap pipeline is then used to identify SNPs between the re-sequenced genotypes and the reference. SNPs identified in both genotypes are subtracted. A list of candidate causal mutations is then generated based on frequency in the pooled susceptible F_2 genome.

5.2 Materials and Methods

5.2.1 Generation of *BGL2:GUS* x *eds11* F₂ mapping population

Pollen from a single *eds11* plant was used to fertilise the emasculated flowers of a single plant from the parental line *BGL2:GUS*. The siliques arising from cross-pollinated flowers were allowed to ripen and then harvested and dried. Seed was stratified at 4°C for 4 days and then sown. Two resulting F₁ plants were allowed to self-fertilise, giving rise to two F₂ populations derived from the same initial cross. These populations were then screened for *F. culmorum* susceptibility (see below). The rationale for initially screening two F₂ populations was that there was no way to verify that the initial cross had been successful, and there was therefore a risk that the F₁ plants had actually arisen from self-fertilisation of the wild-type parent used as the pollen recipient. In this situation all of the resulting progeny would be wild type. Seed from two F₁ plants was therefore used for the F₂ screen in case one population contained no plants with the *eds11* phenotype. Both F₂ populations contained equivalent proportions of plants with the desired phenotype (Table 5.1). Since all F₁ plants arising from the initial cross would be expected to be genetically identical, the two F₂ populations were treated as one during selection, DNA extraction and re-sequencing.

5.2.2 Selection of susceptible F₂ plants

Successful selection of mutant plants from an F₂ population requires a clear and consistent phenotype which can be identified in single plants and is not present in wild type plants.

Since the *eds11* mutant was originally selected for increased susceptibility to *Pseudomonas syringae* pv. *maculicola* strain ES4326, the initial intention was to

use this pathogen to screen for *eds11* mutants in the F₂ population. However, neither this pathogen nor the related pathogen *P. syringae* pv. *tomato* DC3000, to which *eds11* has also been shown to be susceptible, produced a clear and consistent susceptibility phenotype in *eds11* plants under the growth conditions used. While *eds11* plants generally supported more bacterial growth than wild type plants, they did not consistently display more severe disease symptoms than wild type Col-0, irrespective of inoculum titre (Figure 5.3). This made *Pseudomonas* susceptibility an unreliable determinant of the *eds11* mutation in the F₂ population.

Table 5.1: Numbers of plants screened for *F. culmorum* susceptibility and selected based on high susceptibility, from two F₂ pools.

F₂ Pool	screened	selected	% selected
A	308	34	11.0
B	232	24	10.3
TOTAL	540	58	10.7

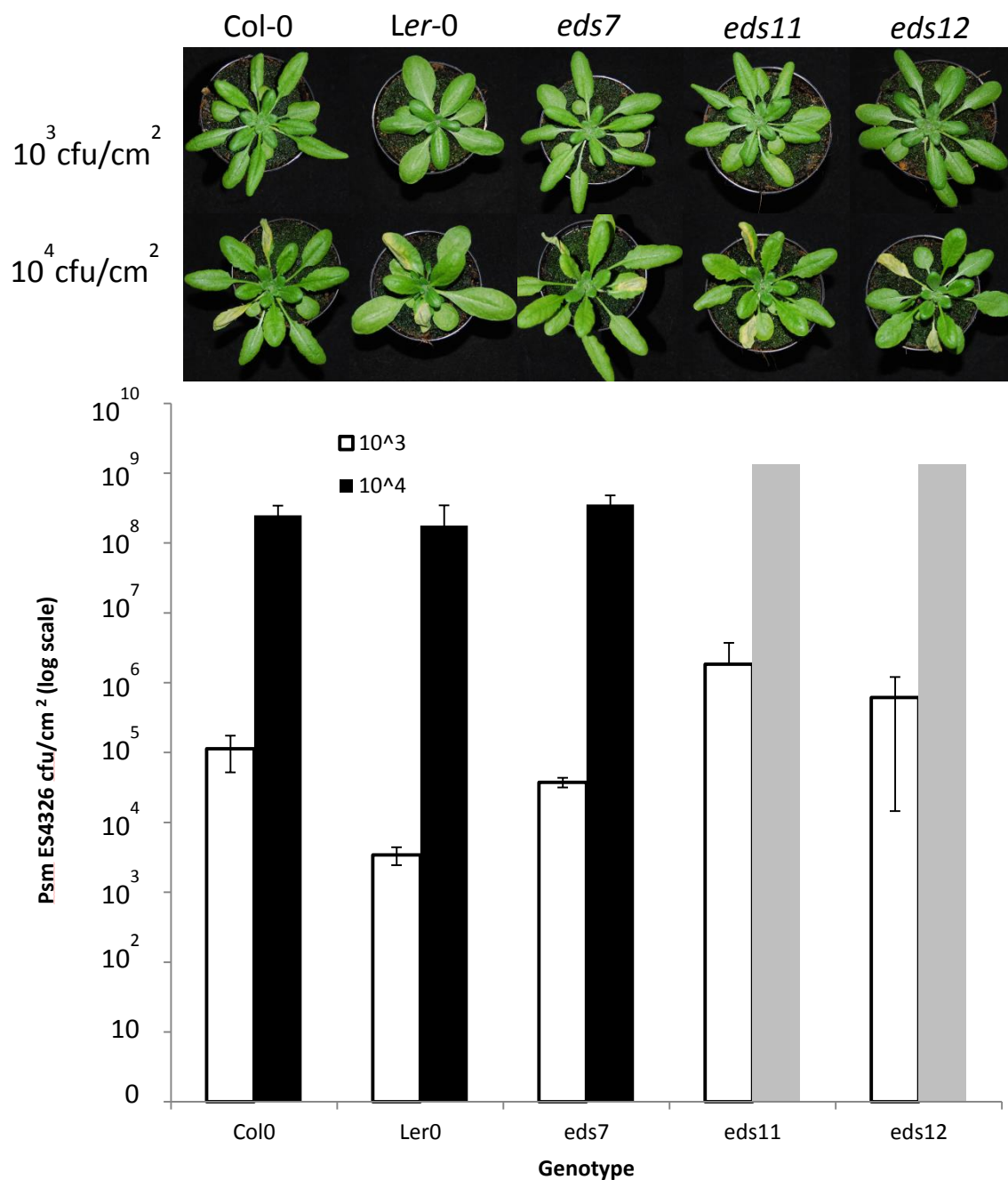


Figure 5.3 Infection of *Arabidopsis enhanced disease susceptibility* mutants with *Pseudomonas syringae* pv. *maculicola* (*Psm*) strain ES4326, with initial inoculum titres of approx. 10^3 cfu (colony forming units)/cm² of leaf tissue and 10^4 cfu/cm². Two leaves per plant were inoculated and disease assessed at 3dpi, by bacterial titre (cfu/cm² of leaf, see graph) and disease symptoms, as shown in photographs. N=7. Bar = standard error. Grey data bars = bacterial titre above assessment threshold. Bacterial titres are higher in genotypes *eds11* and *eds7* compared to wild type Col-0 but disease symptoms are equivalent between genotypes.

F. culmorum susceptibility following spray inoculation was therefore used to identify putative *eds11* mutants from the F₂ populations. However, *F. culmorum* susceptibility is not a fool proof way to identify *eds11* mutants on a plant-by-plant basis, since there is a high level of variation in susceptibility in both wild type and *eds11* plants (Urban *et al.*, 2002, Cuzick *et al.*, 2008a). While *eds11* displays significantly higher mean infection levels across multiple plants and experiments, some plants escape disease, while some wild type plants may become heavily infected. In order to mitigate against this, only F₂ plants with a floral FAD score ≥ 5 were selected, and only from trays where all control *BGL2:GUS* plants had a floral FAD score ≤ 2 . At least 3 parental *eds11* and *BGL2:GUS* plants were used as the controls in each tray of inoculated F₂ plants. Typical disease symptoms of *BGL2:GUS*, *eds11* and susceptible F₂ plants are shown in Figure 5.4. A total of 540 F₂ plants grown from seed from two selfed F₁ plants were screened, and 58 susceptible plants were selected and pooled for DNA extraction and re-sequencing (Table 5.1) Plants were inoculated as described in Chapter 2 and assessed for *Fusarium* infection at 11 dpi.

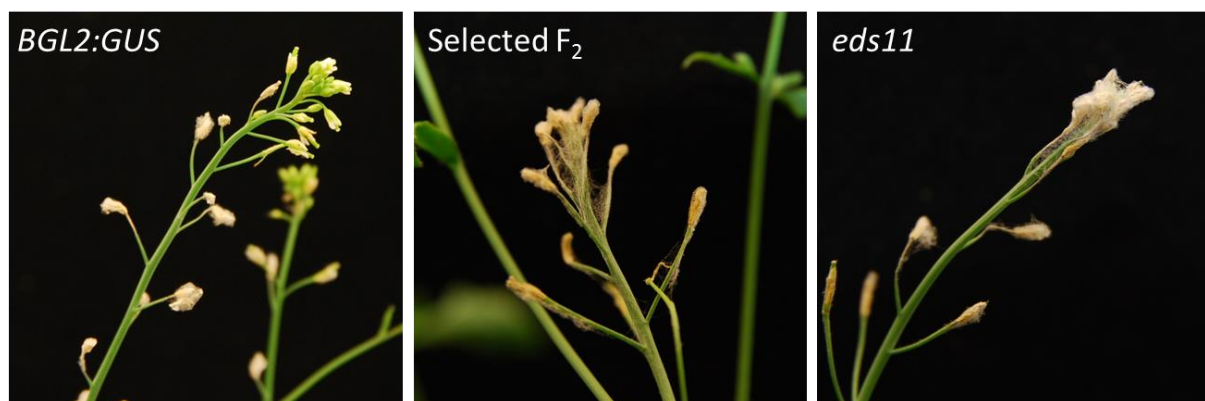


Figure 5.4: Disease symptoms on genotypes *BGL2:GUS* (WT), susceptible F₂ plants and *eds11* plants 11 days after spray inoculation with *F. culmorum*.

5.2.3 DNA extraction and quantification

Floral tissue from 58 susceptible F₂ plants (sample 1), 40 *BGL2:GUS* plants (sample 2) and 40 *eds11* plants (sample 3) were pooled into batches of around 10 plants, harvested and ground in liquid Nitrogen. DNA was then extracted using the Qiagen DNeasy plant tissue kit, as per the manufacturers' instructions, but with water rather than AE buffer used for elution. DNA was then concentrated by ethanol precipitation: eluted DNA was mixed with sodium acetate (final concentration 0.3 M). 2.5 volumes of ice cold 100% ethanol were then added, mixed, and placed on ice for 30 minutes. DNA pellets were spun down in a microcentrifuge for 10 minutes, washed with 70% ethanol and re-suspended in EB buffer (10 mM Tris-Cl, pH 8.5). Final DNA concentration was measured using a Qubit Fluorometer (Life Technologies Ltd., UK) according to the manufacturers' instructions, and DNA quality was assessed by gel electrophoresis on a 0.8% agarose gel. A total of 5µg genomic DNA per sample was submitted for sequencing.

5.2.4 Sequencing, alignment and SHOREmap analysis

Purified DNA from pooled F₂, *BGL2:GUS* and *eds11* plants was submitted to the Exeter DNA sequencing service where it was sequenced using the Illumina HiSeq 2500 Next Generation Sequencing platform, which generates many millions of short, paired end reads with multiple coverage of each region of the genome (Table 5.2). Reads from the three samples were independently aligned to the reference genome for *Arabidopsis* accession Col-0. SHOREmap *backcross* analysis was then used to identify SNPs compared to the reference genome in the *BGL2:GUS* and F₂ reads. SNPs identified in both samples were filtered out. Frequency of SNPs in the F₂ population was then estimated by dividing the number of reads supporting the mutant allele by the total read number. The type of DNA altered by each SNP

(intergenic, intronic or coding) was then analysed using the TAIR10 genome annotation, along with the amino acid changes induced by SNPs in coding regions.

Table 5.2: Summary data from whole genome resequencing of *BGL2:GUS*, *eds11*, and *BGL2:GUS* x *eds11* F₂ plants. *fold coverage calculated as Yield / Arabidopsis genome size.

Sample ID	Yield (Mb)	# Reads	Average fold coverage*
BGL2:GUS_wildtype parent	7,497	74,971,836	56
eds11_mutant parent	575	5,749,220	4
F₂ bulked segregants	17,590	175,898,764	130

5.2.5 Refinement of the SNP call

The SNP call generated by the SHOREmap *backcross* analysis was checked against the sequence reads for the wild type *BGL2:GUS* parent using the ‘Jump to Base’ feature in the Tablet genome browser (Milne *et al.*, 2013). SNPs which were present in any of the *BGL2:GUS* reads were removed from the SNP list (Figure 5.5). The remaining SNPs were also checked against the sequence reads from the mutant *EDS11* parent to confirm their homozygosity in the original mutant stock, however, read coverage for this sample was very poor with no coverage for some of the SNPs. The presence of candidate causal mutations in the *eds11* parent was therefore confirmed using fragment amplification re-sequencing as described in Chapter 2.

5.3 Results

5.3.1 Segregation of the *eds11* phenotype in the F₂ mapping population

Based on previous analysis by Glazebrook *et al.* (1996), the *eds11* phenotype was expected to segregate 1:4 in the F₂ population. However, discounting experiments where the control plants became heavily infected, enhanced susceptibility to *F.*

culmorum was observed in approximately 10% of F₂ plants (Table 5.1). This does not reflect the 1:4 ratio expected of a phenotype caused by a single recessive mutation ($X^2=58.558$, d.f.=1, $p<0.001$), or the 1:16 ratio expected of a phenotype caused by a double recessive mutation ($X^2=18.586$, d.f.=1, $p<0.001$).

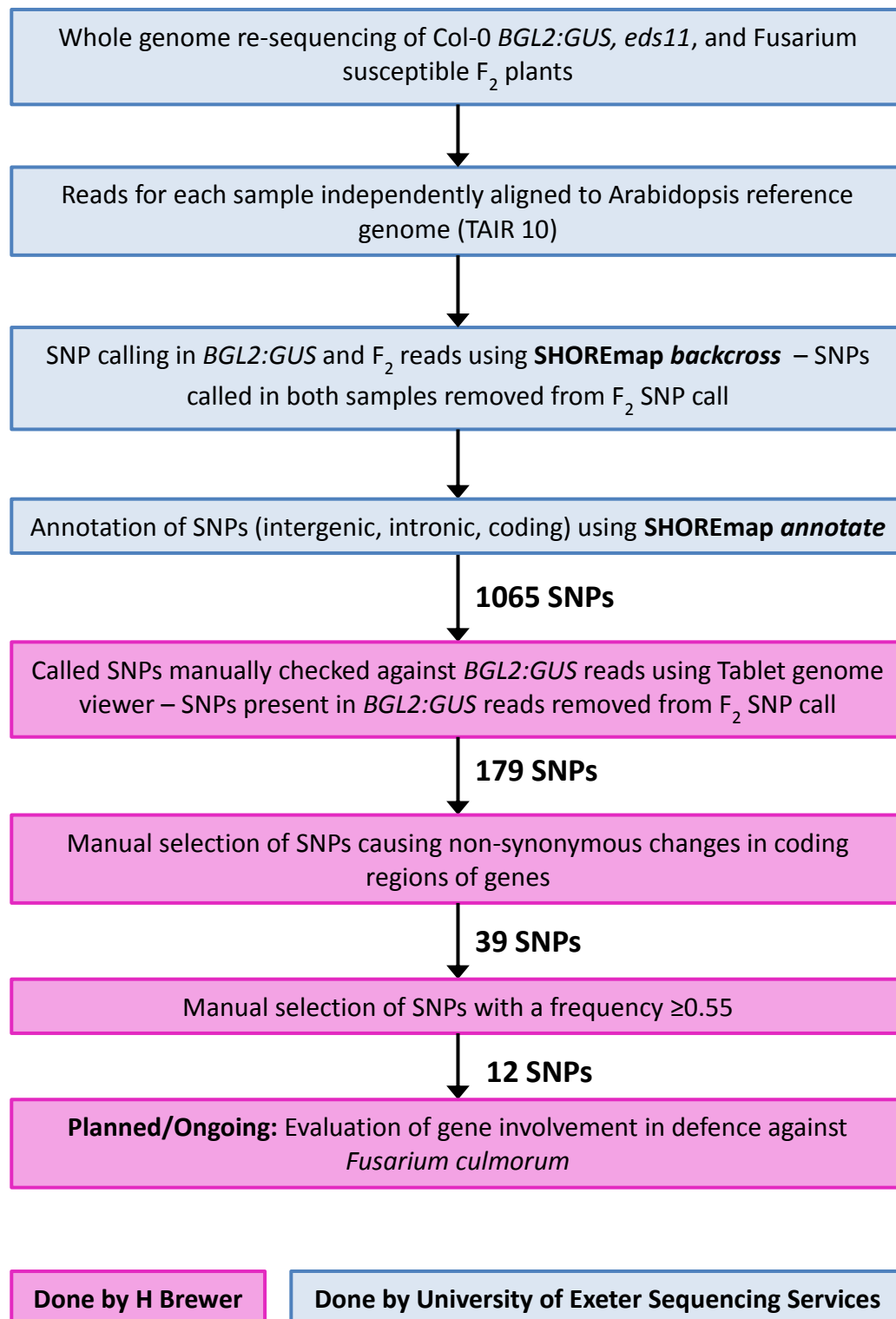


Figure 5.5: Work flow for identification of candidate SNPs. The F₂ SNP call automatically generated by the SHOREmap *backcross* function contained SNPs present in the reference genome *BGL2:GUS*. These were manually removed by checking against the *BGL2:GUS* reads using Tablet genome viewer. SNPs in coding regions of genes with a frequency ≥0.55 have been selected for further analysis.

5.3.2 The majority of called SNPs are present in the parent line *BGL2:GUS*

The SHOREmap *backcross* analysis generated a list of 1,065 SNPs distributed throughout the genome (Figure 5.6, Appendix 2). This included a high number of SNPs with a frequency of 1, distributed across the genome and located in non-coding intergenic DNA (Appendix 2). This suggested that these SNPs were unlikely to be the causal *eds11* mutation. The called SNPs were manually checked against the *BGL2:GUS* reads using the Tablet genome browser and the majority were found to be supported in some or all of the reads, indicating that the analysis pipeline had not successfully identified and removed SNPs present compared to the reference genome in both the *BGL2:GUS* and bulked F₂ reads. The list of SNPs was therefore manually refined, removing all SNPs that were present in the *BGL2:GUS* reads (Figure 5.5). The resulting list contains 179 SNPs (Appendix 3). Their frequencies in the F₂ reads are shown in Figure 5.7.

5.3.3 Distribution and Frequency of SNPs in the F₂ population

The 179 SNPs present in the F₂ reads are predominantly located in 7 clusters across the first four Arabidopsis chromosomes: There are two clusters on the long arm of chromosome 1, two on the long arm of chromosome 2, one on each arm of chromosome 3 and one which covers the length of the short arm of chromosome 4. Each cluster contains SNPs in the coding regions of genes (Figure 5.7, Appendix 4). However, there were no SNPs in any of these clusters with a frequency close to 1. This result could have two explanations. Possibly, the F₂ pool contains some wild type *EDS11* and heterozygous plants, resulting in wild type reads being present at the *eds11* locus. Alternatively, a mutation at more than one genetic locus is required to increase susceptibility to *F. culmorum*.

The highest SNP frequency was observed for the SNPs on the short arm of chromosome 4, where the majority of SNPs had a frequency of around 0.7 (Figure 5.7, Appendix 4). The SNP with the highest frequency (0.82) is found at position 5346521, however this is a region of intergenic DNA and therefore not expected to harbour the *EDS11* locus. The finding that the whole length of the chromosome arm appears to be under selection is also unexpected. The only non-synonymous SNPs in coding regions are found near the telomere of the chromosome. Assuming that only one of these is responsible for the *eds11* phenotype, one would not expect the rest of the chromosome arm to be predominantly inherited from the *eds11* parent during selfing of the F₁ plants. One possible explanation is low recombination frequency in this region, resulting in the whole arm being inherited as a single unit. However, previous work has demonstrated that the short arm of chromosome 4 has a normal recombination frequency (Drouaud *et al.*, 2006). It is however possible that one of the mutations inhibits the formation of chiasmata, lowering the recombination frequency. Alternatively, the *eds11* phenotype may depend on two weakly linked loci on the short arm of chromosome 4.

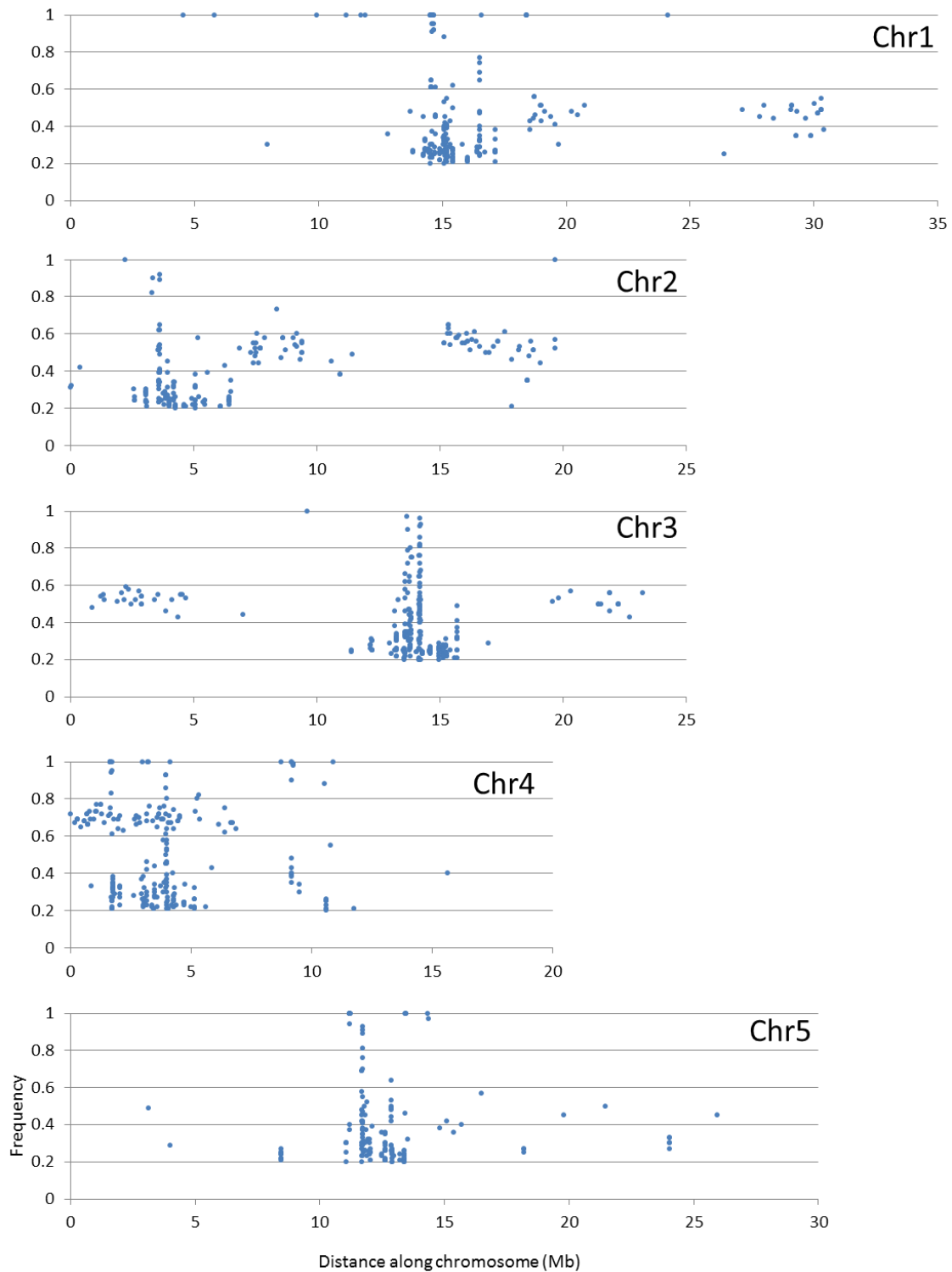


Figure 5.6: Location and frequency of single nucleotide polymorphisms (SNPs) in the *F. culmorum* susceptible F_2 mapping population across the five *Arabidopsis* chromosomes, as identified by SHOREmap *backcross*. Frequency is calculated as the number of Illumina sequencing reads containing a SNP, divided by the total number of reads covering the nucleotide.

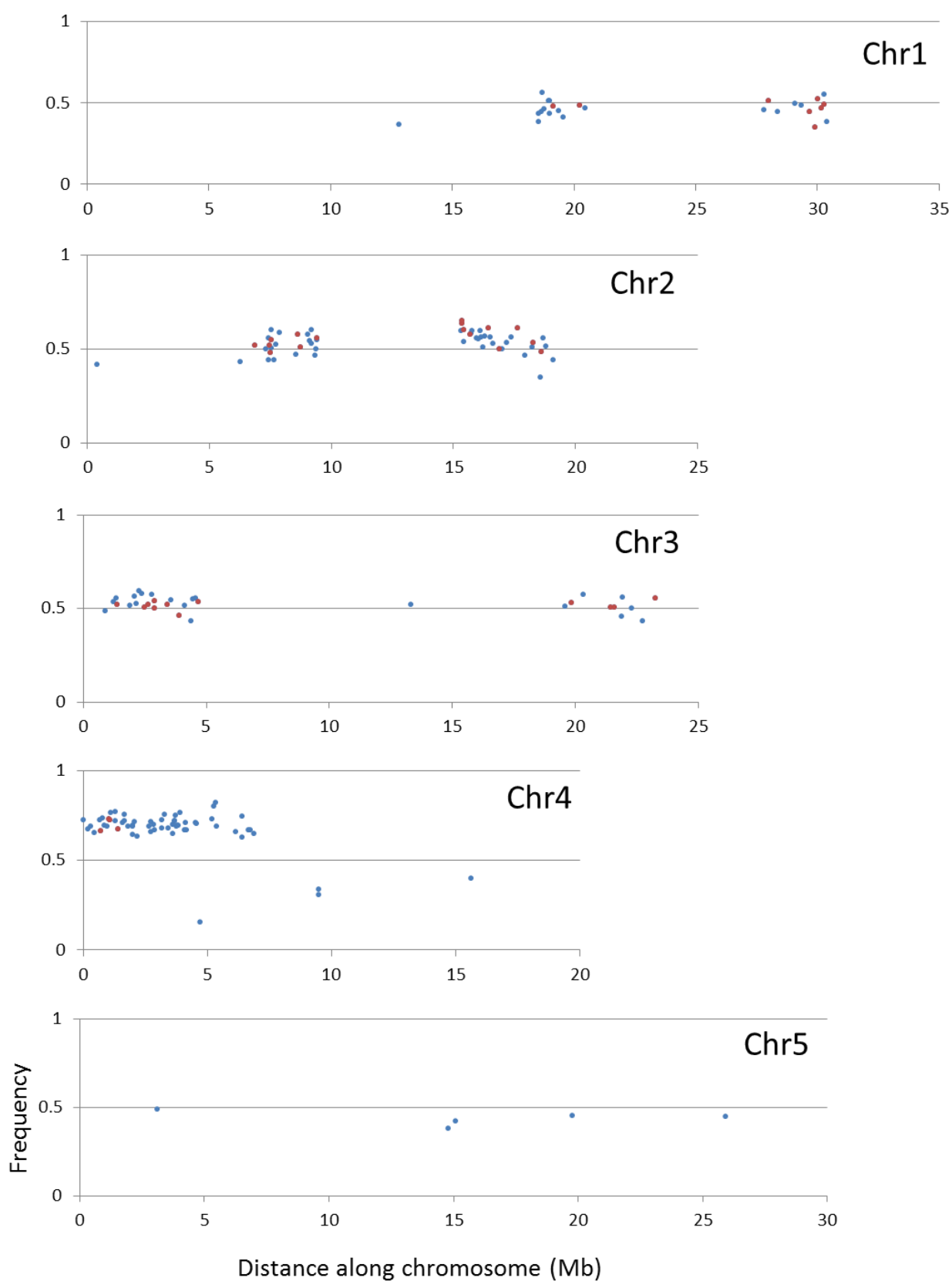


Figure 5.7: Manual refinement of the SNP call presented in figure 5.6 to remove all SNPs which are present in the reads of the wild type parent *BGL2:GUS*. Red dots indicate SNPs which cause non-synonymous changes in coding genes.

5.3.4 Identification of SNPs in candidate genes

Due to the aforementioned possibility of the *eds11* phenotype being caused by more than one mutation (where only one is required to produce the phenotype), and the unexpected frequency pattern on chromosome 4, the frequency of SNPs in other regions of the genome was also subjected to further scrutiny. The majority of other SNPs in coding regions had the expected frequency for a non-causal mutation of ~ 0.5. However, there were exceptions in both clusters on chromosome 2, in addition to the higher frequency observed across the short arm of chromosome 4. A list of all SNPs which cause non synonymous changes in coding genes, that have a frequency ≥ 0.55 , is presented in Table 5.3 and Figure 5.8. It is of note that a number of the genes harbouring SNPs have previously been linked to plant defence signalling. AT2G20010 has a frequency of 0.57 and is predicted to encode an antimicrobial peptide (Silverstein *et al.*, 2007), and expression of AT2G22170 (frequency 0.55) and AT4G02480 (frequency 0.72) are altered in response to viral infection (Ascencio-Ibáñez *et al.*, 2008). Meanwhile AT2G42360 (frequency 0.60) has been flagged in a number of transcriptomic analyses of plant responses to pathogens (Ascencio-Ibáñez *et al.*, 2008, AbuQamar *et al.*, 2006), as has AT4G01680 (frequency 0.66) (Zhao *et al.*, 2007, Ditt *et al.*, 2006).

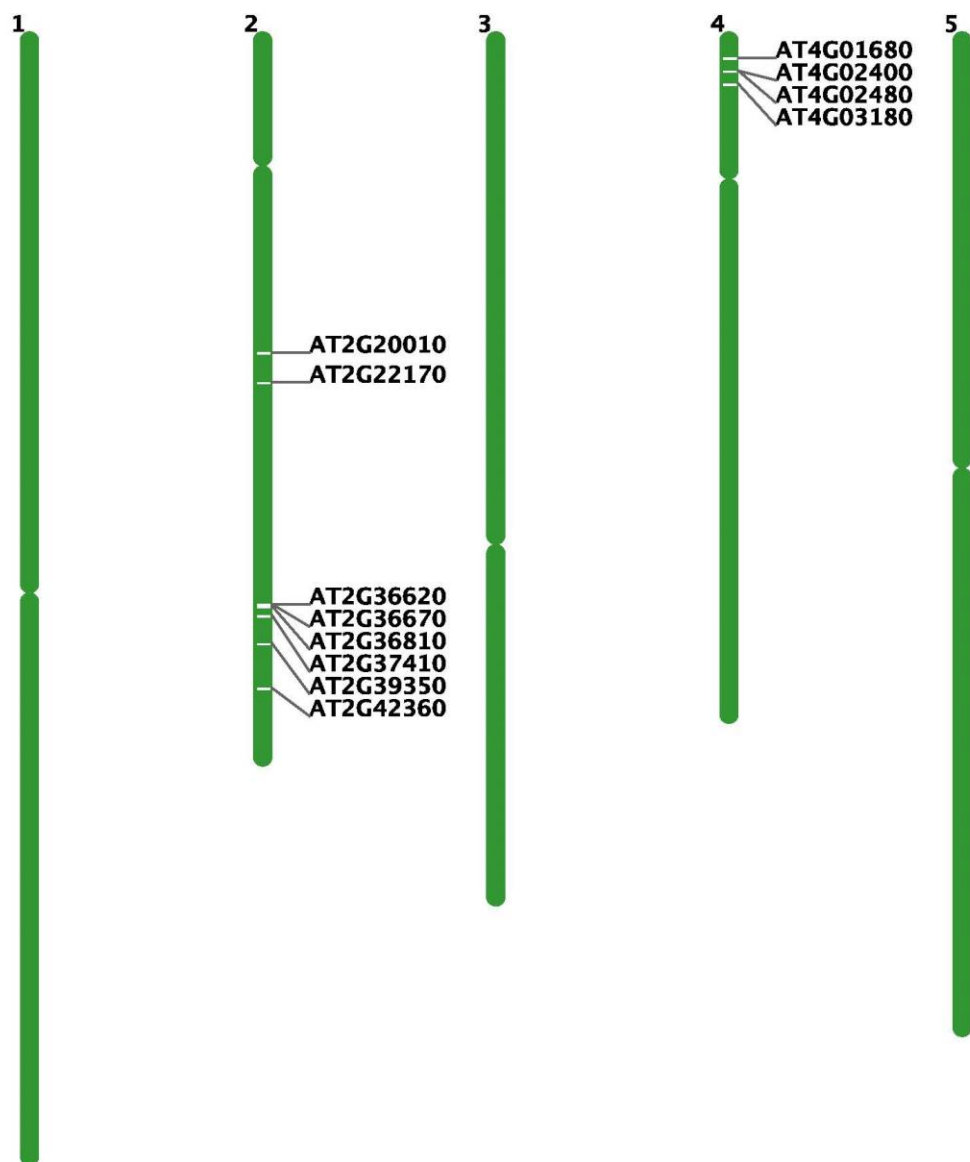


Figure 5.8: Physical genomic location of genes harbouring SNPs with a frequency ≥ 0.55 in the F_2 sequence reads. Image generated using the Chromosome Map Tool at www.arabidopsis.org

Table 5.3: Candidate genes with SNPs in coding regions occurring at a frequency ≥ 0.55 in the *BGL2:GUS* x *eds11* F₂ sequence reads. Annotation, defence association and associated references obtained from locus entries in The Arabidopsis Information Resource (www.arabidopsis.org). ZF = zinc finger, TF = transcription factor

Candidate gene	SNP frequency	Reference	SNP	Position	Amino acid change	Annotation	Defence related?	References
AT2G20010	0.58	G	A	1294	E to *	Putative antimicrobial peptide	Yes	Silverstein <i>et al.</i> (2007)
AT2G22170	0.56	G	A	292	I to L	PLAT domain protein	Yes	Ascencio-Ibáñez <i>et al.</i> , (2008)
AT2G36620	0.63	C	T	313	* to K	Ribosomal protein L24	No	
AT2G36670	0.65	C	T	1310	V to E	Aspartyl protease	Yes	Ascencio-Ibáñez <i>et al.</i> , (2008)
AT2G36810	0.60	C	T	5102	I to N	Gravitropism related	No	
AT2G37410	0.58	C	T	626	L to H	Mitochondrial translocase	No	
AT2G39350	0.61	C	T	929	T to N	ABC-2 type transporter	No	
AT2G42360	0.61	C	T	373	I to L	ZF	Yes	Ascencio-Ibáñez <i>et al.</i> , (2008) AbuQamar <i>et al.</i> (2006)
AT4G01680	0.66	G	A	578	A to V	TF (MYB55)	Yes	Zhao <i>et al.</i> (2007) Ditt <i>et al.</i> (2006)
AT4G02400	0.73	G	A	889	E to K	U3 ribonucleoprotein (Utp) family	No	
AT4G02480	0.73	G	A	3553	R to *	ATPase	Yes	Ascencio-Ibáñez <i>et al.</i> , (2008)
AT4G03180	0.67	G	A	400	A to T	Unknown	No	

5.4 Ongoing work – SNP profiling of susceptible F₃ populations

The screen of F₂ plants for the *eds11* phenotype relied on the mutant phenotype being detectable on a plant by plant basis. As previously explained, while *eds11* plants are on average more susceptible to infection, there is high variation in the susceptibility of both wild type and *eds11* plants. It is therefore likely that some of the pooled F₂ plants were wild type or heterozygous at the *EDS11* locus, inhibiting the identification of the causal mutation. F₃ populations derived from susceptible F₂ plants are therefore being screened for increased *F. culmorum* susceptibility at a population level, as this is a more robust way of identifying F₃ pools that are homozygous for the *eds11* mutation. Fragment re-sequencing of susceptible F₃ pools will then be used to re-analyse the frequency of the candidate mutations in Table 5.3. F₃ pools will be sequenced individually rather than bulked, in order to analyse linkage on chromosome 4.

5.5 Discussion

While many recent papers on mapping by sequencing present the approach as a straightforward pipeline for identifying a causal SNP in an EMS generated mutant, this chapter illustrates that such studies do not always result in identification of a single small region likely to contain the affected gene.

5.5.1 Identification of SNPs between the wild type parent and the reference genome

In particular, this study illustrates that the filtering step in the SHOREmap *backcross* pipeline, designed to filter out SNPs that are present between the ‘wild type’ parent and the reference genome, is not completely effective. In this case this may be due to the sheer number of SNPs between the *BGL2:GUS* reporter line and the Col-0 reference genome. While approximately 6000 SNPs were identified and filtered out using the SHOREmap software, another ~900

SNPs were not called and therefore falsely attributed to the EMS mutagenesis used to generate the *eds11* mutant. This interfered with the identification of candidate mutations and required the SNP list to be further refined manually (Figure 5.5). This illustrates the amount of variation that can exist between so called 'wild type' Col-0 lines and the reference genome. While the Col-0 line used in this study carries a reporter gene insertion, this is unlikely to be responsible for the high number of genome wide SNPs observed. The lack of accurate calling of the SNPs in the wild type parent also raises the question of whether all of the SNPs present in the F₂ population were successfully identified. While this study has identified a number of promising candidate genes for *EDS11*, it is possible that the true causal SNP was not identified.

5.5.2 Is the causal mutation in an exon?

The list of candidate mutations generated in Table 5.3 relies on the assumption that the causal mutation is most likely to affect the coding region of a gene by introducing a non-synonymous change in the translated protein sequence. However, this process has discarded many intronic SNPs, which may affect gene expression or splicing, along with those potentially falling in promoter regions. Likewise it is possible that some of the SNPs that fall in predicted intergenic regions are actually in transcribed regions that have not been identified or annotated. However, since it is not possible to follow up on every mutation, further work will focus on the candidate SNPs in the coding regions of genes, particularly those on the short arm of chromosome 4.

5.5.3 Are all *eds11* phenotypes caused by one mutation?

The *eds11* mutant is associated with a number of phenotypes, namely *Pseudomonas* susceptibility, *Fusarium* susceptibility and altered accumulation of primary sugars, amino acids and flavonoids (chapters 3 and 4). The original

study by Glazebrook *et al* (1996) demonstrated that *Pseudomonas* susceptibility is caused by a single recessive mutation, based on 1:3 segregation of the phenotype in an F₂ population. However, there is no evidence that both the *Pseudomonas* susceptibility phenotype and the *Fusarium* susceptibility phenotype are caused by the same EMS induced mutation. As demonstrated in Table 5.3, several SNPs cause non-synonymous changes in genes associated with plant defence signalling. It is therefore possible that one of these mutations alters *Pseudomonas* susceptibility, while another causes enhanced *Fusarium* floral infection. Furthermore, it is not clear whether the *Fusarium* susceptibility may be the result of more than one mutation. It is unlikely that the phenotype could be caused by either of two or more mutations acting independently, since this should have resulted in an increased number of susceptible plants in the F₂ population (~43.75%). However, reliance on two interdependent mutations for the phenotype would result in 1/16 F₂ plants having increased *Fusarium* susceptibility. The observed susceptibility ratio, disregarding plants from batches where the wild type was heavily infected, was approximately 1/10. However, since it is likely that some of these plants were mis-selected wild type or heterozygous plants it is possible that the true susceptibility ratio is 1/16 and that *Fusarium* susceptibility requires two interdependent mutations. However, this being the case, one would have expected to see two regions of the genome with high frequency SNPs. As previously mentioned, it is possible that the *eds11* phenotype relies on two weakly linked mutations on the short arm of chromosome 4, and that this linkage is responsible for the observed segregation ratios and for the elevated frequency of all SNPs in this region of the genome. The only SNPs causing non-synonymous changes in coding regions of annotated genes are those at the end of the chromosome arm, but it

is possible that an additional SNP is disrupting the function of currently un-annotated genes or other regulatory sequences.

As previously discussed in chapter 2, it is possible that the SNPs responsible for the metabolic profile of *eds11* are unrelated to the susceptibility phenotype. Indeed, some SNPs identified with a frequency close to 0.5 caused non-synonymous changes in genes linked to primary metabolism. These include the glycosyl hydase encoding gene AT3G10900 which is involved in carbohydrate metabolism. Metabolomic analysis of T-DNA insertion lines for these genes might reveal fingerprints similar to that seen in *eds11* in chapter 3. It would also be interesting to analyse the metabolic fingerprints of susceptible F_3 populations in order to identify which of the previously observed metabolic perturbations segregate with the susceptibility phenotype.

5.5.4 The effect of using F_2 pools from two F_1 plants

As previously described, two F_2 populations were screened and combined for re-sequencing, relying on the assumption of high levels of homozygosity in both the *BGL2:GUS* and *eds11* parent plants. This would mean that all F_1 plants would be identical, generating equivalent F_2 pools. However, analysis of the *BGL2:GUS* sequence reads revealed that many SNPs which were not present in all of the reads, indicating lack of fixation in the sequenced *BGL2:GUS* population and likely heterozygosity in the parent plant used for the crosses. They may also not have been present in the single plant that was used to generate the *eds* mutants. This may explain the high number of SNPs with a frequency of 0.25 in the original SNP call prior to manual filtering of SNPs present in the *BGL2:GUS* reads (Fig. 5.6). These SNPs are likely to have been heterozygous in the *BGL2:GUS* parent and absent from the *eds11* parent, and therefore only inherited by one F_1 plant. This would result in a frequency of 0.5

in one F_2 pool and 0 in the other, creating an overall frequency of 0.25.

However, since these SNPs were removed in the manual filtering step, and the remaining SNPs generally had a frequency of around 0.5, it is likely that the *eds11* parent was homozygous for the remaining SNPs and that using two F_2 pools has not inhibited the mapping process.

5.6 Conclusions

This chapter describes the attempted mapping by sequencing of the *eds11* mutation responsible for *F. culmorum* susceptibility, using the SHOREmap *backcross* pipeline. While the mutation could not be narrowed down to a single causal SNP in one gene, the study has identified a number of potential candidate genes, particularly those near the telomere of the short arm of chromosome 4. Ongoing work includes the screening of F_3 populations derived from susceptible plants in order to narrow down the list of candidates, and possibly ascertain whether the high frequency of SNPs along the entire chromosome arm is due to reduced recombination. Future work will involve obtaining T-DNA insertion lines in the candidate genes and analysis of their susceptibility to *Pseudomonas* and *Fusarium*, along with their metabolic fingerprint.

6 Resistance to *Fusarium culmorum* and *F. graminearum* in *Arabidopsis* silique and leaf tissue is mediated by mutations in the homoserine kinase gene *DMR1*[†]

6.1 Introduction

The *Arabidopsis downy mildew resistant* (*dmr*) mutants were isolated from a gain of function screen for resistance to the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*), following ethyl methanesulfonate (EMS) mutagenesis of plants of the susceptible genotype Ler-0 harbouring the *enhanced disease susceptibility* mutation *eds1-2* (van Damme *et al.*, 2005). The *eds1-2* mutation in Ler-0 has previously been shown not to alter the interaction outcome between *F. culmorum* and *Arabidopsis* floral or silique tissue (Cuzick *et al.*, 2009). Of the five *dmr* mutant alleles identified, three (*dmr3*, *dmr4*, *dmr5*) showed constitutive expression of the salicylic acid mediated defence related gene *PR-1*. The remaining mutants, *dmr1* and *dmr6*, were mapped and identified as encoding mutations in the *Arabidopsis* homoserine kinase, and a putative 2-oxoglutarate oxygenase, respectively (van Damme *et al.*, 2008, van Damme *et al.*, 2009). *DMR6* is associated with salicylic acid mediated defence signalling but is required for *H. arabidopsidis* susceptibility. Mutation of *dmr1* results in accumulation of homoserine in non-inoculated plants, and exogenous application of L-homoserine co-incident with *H. arabidopsidis* inoculation confers resistance in wild type plants. However, the role of L-homoserine in resistance is not known.

The *Fusarium-Arabidopsis* pathosystem was used to assess the effects of the *dmr* mutations on *Fusarium* susceptibility in *Arabidopsis* floral, silique and

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rosette leaf tissue. Mutants *dmr1-1*, *dmr1-2*, *dmr5* and *dmr6* (all of which also carry the *eds1-2* mutation) were initially investigated; *dmr3* and *dmr4* have pleiotropic dwarf phenotypes which affect floral morphology and were therefore unsuitable for inclusion in this study.

This chapter presents the finding that reduced function of the Arabidopsis homoserine kinase DMR1 confers resistance to *F. graminearum* and *F. culmorum* in silique and/or rosette leaf tissues, with varying levels of resistance conferred by different *dmr1* mutant alleles. The siliques of *dmr1* plants accumulate homoserine but are not depleted in amino acids such as threonine and methionine which are downstream products of homoserine kinase activity. It was also found that mutation of *DMR1* results in delayed leaf senescence which may be related to the observed resistance phenotype. Exogenous application of L-homoserine reduced floral and silique disease severity in both *eds1-2* and *dmr1* plants, but did not inhibit *in vitro* *Fusarium* growth. A trend was observed towards reduced infection following exogenous L-homoserine application onto wheat, but this finding was not conclusive.

All experiments and analyses were done by the author, with the exception of Gas Chromatography – Mass Spectroscopy (GCMS) and subsequent analysis which was done by Nathan Hawkins at the Rothamsted Metabolomics facility (MeT-RO).

6.2 Materials and Methods

6.2.1 *In planta* amino acid treatment

For amino acid treatment studies on spray inoculated plants, *F. culmorum* inoculated plants were sprayed at the time of inoculation with a solution of 10mM L-homoserine, D-homoserine (Sigma-Aldrich) or L-threonine (Sigma-

Aldrich/Fisher Scientific), or sterile water as a control. This was repeated daily for 5 days post inoculation. For amino acid treatment of single silique wound point inoculated plants, conidial suspensions were supplemented at the time of point inoculation with 20mM L-homoserine or D-homoserine, or sterile water as a control. Amino acid suspensions were then re-applied to the wounded silique tip daily for 6 days post inoculation.

6.2.2 Wheat infection and amino acid treatment

The dwarf wheat cultivar Apogee (Bugbee *et al.*, 1997) was used for wheat infection assays. The 8th and 9th spikelets of ears at anthesis were point inoculated with 5µl of *F. graminearum* conidial suspension at 10⁵ ml⁻¹. In addition, the 6th-11th spikelets were treated with 5µl of either 10mM L-homoserine or D-homoserine, or sterile water. Homoserine/water treatment was repeated daily for 7 days.

The number of bleached spikelets and bent awns (preceding bleaching in infected spikelets) was assessed, and grain weight and number recorded at 10dpi following dissection of the rachis, as per Baldwin *et al.* (2010).

6.2.3 *In vitro* Fusarium growth tests

F. culmorum and *F. graminearum* conidia at a concentration of 2x10⁵ spores ml⁻¹ were cultured for two days in 96 well flat bottomed culture plates in 200µl synthetic nutrient poor liquid media supplemented with either L- or D-homoserine at a concentration range from 0 to 80mM. Absorbance as a surrogate for fungal growth was measured for each homoserine concentration as previously described (Fan *et al.*, 2013). Three biological replicates were included per fungal isolate/amino acid treatment, and the experiment repeated.

6.2.4 Analysis of silique amino acids

The amino acid content of 15mg freeze dried and ground silique samples from *dmr1* mutant and *eds1-2* plants was analysed by Nathan Hawkins at the Rothamsted Metabolomics Facility (Met-RO) using the EZFaast GC-MS physiological amino acid analysis kit, according to the manufacturers' instructions (Phenomenex, UK). The protocol was amended such that the addition of the internal standard supplied with the kit was omitted and the final solvent evaporation step with reconstitution in organic solvent was replaced with a 1:10 dilution with the organic solvent (reagent 6). Samples were analysed on an Agilent 5975 Inert MSD coupled to a 7890A Gas Chromatograph fitted with a Zebron Amino acid ZB-AAA column (10m x 0.25mm I.D. Phenomenex, Cheshire, UK), Gestel MPS2 autosampler and split/splitless injector (fitted with quartz wool packed SGE FocusLiner). For each genotype three biological replicates were analysed, each consisting of siliques from ~8 pooled 6-week old plants. The internal standard, amino acid standard solutions and glutamine standard were obtained from Sigma (Dorset, UK). Homoserine standard was obtained from Koch-Light Laboratories, Colnworth, Bucks, UK.

6.2.5 Statistical analysis

Statistical analysis was done as described in chapter 2, with differences between genotypes and/or treatments considered significant at $p < 0.05$. Statistical outputs are shown in Appendix 5.

6.3 Results

6.3.1 A selection of the Arabidopsis downy mildew resistant mutants have altered susceptibility to *Fusarium culmorum* leaf and silique infection

The Arabidopsis mutants *dmr1-1*, *dmr1-2*, *dmr5* and *dmr6*, which were generated in the Ler-0 *eds1-2* background, were screened for altered susceptibility to *F. culmorum* infection compared to *eds1-2*. Wild type Ler-0 was also included in the assay. Following spray inoculation with *F. culmorum* spores, the plants were scored for floral and silique disease levels, along with rosette leaf infection and number of uninfected green siliques, after 7, 11 and 14 days (Figures 6.1 & 6.2). There was no statistically significant difference in the floral FAD (Fusarium-Arabidopsis Disease) score (Urban *et al.*, 2002) between the genotypes tested (Regression analysis, $F_{4, 93} = 0.7$, $p=0.591$) at any of the time points assessed, with disease progressing at an equivalent rate in all genotypes (Fig. 6.1a). At the time of inoculation, this tissue had been unopened green buds.

By contrast, there was a significant effect of genotype on silique FAD score ($F_{4, 91} = 16.23$, $p<0.01$). The siliques assessed had been open flowers at the time of inoculation. The disease progression in the Ler-0 and Ler-0 *eds1-2* plants was identical (Fig 6.1b). The mutant allele *dmr1-2* had significantly reduced silique disease levels at all time points compared to *eds1-2* (Fig. 6.1b, Fig. 6.2a). This finding was confirmed in multiple independent experiments.

Genotypes *dmr5* and *dmr6* had reduced silique disease symptoms at 7 and 11 days post inoculation (dpi) in the displayed experiment but these findings were not consistent across experiments. The *dmr1-2* plants had significantly higher numbers of uninfected green siliques than *eds1-2* at all time points, whilst for

dmr6 significantly more green siliques were observed at 7 and 11 dpi but not at 14 dpi (Fig. 6.1c).

The number of infected rosette leaves following the initial spray inoculation was also significantly affected by genotype ($F_{4, 93} = 66.06$, $p < .001$). Both *dmr1* alleles had significantly fewer infected rosette leaves than *eds1-2* at all time points (Fig. 6.1d, Fig. 6.2b). Interestingly, Ler-0 had significantly more infected rosette leaves per plant than *eds1-2*, indicating that the *eds1* mutation may have an effect on *F. culmorum* leaf susceptibility that was not identified in the previous floral screen (Cuzick *et al.*, 2009).

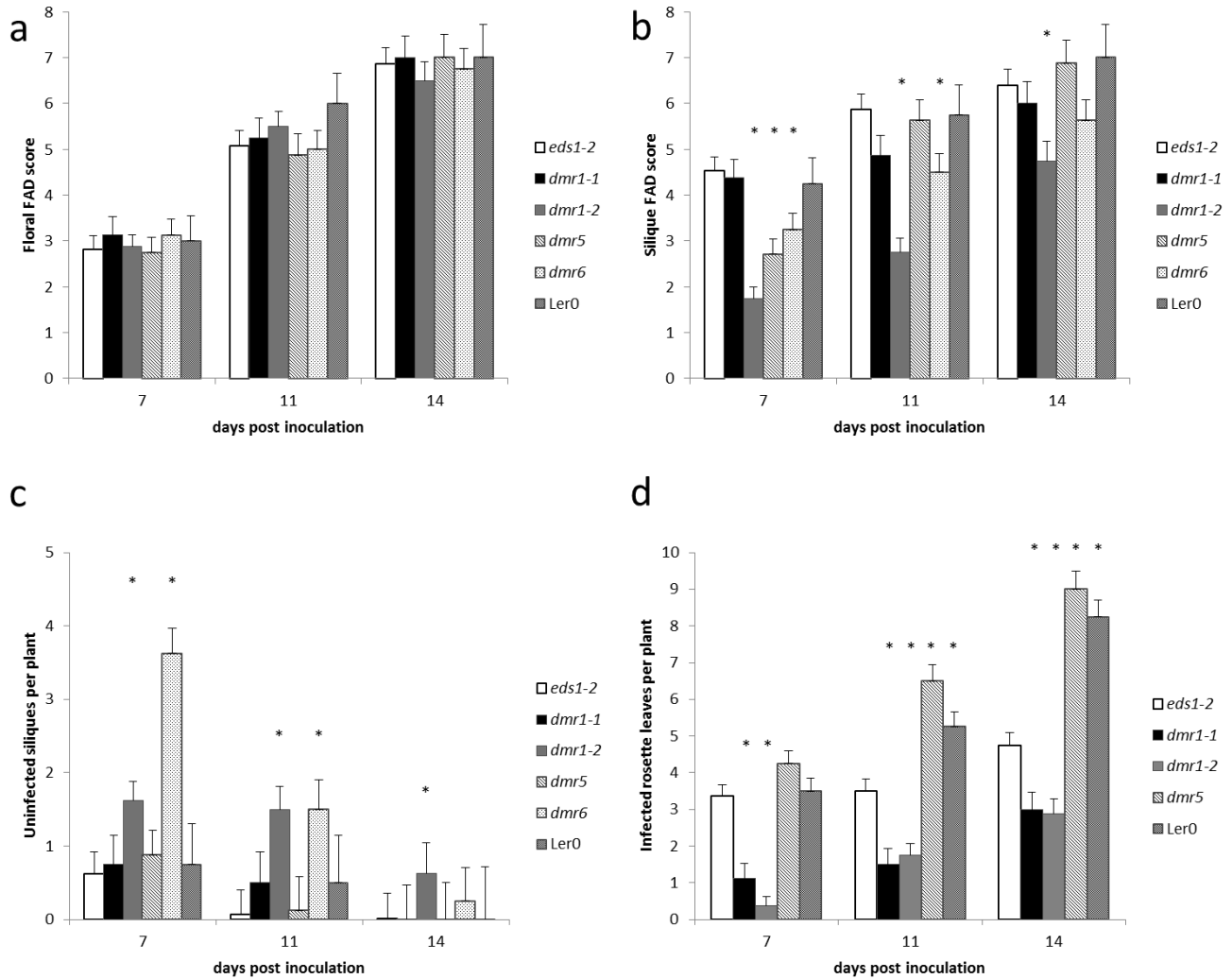


Figure 6.1: Analysis of susceptibility to *F. culmorum* infection in four *downy mildew resistant* (*dmr*) mutant lines, compared to wild-type *Ler-0* and the parental genotype *Ler-0 eds1-2*. Six plants per genotype were scored for (a) floral disease levels, (b) silique disease levels, (c) the number of healthy siliques, and (d) the number of infected rosette leaves, at 7, 11 and 14 days post inoculation (dpi). For the floral and silique evaluations the FAD- Fusarium-Arabidopsis Disease scoring system was used.

Asterisks indicate genotypes significantly different from *eds1-2* at each time point (regression analysis followed by calculation of LSDs, $p < 0.05$). Error bars represent standard error of the mean. The experiment was repeated with similar results. Since the *dmr6* mutant flowers approximately 1 week later than *eds1-2*, *dmr6* plants were used in this experiment were 1 week older than those of other genotypes, and therefore rosette leaf data were not comparable due to increased senescence in the *dmr6* mutant.

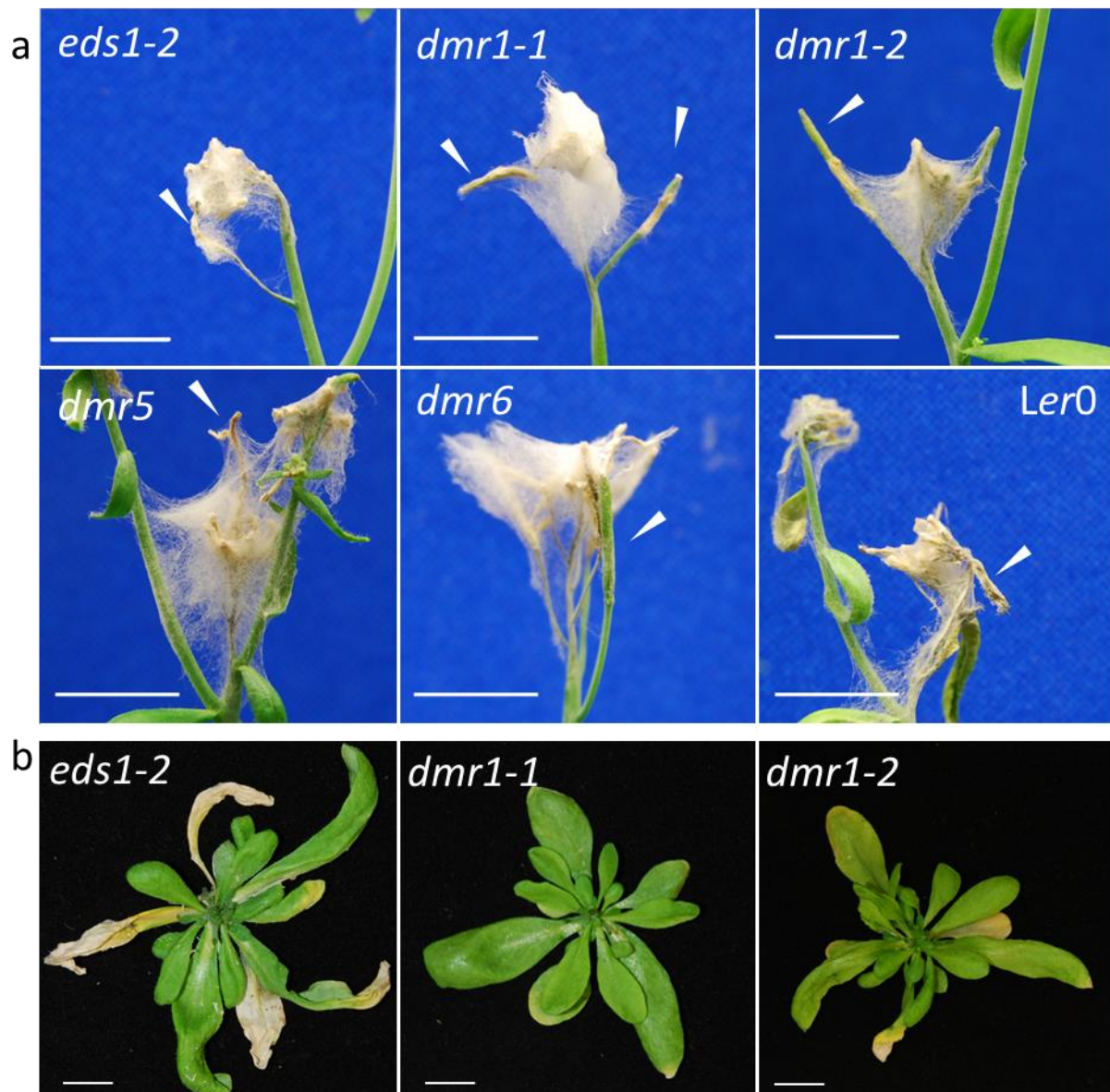


Figure 6.2: Representative images of the floral and rosette leaf *Fusarium culmorum* infections of the *Arabidopsis* downy mildew resistant (*dmr*) mutants at 14dpi, compared to the parental genotype *eds1-2* and wild type *Ler-0*. (a) Infected floral tissue of all genotypes. (b) Rosette leaves of the *dmr1* alleles compared to *eds1-2*. The stem and floral tissue has been removed from each plant in panel b. Bar = 1 cm. White arrows = siliques with different levels of infection.

6.3.2 Mutation of DMR1 reduces susceptibility to *F. graminearum*

FEB disease is caused by several cereal-infecting *Fusaria* species. Therefore, susceptibility to *F. graminearum* infection was compared between the *dmr1* mutant alleles *dmr1-1* and *dmr1-2*, and the parental genotype *eds1-2* at 7, 11 and 14dpi. Results were similar to those obtained for *F. culmorum*: No difference was observed in floral susceptibility ($F_{2, 62} = 2.25$, $p = 0.114$) (Fig. 6.3c). Rosette leaf infection was affected by genotype ($F_{2, 62} = 37.10$, $p < .001$) with both *dmr1* alleles having fewer infected rosette leaves than *eds1-2* (Fig. 6.3b and f). Silique FAD scores and uninfected silique numbers also differed between genotypes ($F_{2, 62} = 48.63$ and 55.31 respectively, $p < .001$). Silique FAD scores were lower in *dmr1-2* than *eds1-2* at all time points, with uninfected green siliques higher in *dmr1-2* at 7 and 11dpi (Fig. 6.3a, d and e). In these *F. graminearum* inoculated experiments, fully infected and very necrotic siliques were visible in the *eds1-2* plants from 7dpi onwards, whereas this extreme silique phenotype was rarely observed from 11dpi onwards for *dmr1-2* plants. Overall these results indicate that both leaf and silique resistance conferred by mutation of *DMR1* is conserved across at least two cereal infecting *Fusarium* species.

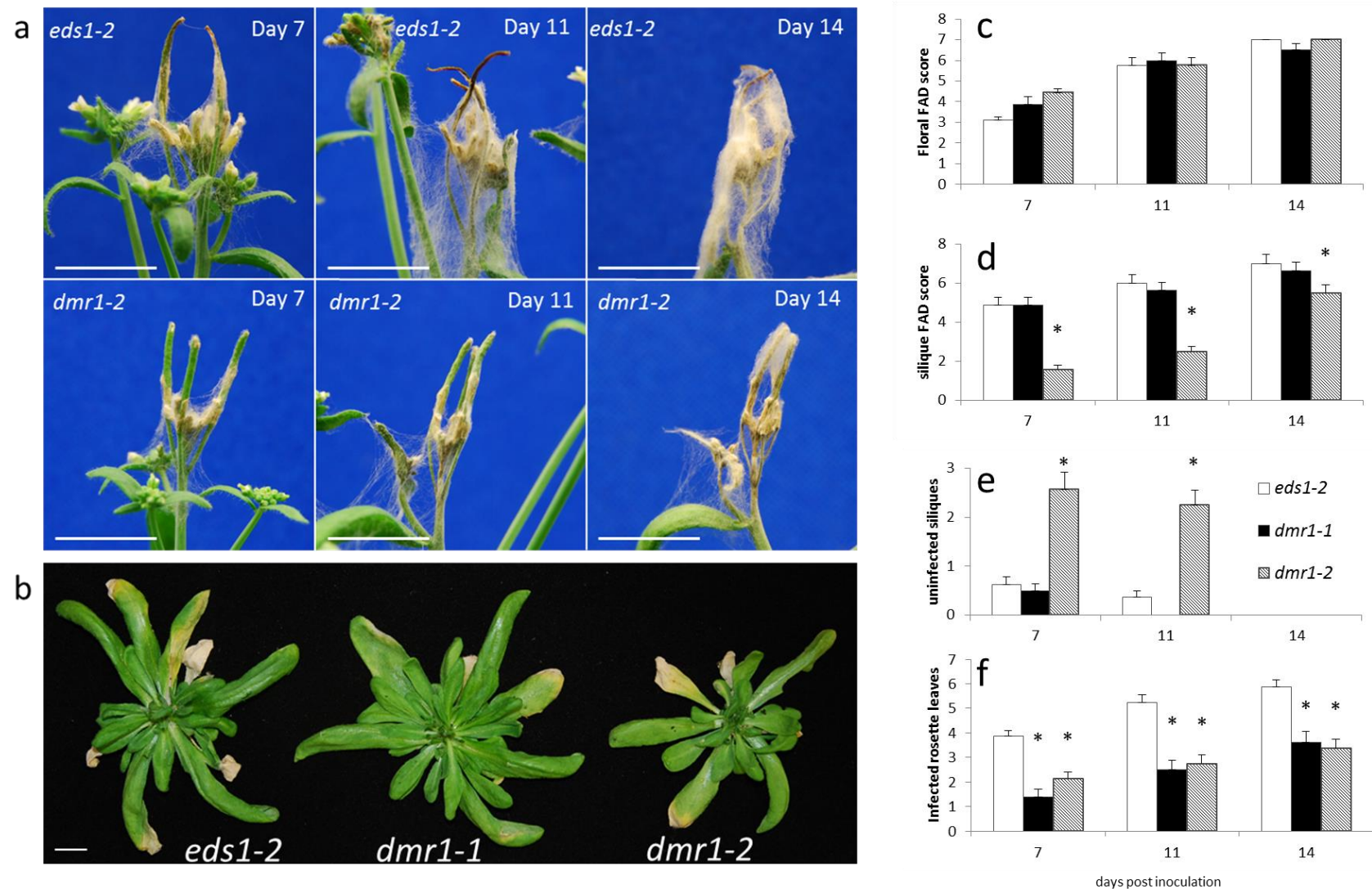


Figure 6.3: Analysis of susceptibility to *F. graminearum* infection in plants harbouring different alleles of the *dmr1* mutation, *dmr1-1* and *dmr1-2*, compared to the parental line *eds1-2*. Panel (a) shows infection of the apical inflorescence and siliques in *eds1-2* and *dmr1-2* at 7, 11, and 14 days post inoculation. In panel b the rosette leaves of the *dmr1* alleles are compared to *eds1-2*, and the stem and floral tissues have been removed. Bar = 1cm. Eight plants per genotype were scored for (c) floral disease levels, (d) silique disease levels, (e) number of green, uninfected siliques and (f) number of infected rosette leaves and at 7, 11 and 14 days post inoculation (dpi). Asterisks indicate genotypes significantly different from *eds1-2* at each time point (regression analysis followed by calculation of LSDs, $p < 0.05$). Error bars represent standard error of the mean. The experiment was repeated with similar results.

6.3.3 Multiple *dmr1* alleles have increased resistance to *F. culmorum*

In order to verify that the silique resistance phenotype observed in *dmr1-2* is a result of mutation of *DMR1* and not caused by a second EMS induced mutation, three additional alleles of *dmr1* (*dmr1-3*, *dmr1-4* and *dmr1-6*) were tested for altered resistance to *F. culmorum* (Fig.6.4). The *dmr1-2*, *dmr1-3*, *dmr1-4* and *dmr1-6* mutants all had lower silique disease levels than *eds1-2* (Fig. 6.4a, c) ($F_{5, 49} = 2.31$, $p = 0.005$), whilst no differences in floral susceptibility were observed between the various *dmr1* genotypes and *eds1-2*. This again indicates that the open flowers and very immature siliques at the time of inoculation of the *dmr1* mutant plants were more resistant to *F. culmorum* infection than the green unopened buds. Rosette leaf infection levels were also different between genotypes ($F_{5, 49} = 15.04$, $p < 0.001$) with fewer rosette leaves per plant infected in genotypes *dmr1-1*, *dmr1-2*, *dmr1-3* and *dmr1-4* compared to *eds1-2* (Fig. 6.4b, d). For *dmr1-6*, there was also a trend towards less leaf disease, but this was not statistically significant. Collectively, these results confirm that increased silique and leaf resistance occurs in multiple *dmr1* alleles and is therefore likely a result of disruption of *DMR1* function.

Mutant *dmr1-1* and *dmr1-2* plants were also assessed for altered susceptibility to *F. culmorum* using a second inoculation method, namely the spore droplet, single silique point inoculation assay. This assay involves initially removing 1mm of tissue from the tip of each immature silique and then placing the 1 μ l spore droplet onto the cut surface. No clear differences were seen in the distance of visible disease progression through the silique and pedicel between genotypes. However, necrosis in siliques of *dmr1-2* appeared less severe than in *eds1-2* or *dmr1-1*, with more green tissue visible at 7dpi (Fig. 6.5).

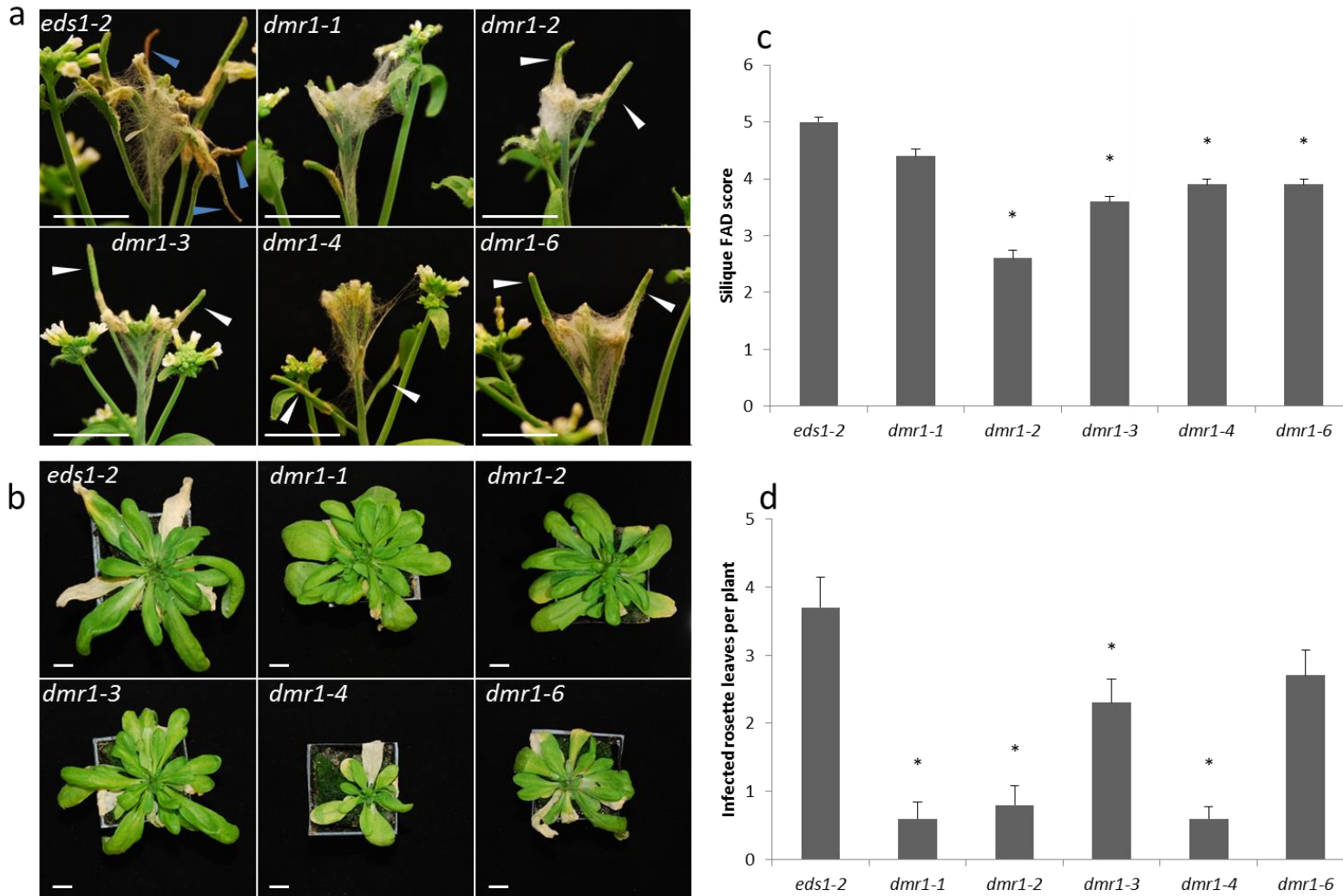


Figure 6.4: Multiple *dmr1* alleles have reduced disease symptoms of *Fusarium culmorum* infection in siliques and rosette leaves. Plants were spray inoculated with *F. culmorum* conidia at early flowering, and disease levels were assessed at 7dpi. Representative images of apical inflorescences at 7dpi for (a) *eds1-2*, *dmr1-1*, *dmr1-2*, *dmr1-3*, *dmr1-4* and *dmr1-6*. Representative images of rosette leaves at 13dpi for (b) *eds1-2*, *dmr1-1*, *dmr1-2*, *dmr1-3*, *dmr1-4*, and *dmr1-6*—minus floral and stem tissues. . Bar = 1cm. Blue arrow head – severely necrotic siliques visible in *eds1-2*. White arrow head – green siliques of *dmr1* plants. Silique FAD scores (c) and infected rosette leaves per plant (d) are shown at 7dpi. Asterisks indicate genotypes significantly different from *eds1-2* (regression analysis followed by prediction of LSDs, $p < 0.05$). Error bars represent standard error of the mean. Data shown are pooled from two independent experimental replicates. $n = 10$ (*eds1-2*, *dmr1-3*, *dmr1-4*, *dmr1-6*), $n = 5$ (*dmr1-1*, *dmr1-2*).

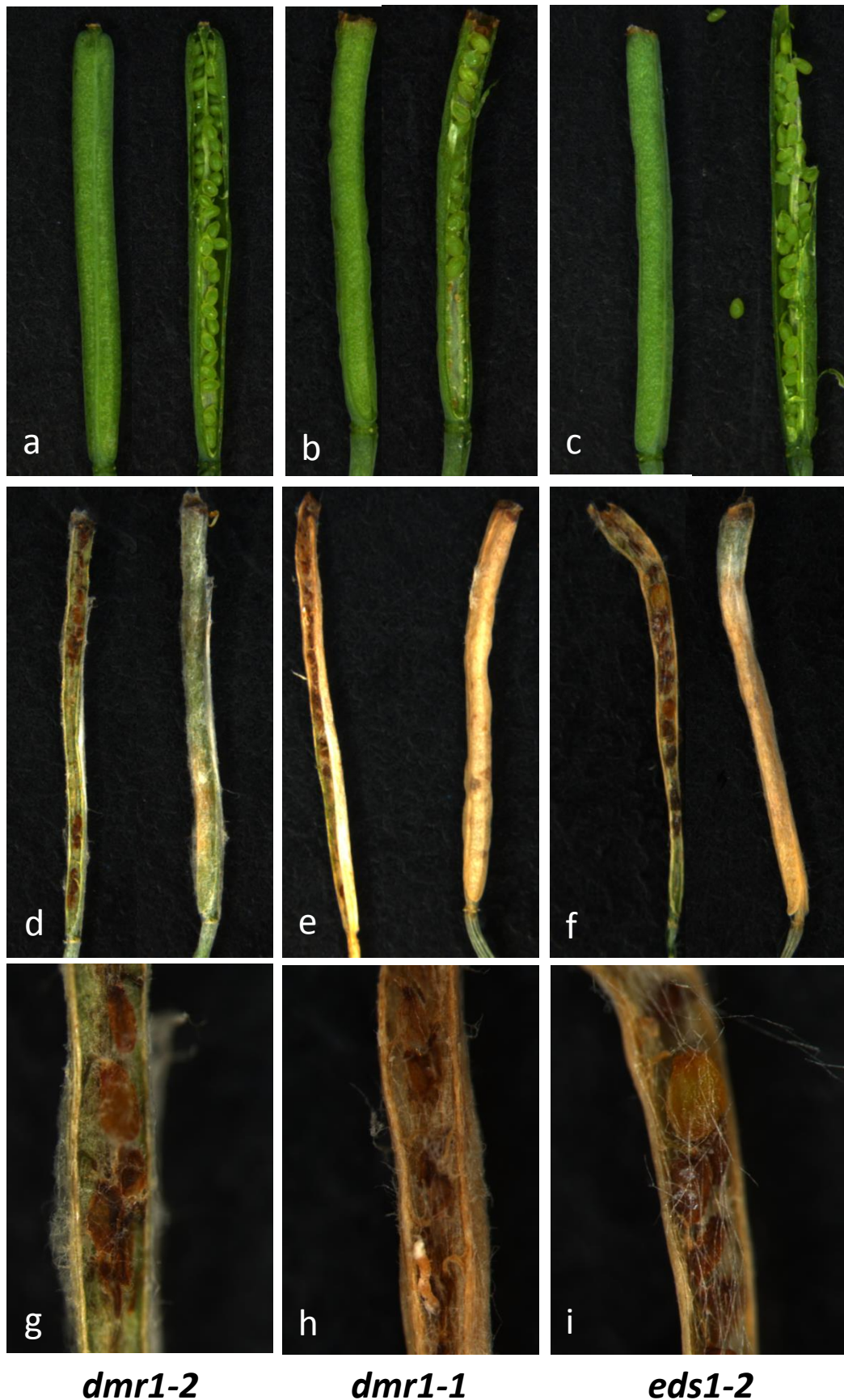


Figure 6.5: Images of individual siliques point inoculated at the cut tip. (a-c) Water inoculated controls show comparable development and seed set between *eds1-2* and *dmr1-1* and *dmr1-2* genotypes. (d-i) Comparable levels of *Fusarium culmorum* infection of *dmr1* mutant and *eds1-2* siliques 7 days post inoculation. In panels d through f, whole infected split (left) and intact (right) siliques are shown. In panels g through i are close-up images of infected seeds. Shown are representative images present in multiple biological replicates.

6.3.4 Homoserine accumulates in the siliques of *dmr1* mutant plants

Resistance of *dmr1* leaves to *H. arabidopsidis* infection was previously linked to elevated homoserine levels in 10 day old seedlings (van Damme *et al.*, 2009).

The amino acid composition of the siliques of three *dmr1* mutant alleles was therefore analysed and compared to *eds1-2* in order to identify whether homoserine also accumulates in *dmr1* siliques (Fig. 6.6). Homoserine was not detectable in *eds1-2* siliques, but was abundant in *dmr1* siliques (Fig. 6.6a). Homoserine levels were comparable between all three mutant alleles, but were higher on average in *dmr1-2* and *dmr1-3* siliques, which are resistant to *F. culmorum*, compared to *dmr1-1* siliques, which have wild type resistance levels. As previously observed in seedling tissue by van Damme and colleagues, mutation of homoserine kinase does not reduce levels of downstream amino acids (Fig. 6.6b-e). Threonine levels were elevated in *dmr1-2* siliques, while methionine was more abundant in *dmr1-1* siliques. These changed levels observed in siliques correlate well with the levels of these amino acids in young seedlings. Glycine (which can be synthesised from threonine) was more abundant in all *dmr1* siliques than in *eds1-2*. A highly abundant unidentified amino acid was also detected in *dmr1* samples but absent from *eds1-2* (Fig. 6.6f).

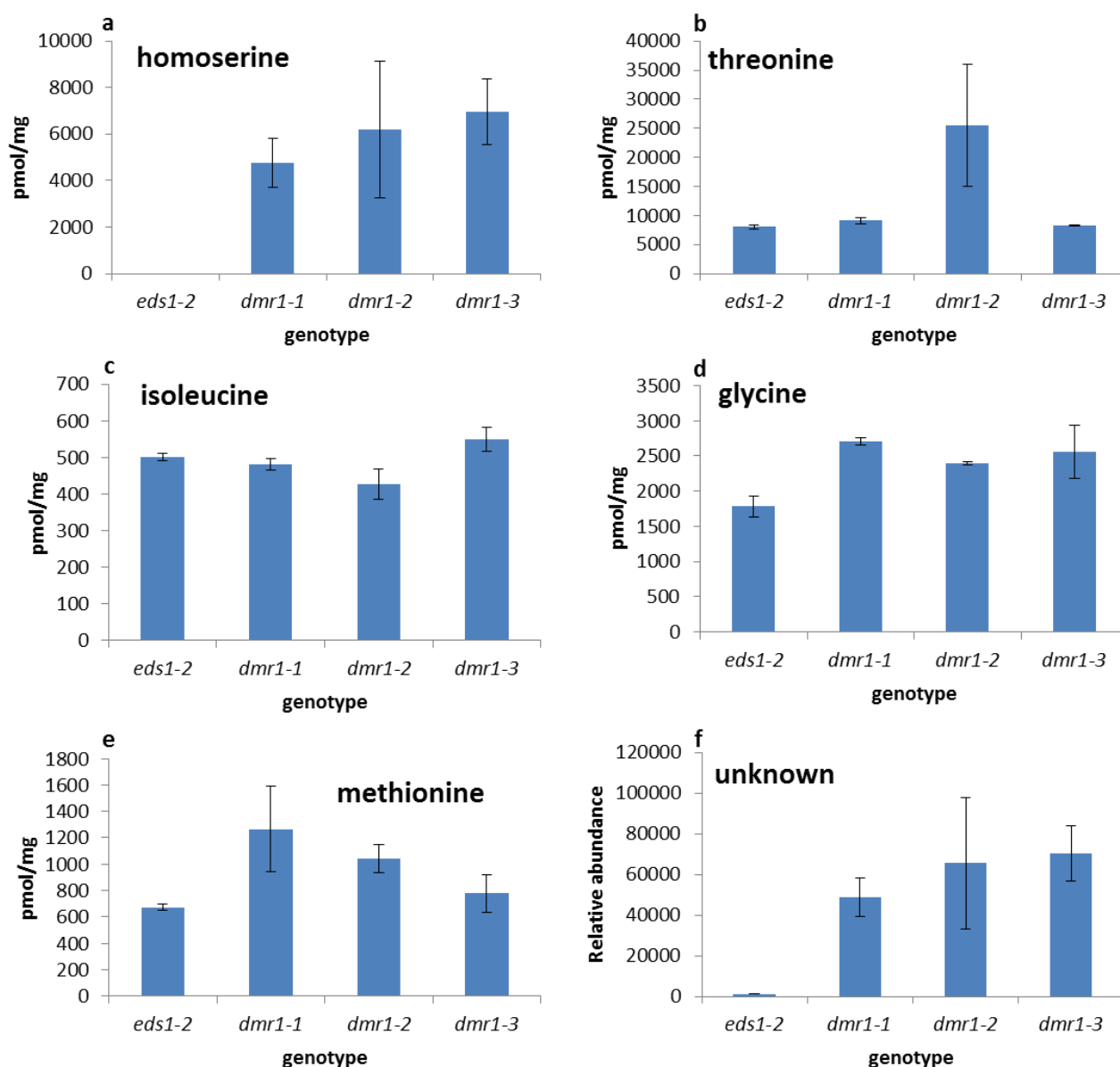


Figure 6.6: Silique amino acid composition of three *dmr1* mutant alleles. Gas chromatography mass spectroscopy (GCMS) was used to identify and quantify the amino acids present in *dmr1-1*, *dmr1-2* and *dmr1-3* compared to *eds1-2* in the absence of *Fusarium* infection. Homoserine (a) was not detectable in *eds1-2* siliques but was abundant in the siliques of the *dmr1* mutants. Despite the absence of a functional homoserine kinase in the *dmr1* mutants, levels of threonine (b) isoleucine (c) glycine (d) and methionine (e), downstream products of homoserine phosphorylation, were not reduced in the *dmr1* mutants compared to *eds1-2*. The level of an uncharacterised amino acid (f) was also elevated in all three *dmr1* mutants. Analysis was done on three independent biological silique samples per genotype. Bar = standard error.

6.3.5 Exogenous homoserine application reduces *F. culmorum* infection in Arabidopsis buds and siliques

Exogenous application of L-homoserine, but not D-homoserine, was previously shown to increase resistance in Arabidopsis and tomato to the obligate biotrophs *Hyaloperonospora arabidopsidis*, and *Oidium neolycopersici*, respectively, but homoserine did not inhibit spore germination or germling *in vitro* growth of either the oomycete or ascomycete pathogen (van Damme *et al.*, 2009, Huibers *et al.*, 2013). The effect of application of either enantiomer of homoserine on *in vitro* and *in planta* growth of *Fusarium* was therefore investigated. The effect of *in planta* threonine treatment on *Fusarium* growth was also tested, since threonine is elevated in some *dmr1* alleles and was previously shown to reduce *H. arabidopsidis* growth (Stuttman *et al.*, 2011).

No strong inhibitory effect of either homoserine isoform on *in vitro* growth was found for *F. culmorum* or *F. graminearum*, following 48h incubation in synthetic nutrient poor media supplemented with L- or D-homoserine at concentrations ranging from 0 to 80mM (Fig. 6.7).

To assess the *in planta* effects of amino acid treatment on *F. culmorum* growth, flowering Arabidopsis plants were sprayed with either 10mM L- or D-homoserine (LHS, DHS), L-threonine (THR) or water, concurrent with spray inoculation with *F. culmorum* at early flowering. Amino acid/water treatments were repeated daily for 5dpi. Significant differences in *F. culmorum* infection between treatments were found for unopened buds ($F_{3, 31}=41.38$, $p < 0.001$), open flowers ($F_{3, 31}=7.31$, $p < 0.001$), siliques ($F_{3, 31}=1.68$, $p < 0.001$) and rosette leaves ($F_{3, 31}=7.71$, $p < 0.001$). At 7dpi, LHS treated buds showed little or no infection, and infection of opened flowers was also reduced, compared to

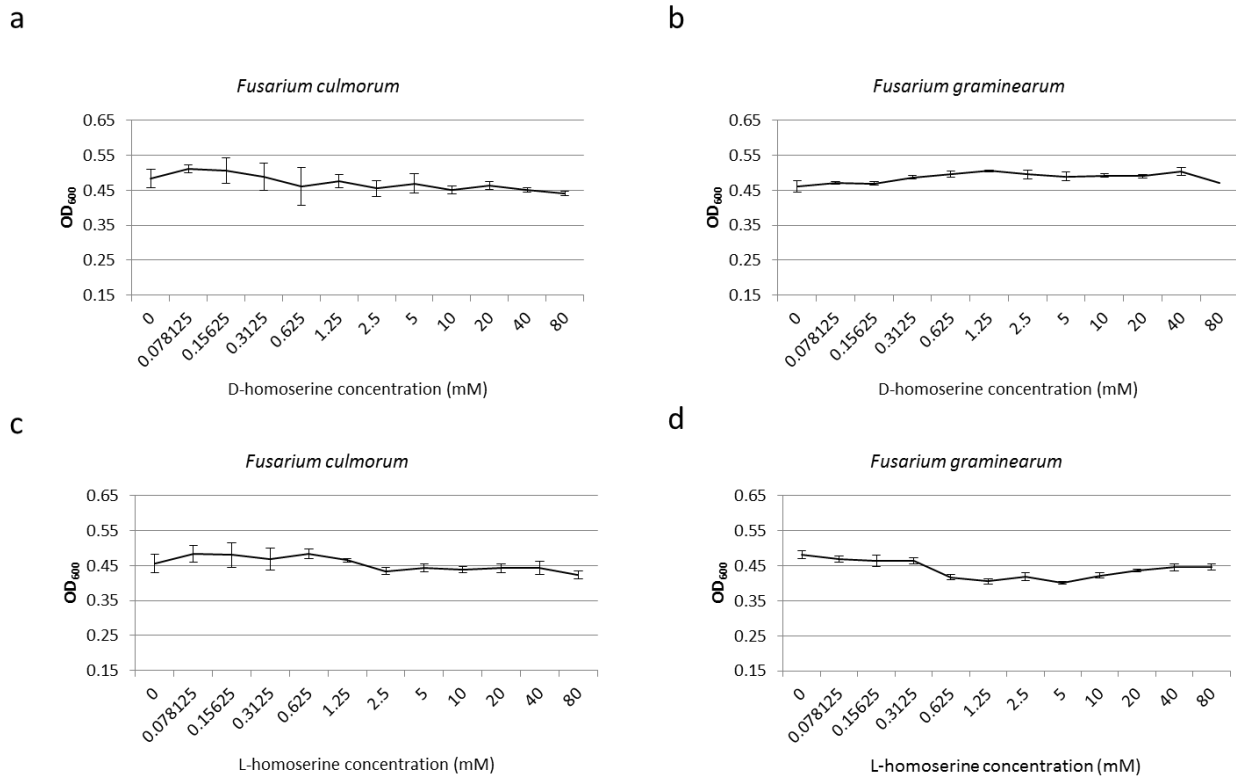


Figure 6.7: Homoserine does not inhibit *Fusarium* hyphal growth *in vitro*. Spores of either *F. culmorum* or *F. graminearum* were cultured for 2 days in synthetic nutrient poor media supplemented with (a, b) D-homoserine and (c, d) L-homoserine at concentrations ranging from 0 to 80mM. Graphs show the optical density at 600nm of fungal colonies after 2 days growth. The experiment was repeated with similar findings.

DHS and water treated control plants (Fig. 6.8a, b, c). Silique infection levels were slightly elevated in all amino acid treated plants compared to water controls in these experiments (Fig. 6.8d). Threonine treatment increased *F. culmorum* colonisation in both open flowers and rosette leaves (Fig. 6.8a and e).

Plants treated with threonine also exhibited leaf chlorosis and lesion formation in the absence of *F. culmorum* infection, indicating that threonine spray treatment at and above 10mM may induce a cell death response and /or premature senescence. This result was consistent in both *eds1-2* and wild type *Ler-0* plants (Fig. 6.9).

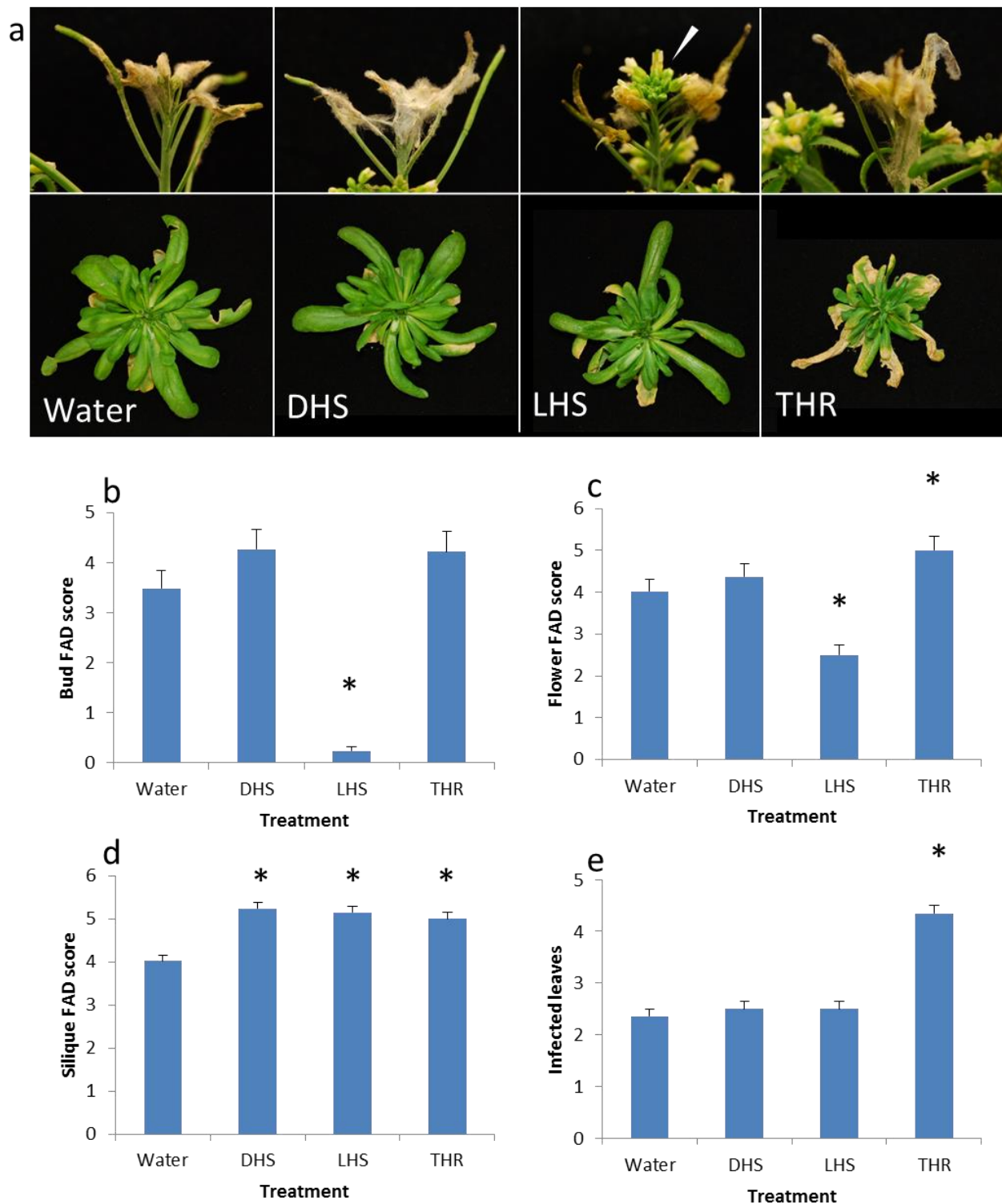


Figure 6.8: The effect of exogenous amino acid treatments on *Fusarium* susceptibility in *Arabidopsis* floral, silique and rosette leaf tissue. *Arabidopsis* plants of genotype *eds1-2* were sprayed at early flowering with either 10mM D-homoserine (DHS), L-homoserine (LHS), threonine (THR) or sterile water, co-incident with *F. culmorum*. Amino acid/water treatments were then repeated daily for 6 dpi. Disease was assessed at 7dpi. A) Images show infected apical inflorescences (upper panel) and rosette leaves (lower panel) – stem and floral tissue have been removed from rosettes. White arrow – green and opening buds present in LHS treated plants. Plants were scored for (b) bud disease, (c) open flower disease (d) silique disease and (e) infected rosette leaf number. Asterisks indicate statistically significant differences from H₂O treated plants (regression analysis followed by prediction of LSDs, $p < 0.05$, $n = 8$). Results are representative of two independent experiments.

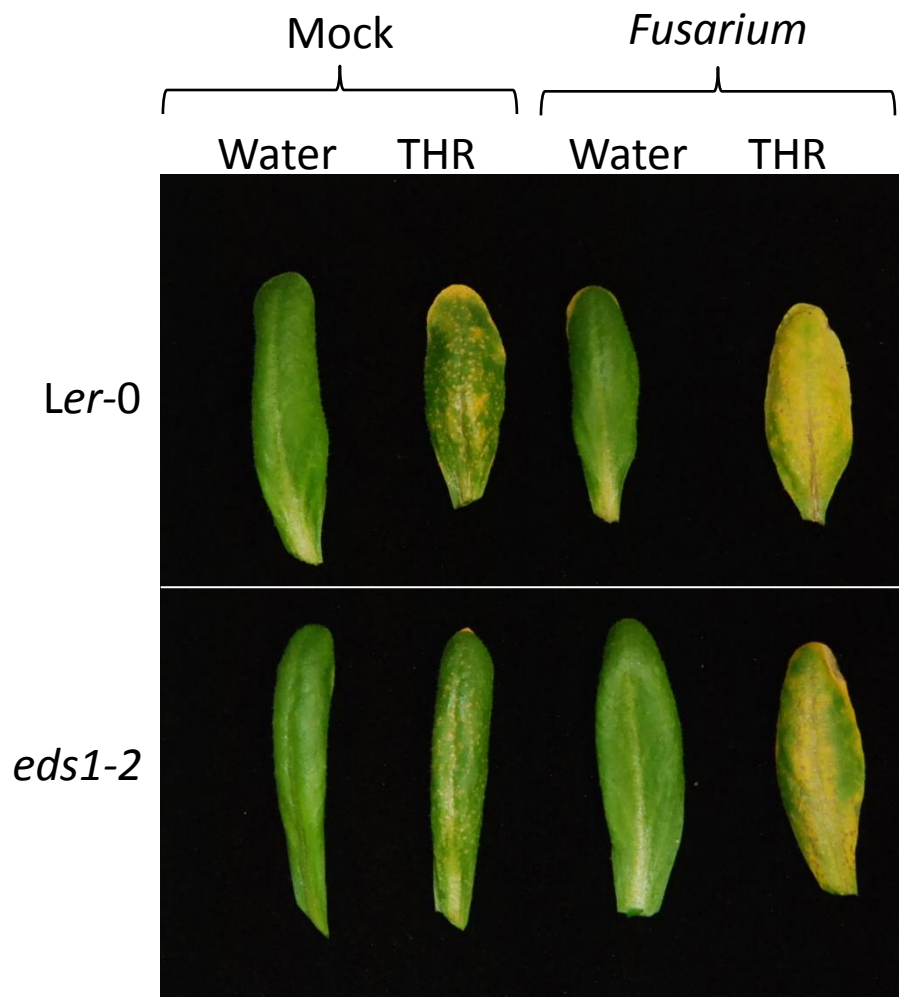


Figure 6.9: Threonine (THR) mediated chlorosis in rosette leaves of *Arabidopsis* genotypes *Ler-0* and *eds1-2*. Plants were sprayed with 10mM threonine or water daily for 5 days, first treatment coincident with *F. culmorum* or mock (water) spray inoculations. The effect of threonine was most pronounced in *Fusarium* inoculated leaves. Threonine from two different commercial suppliers was tested with identical outcomes.

The effect of D- and L-homoserine on *F. culmorum* infection of *eds1-2* siliques following single silique wound point inoculations was also analysed (Fig. 6.10). Siliques were droplet inoculated with water, DHS or LHS for 5 days following *F. culmorum* inoculation. There was a significant difference in *F. culmorum* infection development between treatments (ANOVA, $p = <0.001$). DHS treatment resulted in a modest reduction in *F. culmorum* growth along inoculated siliques compared to water treatment, while LHS treatment resulted in significantly less *Fusarium* growth than either water or DHS treatment, with most plants showing no externally visible infection. However, *F. culmorum* hyphae were present on and between seeds within some LHS treated siliques with externally uninfected pericarps (Fig. 6.11).

The effect of LHS treatment on *dmr1* mutants was also analysed using the spray treatment method (Fig. 6.12). We found that exogenous LHS application, compared to DHS application, conferred *F. culmorum* resistance in *dmr1-2* buds (which are not resistant) equivalent to that seen in LHS treated *eds1-2* buds (Fig. 6.12a). Furthermore, LHS treatment afforded a further increase in silique resistance in *dmr1-2* siliques, despite a high level of resistance already being conferred by the mutation. By contrast, LHS did not increase silique resistance in *eds1-2* (Fig. 6.12b, Fig. 6.8d).

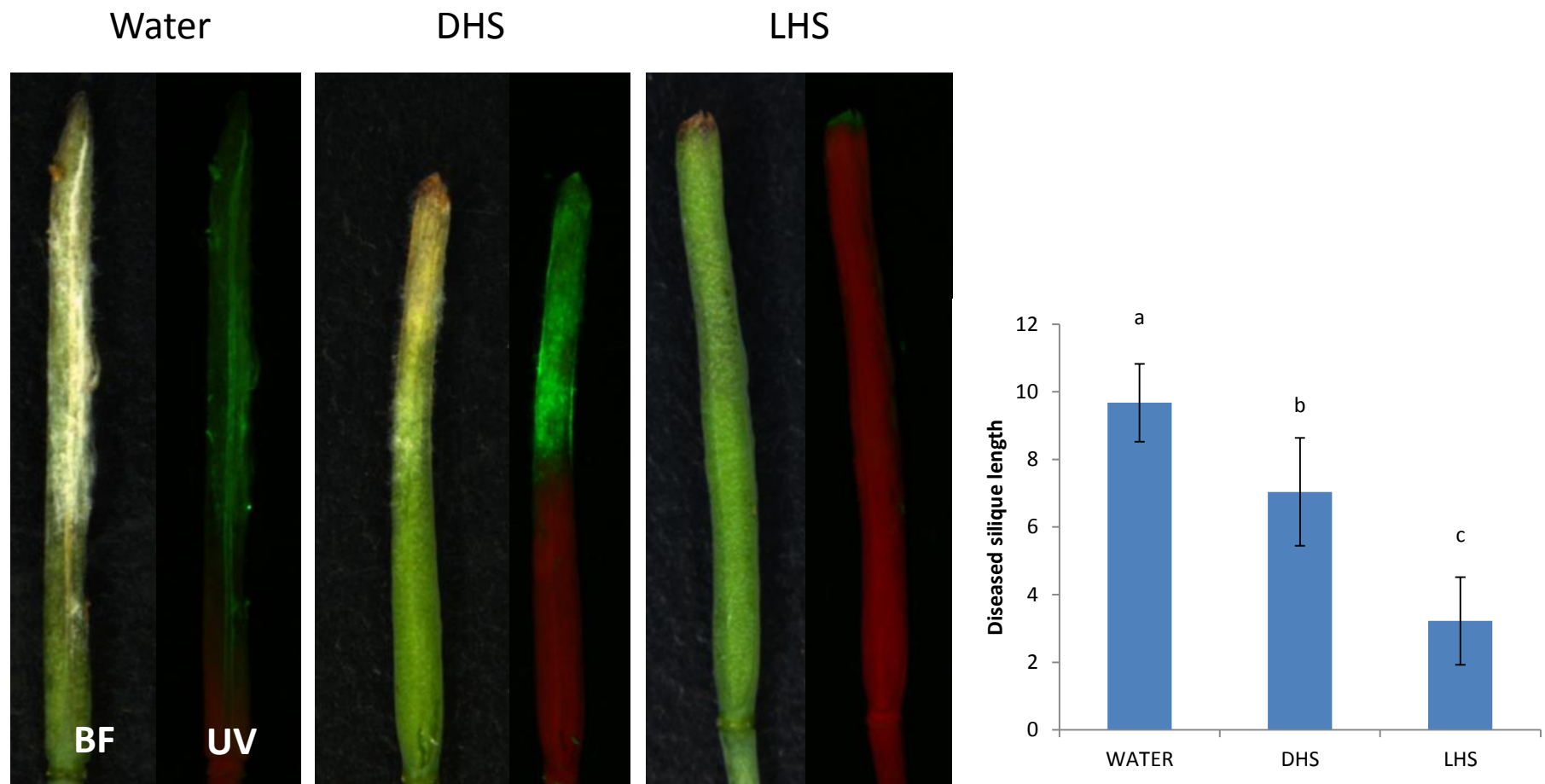


Figure 6.10: Homoserine treatment reduces *F. culmorum* growth in point inoculated *eds1-2* siliques. Tip-wounded *eds1-2* siliques were treated with 10mM L-homoserine (LHS), D-homoserine (DHS) or sterile water coincident with *F. culmorum* inoculation. Amino acid/water treatment was repeated for 5dpi. Images show infected siliques 8dpi under brightfield (BF) and UV light with a violet filter. Red fluorescence indicates healthy tissue, green fluorescence indicates infected tissue. The length of infection along three siliques per plant was assessed at 8dpi. Different letters indicate statistically significant differences between treatments (analysis of variance followed by prediction of LSDs, $p < 0.01$, $n = 12$). Data were pooled from three independent experiments.



Figure 6.11: Homoserine treatment reduces *F. culmorum* growth in point inoculated *eds1-2* siliques. Tip-wounded *eds1-2* siliques were treated with 10mM L-homoserine (LHS), D-homoserine (DHS) or sterile water coincident with *F. culmorum* inoculation. Amino acid/water treatment was repeated for 5dpi. Images show opened silique sections at 8dpi. Tissue necrosis and fungal growth is evident in the pericarp (P) and seed (S) of water and D-homoserine (DHS) treated siliques. L-homoserine (LHS) treated siliques have predominantly uninfected pericarps, but some externally uninfected LHS treated siliques revealed, when opened, the presence of fungal colonisation within the silique (far right).

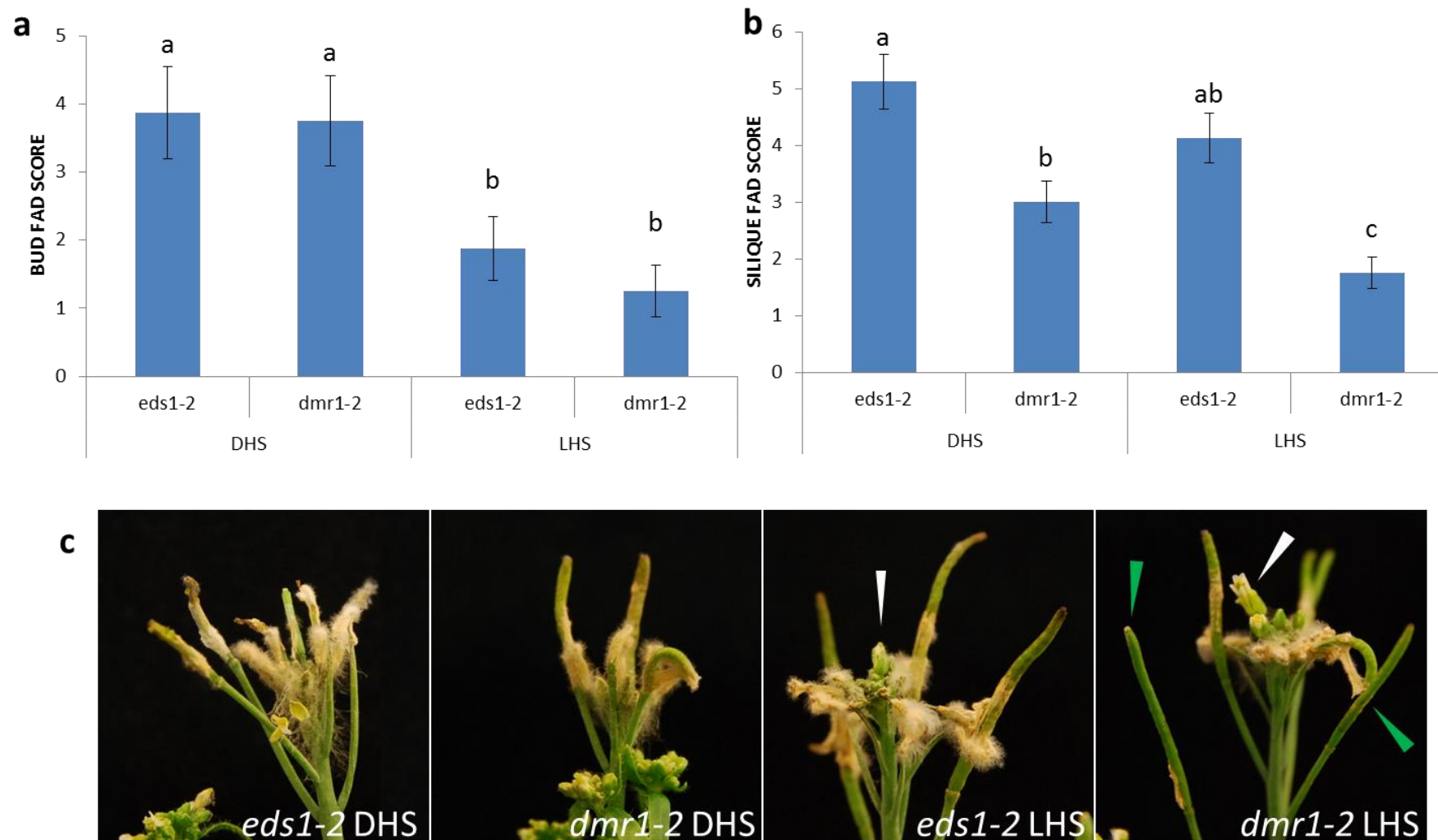


Figure 6.12: Treatment of *dmr1-2* with LHS further reduces *Fusarium* silique susceptibility compared to DHS treated controls. Arabidopsis plants of genotype *eds1-2* and *dmr1-2* were treated at early flowering with 10mM L-homoserine (LHS) or D-homoserine (DHS) as a control, 1-7dpi with *F. culmorum*. Bud (a) and silique (b) disease levels were assessed at 7dpi. Different letters indicate statistically significant differences between treatments (regression analysis followed by prediction of LSDs, $p < 0.05$, $n=8$). C) Images show inoculated inflorescences 7dpi. LHS treatment reduced bud disease levels in both *eds1-2* and *dmr1-2*. Silique disease levels were reduced significantly more by LHS treatment in *dmr1-2* plants. White arrow – opening uninfected buds. Green arrow – green uninfected siliques. Analysis based on pooled data from two independent experiments.

6.3.6 Mutation of DMR1 affects plant growth and senescence

During the growth of the experimental plants, *dmr1-2* plants appeared to be slightly smaller in size than *eds1-2* plants. Therefore, the rosette diameter and leaf number were measured and compared between 5-week old plants of genotypes *dmr1-1*, *dmr1-2* and *eds1-2*. The quantification of growth confirmed that the *dmr1-2* plants have approximately ~ 25% smaller rosettes on average than *eds1-2* (Fig. 6.13a & b), but that leaf number is similar between genotypes (Fig 6.13c). This supports the recent findings by Huibers *et al.* (2013) that some *Arabidopsis dmr1* mutants have reduced fresh weight compared to *eds1-2*. Leaf senescence between genotypes was also assessed and found to be delayed in both *dmr1-1* and *dmr1-2* compared to *eds1-2* (Fig 6.13d & e).

Silique number were compared between genotypes at 7, 11 and 14 days post flowering (corresponding to assessment of infected plants at 7, 11 and 14dpi) to ensure that the increased number of uninfected siliques observed in *dmr1-2* was not due to more siliques being produced in this genotype. No difference was found between genotypes at any of the time points assessed (Fig 6.13f). There was no evidence of increased silique number in the other *dmr1* alleles investigated in this study, although this was not formally assessed.

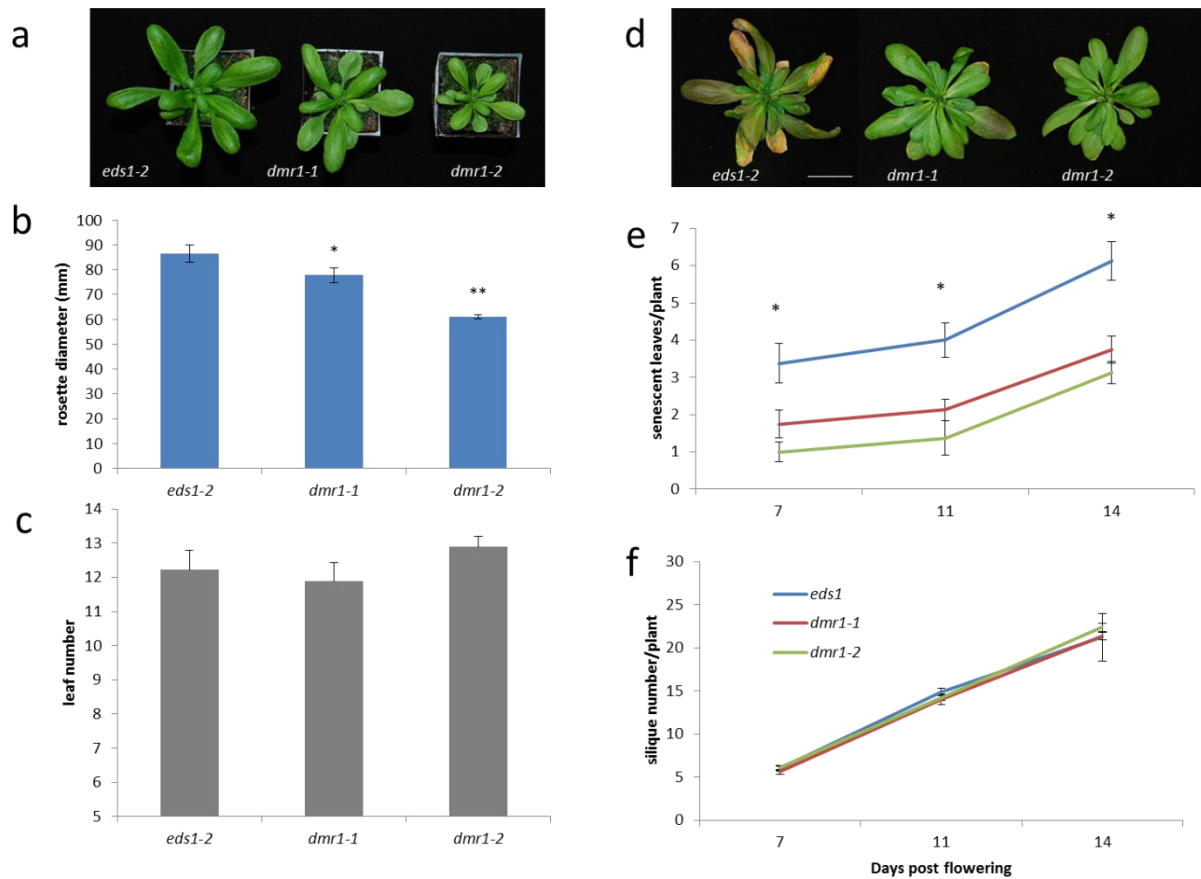


Figure 6.13: Differences in developmental morphology and senescence between the *dmr1-1* and *dmr1-2* mutant alleles and *eds1-2*. (a, b) Rosette diameter is reduced in 5-week old plants of genotype *dmr1-2* compared to *eds1-2*. (c) Leaf number is comparable between genotypes. (d, e) Leaf senescence is delayed in both *dmr1-1* and *dmr1-2*. Panel d shows the appearance of the rosettes of flowering plants at 14 days post flowering. (f) Silique number was equivalent between all genotypes throughout seed set. These phenotypes were observed across multiple experimental replicates. Asterisks indicate significant difference from *eds1-2*. * $p < 0.05$, ** $p < 0.01$ (b – ANOVA, e – Regression analysis).

6.3.7 Exogenous application of L-homoserine does not consistently significantly affect *Fusarium* colonisation of wheat ears

The effect of exogenous L-homoserine application on *Fusarium* infection in wheat was assessed by treating *F. graminearum* infected wheat spikelets with L-homoserine, D-homoserine or sterile water daily for 7dpi. The fully *Fusarium* susceptible spring wheat cultivar Apogee was used for these experiments. The number of bleached spikelets and bent awns (which precedes spikelet bleaching in infected spikelets), along with grain number and weight, were assessed at 10dpi (Fig. 6.14, Fig. 6.15). The experiment was then independently replicated. Fewer mean bleached spikelets and bent awns and higher grain number and weight were observed in L-homoserine treated plants compared to the other treatments. However, only the reduced number of bleached spikelets was statistically significant, and only in the first experimental replicate (Fig. 6.14) ($p=0.03$).

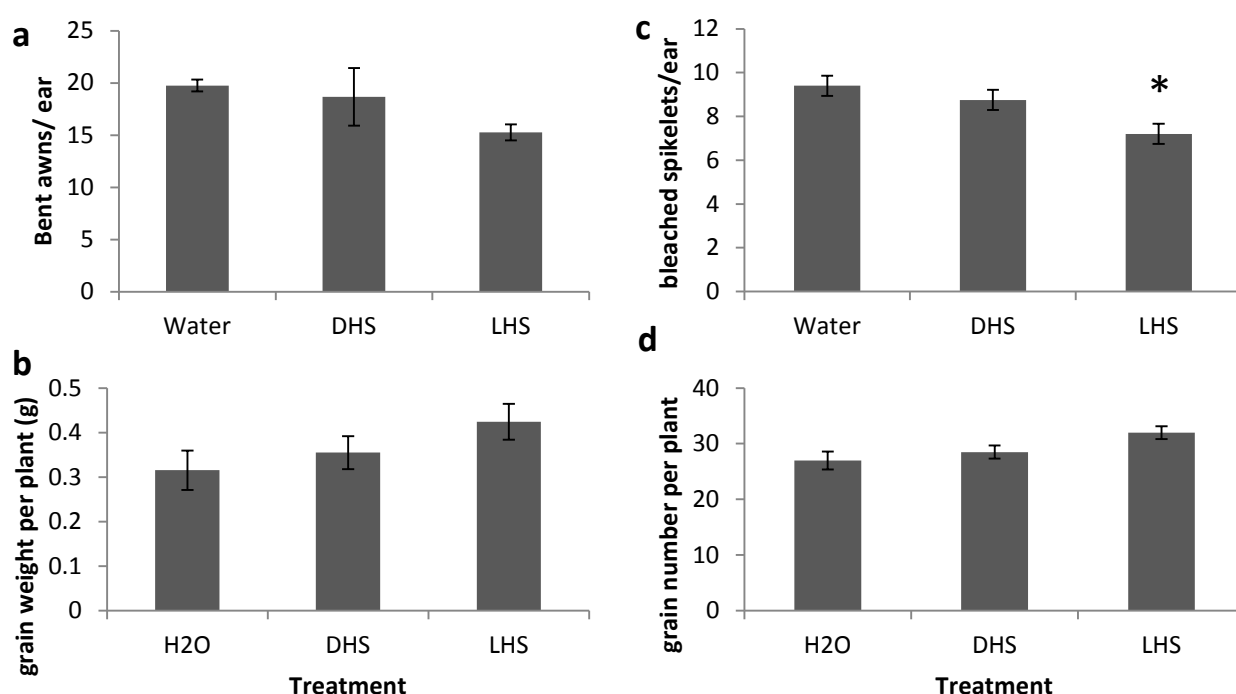


Figure 6.14: Effect of L-homoserine application on *Fusarium* infection of wheat. Spikes of wheat cultivar Apogee were point inoculated with *F. graminearum* and then treated with either L-homoserine (LHS) D-homoserine (DHS) or water for 7 days. The number of bent awns (a) and bleached spikelets (b) along with grain weight (c) and number (d) per plant were assessed at 10dpi. (*) The number of bleached spikelets was significantly lower in LHS treated plants (ANOVA $p=0.03$, followed by LSD calculation at $p=0.05$). No statistically significant difference between treatments was found for other parameters (ANOVA, $p > 0.05$). Bar = standard error

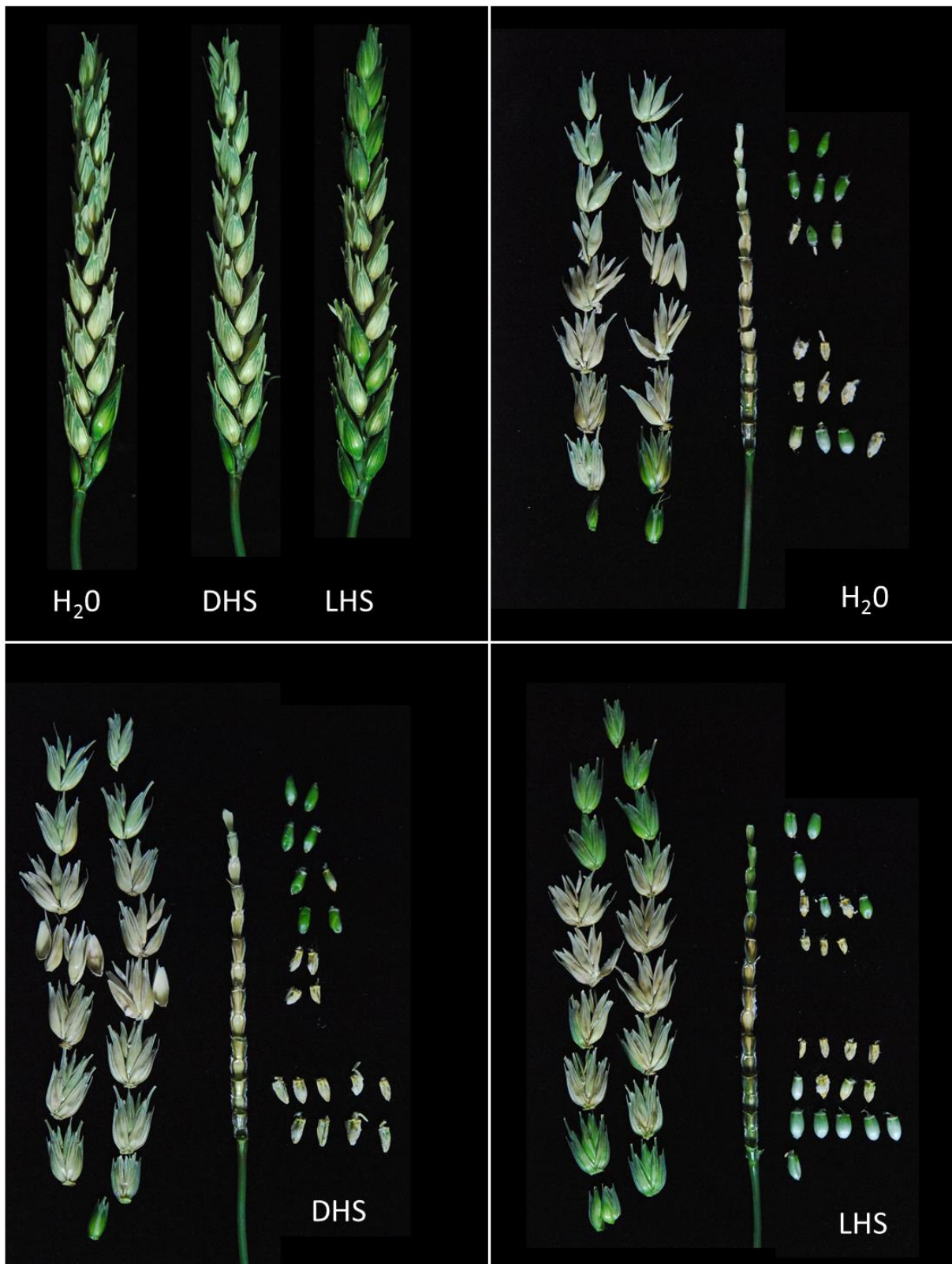


Figure 6.15: Effect of L-homoserine application on symptomatic *Fusarium* infection of wheat. Images taken 10dpi with *F. graminearum* and subsequent treatment with either L-homoserine (LHS), D-homoserine (DHS) or sterile H₂O.

6.4 Discussion

In order to identify additional host genes controlling the outcome of the *Fusarium*–*Arabidopsis* interaction, as well as highlight components of defence signalling which are conserved in response to different pathogen types, a number of recessively inherited *downy mildew resistant* (*dmr*) mutants were screened for altered susceptibility to the fungal pathogens *F. culmorum* and *F. graminearum*, which infect floral tissue in cereals and *Arabidopsis*. It was found that multiple loss of function mutant alleles of the *Arabidopsis* *HOMOSERINE KINASE* gene *DMR1* have increased resistance to *Fusarium* infection in silique and/or leaf tissue: Siliques of *dmr1-2*, *dmr1-3*, *dmr1-4* and *dmr1-6*, and leaves of *dmr1-1*, *dmr1-2*, *dmr1-3* and *dmr1-4*, are more resistant to *F. culmorum* infection. Genotypes *dmr1-1* and *dmr1-2* were also tested for altered *F. graminearum* susceptibility and found to have increased leaf or silique resistance, respectively.

These results indicate that there is potentially a common mechanism of susceptibility occurring in response to infection by both the downy mildew oomycete pathogen *H. arabidopsidis*, which is a leaf adapted obligate biotroph, and fungal hemi-biotrophic *Fusarium* species which are floral adapted. Mutation of *AtDMR1* and its tomato ortholog *SIDMR1* has also recently been shown to increase resistance to the obligate biotrophic fungal mildew *Oidium neolycopersici*, but has not been found to alter resistance to any other pathogens assessed, including the facultative biotrophic bacterium *Pseudomonas syringae* (van Damme *et al.*, 2009, Huibers *et al.*, 2013).

6.4.1 Tissue specific resistance caused by *DMR1* mutation and homoserine application

Mutation of *DMR1* was not found to alter susceptibility to infection of unopened buds and young flowers, despite affecting both leaf and silique infection.

Analysis of Arabidopsis *DMR1* expression using GENEVESTIGATOR (Hruz *et al.*, 2008) shows that *DMR1* is expressed at lower levels in some floral tissues than it is in vegetative tissue, namely in the stamens, anthers, stigma and sepals (Fig. 6.16a). Since homoserine kinase activity has been shown to be driven by homoserine accumulation (Lee *et al.*, 2005), it may be that these tissues do not produce high levels of homoserine and are therefore unaffected by decreased *DMR1* activity. Susceptibility of sepal and male reproductive tissue during early floral development pre-fertilisation may result in loss of the flower, whereas infection of these tissues post-pollination would have little effect on the siliques of resistant *dmr1* plants, as this tissue is shed during silique development.

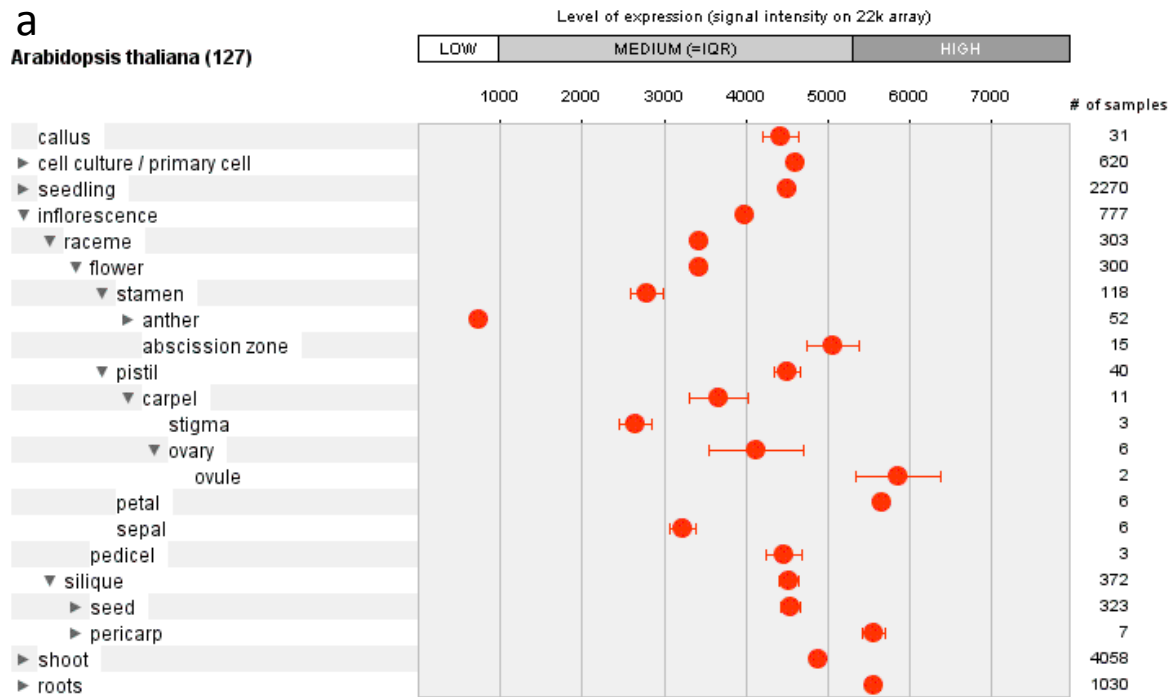
Since *dmr1* induced resistance to *H. arabidopsidis* is proposed to be mediated by homoserine accumulation and can be mimicked by exogenous L-homoserine application in wild type plants, we investigated the effects of homoserine application on Fusarium growth *in vitro* and *in planta*. Treatment of *eds1-2* plants with L-homoserine (LHS) following spray inoculation with *F. culmorum* resulted in significantly decreased bud and flower colonisation by the fungus. This contrasts with the phenotype of *dmr1* mutants, which have increased silique and leaf, but not floral, resistance. As previously discussed, Genevestigator analysis suggests that some floral organs may have lower HSK expression than other plant tissues (Fig. 6.16). This may result in longer persistence of the applied homoserine in buds than in siliques and other

tissues, resulting in reduced fungal growth compared to other tissues. However, it was found that more direct application of both the fungus and the LHS onto the tips of wounded siliques resulted in decreased fungal growth along the silique compared to water and DHS treated controls.

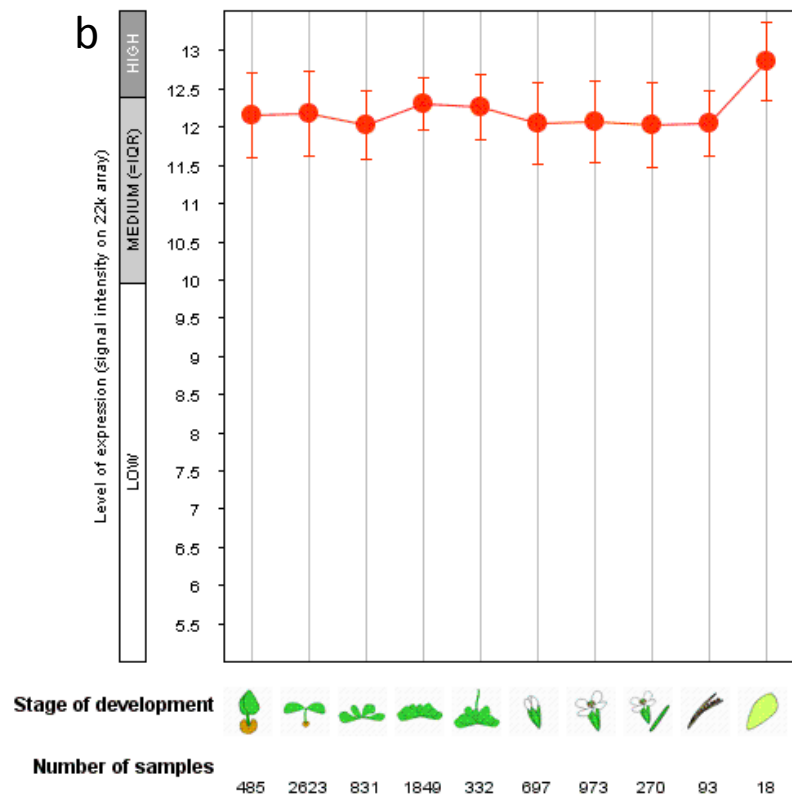
● AT2G17265

a

Arabidopsis thaliana (127)



b



created with GENEVESTIGATOR

Figure 6.16: GENEVESTIGATOR analysis of the expression profile of *Arabidopsis DMR1*. A) Tissue specific expression levels across different floral tissues. B) Development stage specific expression levels.

6.4.2 Investigation of homoserine mediated resistance in wheat

The effect of exogenous homoserine application on *F. graminearum* infection of the wheat cultivar Apogee was also assessed. While there was evidence of reduced infection following L-homoserine treatment, this was not statistically significant across multiple experimental replicates. This may be due to lack of uptake of homoserine into the wheat ear tissue, or to rapid metabolism of homoserine by the wheat homoserine kinase, negating its effect on *Fusarium* colonisation. Planned future work therefore includes Virus Induced Gene Silencing (VIGS) of the wheat homoserine kinase gene using Barley Stripe Mosaic Virus (BSMV) (Lee *et al.*, 2012, Lee *et al.*, 2013) and investigation of the effect of this silencing on homoserine accumulation and *Fusarium* susceptibility. Alternatively a stable RNAi construct could be used to silence the wheat homoserine kinase gene. Or, the effect of homoserine on the infection of ears of a semi-resistance wheat cultivar could be explored.

6.4.3 The effect of exogenous threonine application

This study also presents the novel finding that exogenous application of threonine induces host cell death in Arabidopsis leaves and increased *F. culmorum* colonisation. This raises further questions about the effects of amino acid metabolism on plant defence against different pathogen species and lifestyles. van Damme and colleagues did not find an effect of exogenous threonine application on *H. arabidopsidis* susceptibility when amino acids were applied by vacuum infiltration. However, Stuttmann *et al.* (2011) found that spray application of 1-5mM threonine resulted in decreased *H. arabidopsidis* sporulation in Ler-0 *eds1-2* plants. These contrasting outcomes are interesting. *H. arabidopsidis* is a classic obligate biotroph and would therefore be sensitive to any host induced cell death which would limit this pathogen's access to living

tissue. By contrast *Fusarium* has been shown to have a switching *in planta* lifestyle with host cell death an integral feature of the later disease formation process (Brown *et al.*, 2010, Desmond *et al.*, 2008, Thaler *et al.*, 2004). Cereal infecting *Fusaria* are also able to saprophytically colonise dead plant tissue. Threonine mediated chlorosis may therefore facilitate *Fusarium* colonisation while preventing growth of obligate biotrophic pathogens.

6.4.4 Delayed senescence in *dmr1* mutants

Related to this is the finding that mutation of DMR1 results in delayed senescence. Analysis of DMR1 expression during plant development using GENEVESTIGATOR (Hruz *et al.*, 2008) shows that expression is fairly static throughout plant development but increases during senescence (Fig. 6.16b). This suggests that DMR1 function could have a role in programmed cell death and senescence. The delayed DMR1 dependent cell death in the *dmr1* mutants may restrict *Fusarium* disease progression and prevent its successful exploitation of host cell death (Thaler *et al.*, 2008). For pathogens with a hemibiotrophic life style strategy, delayed cell death could prevent full tissue exploitation and the gaining of additional nutrition from the cellular debris. However, the delayed cell death may not be the underlying cause of the enhanced resistance. For example, the manner in which delayed cell death might help protect plants against obligate biotrophic pathogens such as *H. arabidopsidis* is not clear. It is formally possible that the normal amino acid ratios found in healthy *Arabidopsis* tissue are modified in the *dmr1* mutants and this alters the efficiency of nutrient acquisition via the haustoria interfaces in obligate biotrophic interactions as well as altering the switching lifestyle of hemibiotrophic pathogens. In this regard, the formal identification of the novel

accumulating amino acid in the three *dmr* mutants, but not *eds1-2* (Figure 6.6) remains a priority.

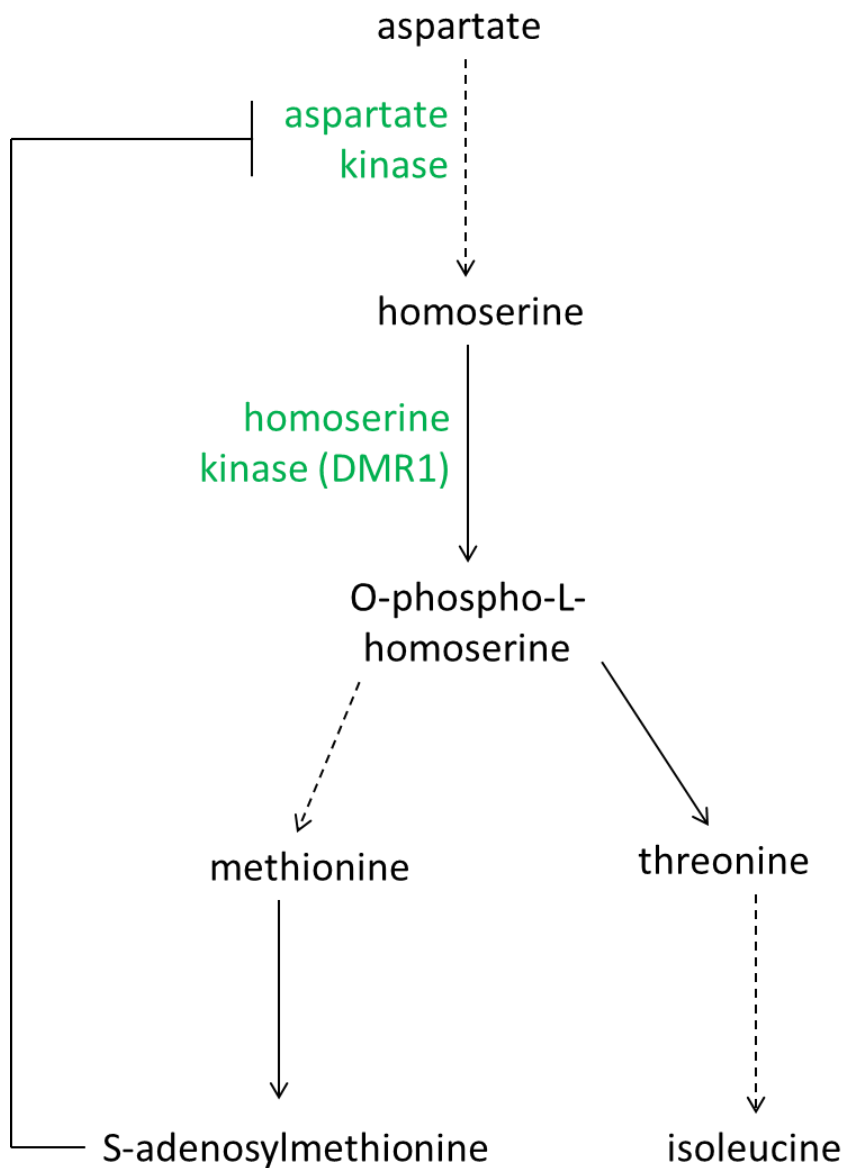


Figure 6.17: Scheme showing biosynthesis of homoserine derived amino acids in Arabidopsis. Enzymes are shown in green. Dashed arrows indicate synthesis via intermediates.

6.4.5 Synthesis of downstream amino acids in *dmr1* mutants

It was also found both in this study and that of van Damme *et al.* (2009), that *dmr1* plants have wild type or elevated levels of the amino acids methionine, threonine and isoleucine, in both foliar and silique tissue (Fig. 6.6). These amino

acids are understood to be synthesised directly via the activity of homoserine kinase (Fig. 6.17). Their abundance in plants with severely reduced homoserine kinase function therefore challenges the current understanding of amino acid biosynthetic pathways. It may be the case that these amino acids are being synthesised via alternative, currently unidentified pathways in the *dmr1* mutants, which are independent of homoserine kinase. Alternatively, mutated homoserine kinase may retain some residual function: Homoserine is synthesised via the activity of Arabidopsis aspartate kinases, which are negatively regulated by accumulation of S-adenosylmethionine (SAM), synthesised from methionine (Curien *et al.*, 2005, Curien *et al.*, 2007). Reduced methionine biosynthesis may therefore result in increased aspartate kinase activity, shunting of more homoserine into the pathway. Some of the accumulating homoserine may then be phosphorylated by the mutated homoserine kinase, restoring equilibrium in the pathway. However, no changes were observed in aspartate levels between wild type and *dmr1* mutant plants. Comparison of aspartate kinase expression and activity between genotypes would be needed to test this hypothesis.

6.4.6 The effect of *dmr1* mutation on plant growth

Huibers *et al.* (2013) found a correlation between reduced Arabidopsis plant fresh weight in different *dmr1* mutant alleles and the level of resistance conferred to *O. neolycopersici*. The authors concluded that it might be difficult to obtain *dmr1* alleles in crop species which conferred enhanced resistance to this pathogen in the absence of a fitness cost. While the *dmr1-3* mutant allele did not confer a significant growth penalty, likewise this mutant did not confer resistance to *O. neolycopersici*. However, in the current study, *dmr1-3* conferred resistance to *F. culmorum* in both the leaf and silique tissue of Arabidopsis,

although the leaf resistance phenotype was not as strong as in other alleles.

Investigation into the effects of homoserine kinase disruption in FEB-susceptible cereal crops is therefore warranted.

6.4.7 The role of homoserine kinase in human-pathogenic fungi

As shown in Figure 6.7, homoserine did not inhibit *Fusarium* growth *in vitro*, and mutation of homoserine kinase in plants does not greatly impact plant development and morphology. This is in contrast to findings related to homoserine kinase function in human pathogens: Kingsbury and McCusker (2008, 2010a, b) investigated the importance of homoserine kinase for growth and infection of human fungal pathogens in order to explore its potential as an antifungal drug target. It was found that homoserine kinase is essential for growth of *Cryptococcus neoformans*; mutation of the *C. neoformans* homoserine kinase was found to be lethal, and associated with depletion of the downstream amino acids threonine and methionine. In addition, reduced virulence but not lethality was associated with homoserine kinase mutation in *Saccharomyces cerevisiae* and *Candida albicans*. This was attributed to the toxicity of accumulating homoserine rather than threonine auxotrophy. It would therefore be interesting to test the effect of mutation of the *Fusarium* homoserine kinase on growth and pathogenicity.

6.4.8 Possible direct effects of homoserine accumulation on *Fusarium* pathogenicity

Homoserine accumulation does not affect *Fusarium* growth *in vitro*, but does affect the ability of the fungus to colonise Arabidopsis tissue. While this could be due to the effects of homoserine on the plant's defence response, it is also possible that homoserine directly affects the virulence of the fungus. For example, the requirement of the fungus to metabolise excess homoserine could

deplete energy or resources required to secrete virulence factors such as cell wall degrading enzymes and/or effectors. Alternatively, homoserine may act as a signal which regulates *Fusarium* gene expression, reducing pathogenicity.

However, homoserine has been shown to have the opposite effect on *Fusarium solani*, which is a pathogen of pea plants: Pea plants naturally accumulate high levels of homoserine, and this has been identified as the signal which triggers the expression of the *F. solani* gene *PeID*, which is essential for pathogenicity and is only expressed *in planta* (Yang *et al.*, 2005). It would be interesting to investigate whether *F. graminearum* or *F. culmorum* have *peID* homologues, and if so, whether their expression is affected by homoserine. The fact that homoserine level acts as an indicator of a plant species' suitability to be a host for other plant-pathogenic fungal species may also be important. It could be that efforts to increase crop resistance to one set of pathogens via homoserine kinase silencing might lead to novel infections by pathogens for which the crop was previously not a suitable host.

6.4.9 Conclusions

This study has identified that a series of mutations in the Arabidopsis homoserine kinase gene *DMR1* confers resistance in both vegetative and reproductive plant tissue to the primary causal agents of cereal FEB disease, a source of crop yield losses and grain contamination. Siliques of the *dmr1* mutants accumulate homoserine, and exogenous application of L-homoserine confers resistance to the floral and silique tissues of both mutant *dmr1* and wild type *DMR1* plants. These findings offer the possibility of developing a novel source of resistance to an economically important floral crop disease for which few other resistance mechanisms exist. However, application of these findings could be limited by the effect of homoserine kinase mutation on plant growth,

and the role of homoserine in pathogenicity of other plant-infecting fungi.

Further work will use virus induced gene silencing of the wheat *DMR1* ortholog to explore the potential of homoserine in *Fusarium* resistance in wheat.

However, the mechanism by which homoserine accumulation in plant tissue mediates resistance is still not fully understood, and may be key to fully exploiting *dmr1* based resistance which has the potential for use in multiple crop species.

7 Additional Arabidopsis mutant/transgenic lines screened for altered *Fusarium* susceptibility

7.1 Introduction

The previous chapter examined the effect of the *downy mildew resistant* mutations on Arabidopsis susceptibility to *F. culmorum*. This chapter examines the susceptibility of a further eight mutant or transgenic Arabidopsis lines to *Fusarium* infection following spray and/or silique wound inoculation. The rationale for investigation of each mutant varied but was largely based on their susceptibility to two or more other Arabidopsis pathogens, and their existence in an accession which is not Col-0, due to low floral infection levels in this accession following spray inoculation. The rationale for the selection of each of the eight lines is described below.

7.1.1 Mutations affecting oxidative burst mediated defence signalling in accessions Ws-2 and Ws-0

The oxidative burst, characterised by rapid release by plant cells of reactive oxygen species (ROS) such as superoxide anions and H₂O₂, is one of the first steps in the plant defence response following pathogen recognition. ROS contribute to plant defence by direct oxidative damage to the invading pathogen, along with their role in cell wall strengthening and as signalling molecules triggering downstream defence responses (O'Brien *et al.*, 2012, Lamb and Dixon, 1997, Torres, 2006, Vellosillo *et al.*, 2010).

Much research has focussed on the role in plant defence of ROS generated via the activity of NADPH oxidases (Torres, 2001, Torres *et al.*, 2005). This has included the identification of *OXI1* (*OXIDATIVE BURST INDUCIBLE 1*). *OXI1* encodes a serine-threonine protein kinase which is induced by NADPH mediated H₂O₂ production, and required for downstream defence responses.

Both knock-out mutation and overexpression of *OXI1* result in increased susceptibility to the biotrophic oomycete pathogen, *Hyaloperonospora arabidopsidis*, and the hemibiotrophic bacteria *Pseudomonas syringae* but not the necrotrophic fungal pathogen *Botrytis cinerea* (Rentel *et al.*, 2004, Petersen *et al.*, 2009).

Apoplastic peroxidases have also been identified as a source of ROS required for plant defence. Bindschedler *et al.* (2006) found that plants exhibit an extracellular peroxidase mediated oxidative burst in response to *Fusarium oxysporum* cell wall extract. Antisense expression of the French Bean peroxidase gene *FBP1* in Arabidopsis silenced the peroxidase genes *PRX33* and *PRX34*, resulting in broad spectrum pathogen susceptibility (Bindschedler *et al.*, 2006). Mutants in these peroxidases show reduced callose deposition in response to pathogen attack and decreased resistance to *P. syringae* (Daudi *et al.*, 2012). The importance of both NADPH – and peroxidase – mediated oxidative burst was evaluated by testing the mutants *oxi1*, *prx33* and *prx33/prx34* (*prx33* + *PRX34* RNAi, hereafter referred to as *prx34*) for altered susceptibility to *F. culmorum* infection following spray and silique point wound inoculations. The *oxi1* mutant is in the Ws-2 background, while *prx33* and *prx34* are in the Ws-0 background.

7.1.2 Mutations in disease *resistance* genes required for defence against multiple pathogens in accession Ws-0

The original gene for gene model postulates that each disease resistance (*R*) gene recognises the presence of a single avirulence gene (Flor, 1974).

However, it has been demonstrated that in many cases, a single *R* gene can recognise multiple avirulence proteins (effectors) from the same or unrelated pathogens (a so-called ‘gene-for-genes’ interaction), indicating a role for *R*

genes in broad spectrum resistance (Bisgrove *et al.*, 1994, Narusaka *et al.*, 2009, Jones and Dangl, 2006, Nombela *et al.*, 2003).

Two such *R* genes are *RRS1* and *RPS4*, which are adjacent in the Arabidopsis genome and encode NB-LRR proteins conferring resistance to the bacterial pathogens *Ralstonia solanacearum* expressing *PopP2* and *P. syringae* pv. *tomato* expressing *avrRps4* (Hinsch and Staskawicz, 1996, Gassmann *et al.*, 1999, Deslandes *et al.*, 2002, Deslandes *et al.*, 2003). The genome of Arabidopsis accession Ws-0 encodes alleles of these *R* genes which confer resistance to the hemi-biotrophic fungal pathogen *Colletotrichum higginsianum*. Mutations in either one of these genes increase susceptibility to *C. higginsianum*, along with *R. solanacearum* and *P. syringae* expressing *avrRps4* (Narusaka *et al.*, 2009). This indicates that both of these genes are required for recognition of effectors from three different pathogens with divergent infection strategies. The effect of mutation of these genes on *F. culmorum* susceptibility was therefore assessed.

It is now understood that *RRS1* and *RPS4* form a heterodimeric 'paired plant immune receptor' complex which aids detection of diverse pathogen effectors. Recognition of an effector by one protein in the pair activates the other protein and triggers downstream defence responses (Williams *et al.*, 2014).

7.1.3 Transgenic lines constitutively expressing the *Cladosporium fulvum* effector *ECP6*

The *Cladosporium fulvum* effector *ECP6* (*EXTRACELLULAR PROTEIN 6*) encodes a protein with three LysM domains, which bind chitin, preventing its recognition as a MAMP by the host plant and the triggering of downstream defence signalling (de Jonge *et al.*, 2010, de Jonge and Thomma, 2009, Bolton *et al.*, 2008). Functional LysM effectors have also been identified in other fungal

pathogen species, but there is little evidence for functional chitin binding effectors in the genomes of *F. graminearum* and *F. culmorum* (Brown *et al.*, 2012, de Jonge and Thomma, 2009, Marshall *et al.*, 2011). Previous work by Thomma *et al.* (*pers comm*) has shown that heterologous expression of *CfECP6* in *Arabidopsis* increases susceptibility to the fungal wilt pathogen *Verticillium dahliae*. Preliminary studies by Hammond-Kosack and colleagues suggested that this also increased *F. culmorum* susceptibility. This was formally assessed in this chapter.

7.1.4 A mutation blocking biosynthesis of scopoletin

As described in Chapter 4, the phenylpropanoid derived coumarin compounds scopolin and scopoletin have been found to accumulate at the *Arabidopsis* stem/pedicle junction following silique infection with *F. culmorum*, and in seedling shoots following inoculation with *Fusarium oxysporum* (Kai *et al.*, 2006). These compounds have been shown to have antifungal activity in a number of plant pathosystems (Sun *et al.*, 2014, Gnonlonfin *et al.*, 2012, Carpinella *et al.*, 2005, Prats *et al.*, 2006, Chong *et al.*, 2002, Valle *et al.*, 1997).

The Fe(II)- and 2-oxoglutarate dependent dioxygenase (2OGD) family gene *F6'H1* is required for the biosynthesis of scopoletin in *Arabidopsis*. Mutants in this gene in ecotype Col-0 show reduced accumulation of scopolin and scopoletin, but do not have a pleiotropic growth phenotype unlike other scopoletin biosynthetic mutants identified (Kai *et al.*, 2008, Kai *et al.*, 2006). Silencing of *F6'H1* in tobacco also prevented scopoletin accumulation and increased susceptibility to the necrotrophic fungus *Alternaria alternata* (Sun *et al.*, 2014). The susceptibility of the mutant allele *f6'h1-2* to *F. culmorum* and *F. graminearum* pedicle infection was therefore assessed, following single silique wound inoculation.

7.1.5 Mutation of *ERECTA* in ecotype Col-0

The Arabidopsis *ERECTA* gene encodes a serine-threonine protein kinase with a role in plant development. Mutation of *ERECTA*, such as that seen in the widely used Arabidopsis accession Landsberg *erecta* (Ler-0), results in plants with a shorter stature, rounded leaves, and more compact floral morphology (van Zanten *et al.*, 2009, Torii *et al.*, 1996). Both the floral and leaf tissues of Ler-0 have been shown to be more susceptible to *Fusarium* infection than Col-0, using spray inoculation of intact plants and a detached leaf assay, respectively (Urban *et al.*, 2002, Chen *et al.*, 2006). The increased floral susceptibility was attributed to the *erecta* mutation, resulting in more compact floral morphology compared to Col-0, facilitating hyphal growth across the surface of the inflorescence. However, leaf susceptibility of Ler-0 was mapped to two QTLs, neither of which encompassed the *erecta* mutation.

This raised the question of whether the differences in floral susceptibility between ecotypes was purely due to the *erecta* mutation, either via its effect on floral morphology and / or by wider effects on defence signalling: Mutation of *ERECTA* has been found to increase susceptibility to a number of other pathogens including the necrotrophic fungal pathogens *Verticillium longisporum* and *Plectosphaerella cucumerina* and the bacterial wilt pathogen *Ralstonia solanacearum*, indicating a role for *ERECTA* in broad spectrum defence signalling (Haeffner *et al.*, 2014, Llorente *et al.*, 2005, Godiard *et al.*, 2003). The floral susceptibility of Col-0 harbouring a mutation in *ERECTA* (Col-*er*) to *F. culmorum* was therefore assessed.

7.2 Methods

Plant and fungal growth and maintenance, infection assays and disease assessment were done as described in Chapter 2. The identity of mutant and transgenic lines was confirmed via PCR and sequencing where required, using the primers outlined in Table 7.1.

Table 7.1: Primers used for confirmation of mutations and transgenes in investigated Arabidopsis lines

Line	Primers (5' – 3')
<i>oxi1</i>	TATCCGTCAACAAACTCGCCA CCACAGCAGTAGTGACGTTCT
<i>prx33/prx34</i>	ATGCAATTCTCTTCATCTTC ATGCAATCGATATCAGCAGCCAATTTTA
<i>ECP6</i>	TATCCGTCAACAAACTCGCCA CCACAGCAGTAGTGACGTTCT
<i>rrs1-1</i>	ACATGAAGCCATTTACAATTGAATATATCC TGATGGGTTTACAGTTTGGGGAGGACTGGTAATTG
<i>rps4-21</i>	TAAGCTACCATTGAAAGAAGTTTCG TTAACCATTACAAAAGCAATCAACAG
<i>f6'h1</i>	TAGCATCTGAATTTCATAACCAATCTCGATACAC ATGGCTCCAACACTCTTGACAACC

7.3 Results

7.3.1 There is no evidence of altered *F. culmorum* susceptibility in the Ws-background mutants *oxi1*, *prx33*, *prx34*, *rps4* or *rrs1*

A selection of defence related mutants in the Arabidopsis Ws-0 (*prx33*, *prx35*, *rps4*, *rrs1*) and Ws-2 (*oxi1*) backgrounds were screened for altered susceptibility to *F. culmorum* following spray and silique point inoculations (Fig. 7.1 and 7.2, respectively). None of the mutants showed statistically significant differences in floral, silique or leaf infection compared to equivalent wild type plants 14 or 20 days after spray inoculation with *F. culmorum* conidia (Regression analysis, $p = >0.05$). The mean silique disease score for *oxi1* was slightly lower at 14 dpi than wild type Ws-2 (FAD=4 and 5.25, respectively) but this difference was not statistically significant. All plants in the Ws-0/Ws-2 backgrounds bolted rapidly after inoculation resulting in low levels of floral disease (Fig. 7.1d and e).

Infection of the pedicel tissue following silique wound point inoculations was also equivalent between genotypes at 14 dpi (Fig. 7.2). Total infected pedicel length was slightly greater in the *oxi1* mutant compared to wild type Ws-2, but this was due to the longer pedicels in the *oxi1* mutant, resulting in the proportion of pedicel tissue infected being slightly lower in *oxi1* than Ws-2 (Fig. 7.2b). Blue-green autofluorescence indicative of scopoletin accumulation was observed under UV light in all plants where infection had reached the stem-pedicel junction, irrespective of genotype (Fig. 7.2c).

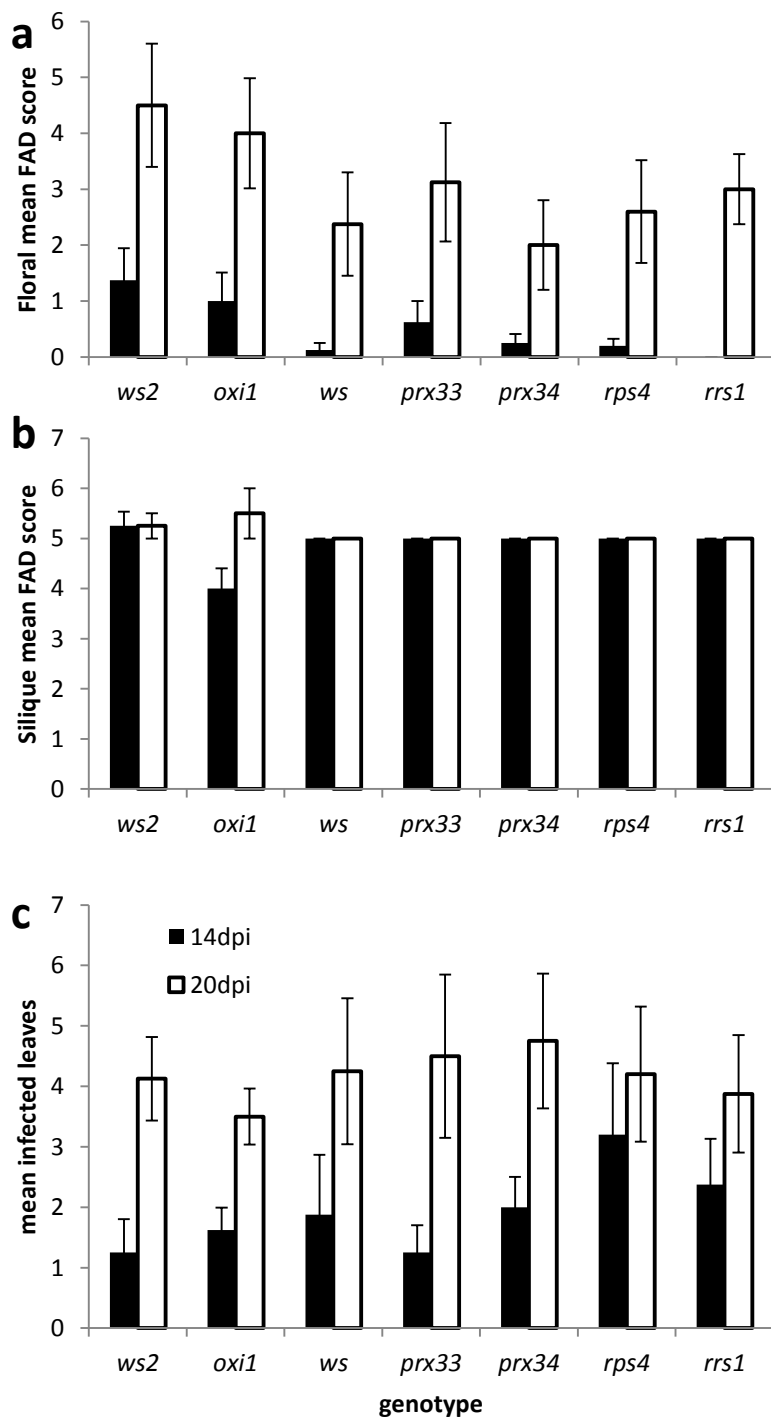


Figure 7.1: *F. culmorum* infection of *Arabidopsis* genotypes *Ws-2* (*ws2*) *oxi1*, and *Ws-0* (*ws*) *prx33*, *prx34*, *rps4* and *rrs1* compared to wild type *Ws-2* and *Ws-0*. Plants were assessed for floral (a), silique (b) and leaf (c) disease at 14 and 20 days after spray inoculation with *F. culmorum*. N= 8, Bar= SE. A representative inoculated apical inflorescence at 14 dpi is shown in (d) (bar = 1cm); a whole plant is shown in (e) (bar=3cm). This experiment was repeated with similar results.

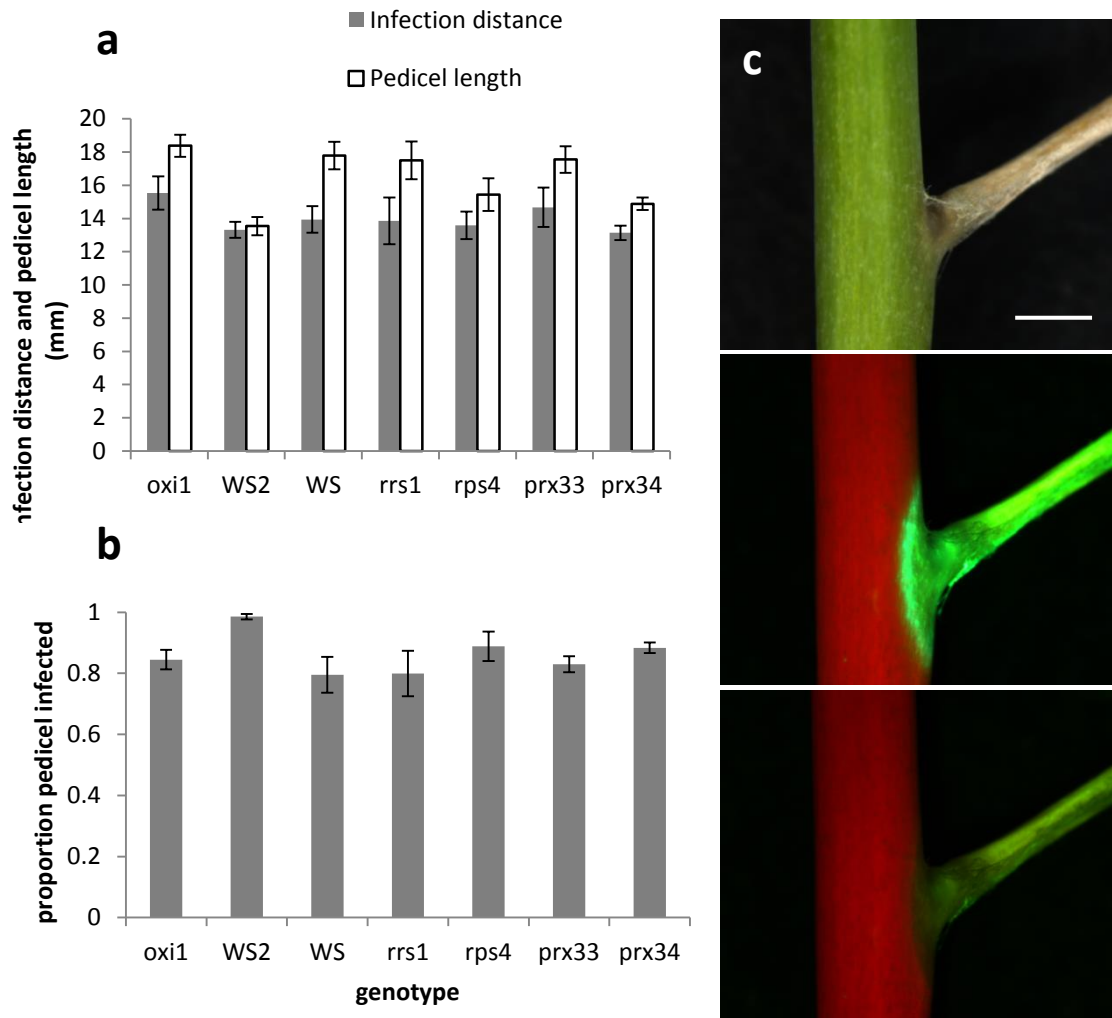


Figure 7.2: *F. culmorum* pedicel infection in Arabidopsis *Ws-2* mutant *oxi1* and *Ws-0* mutants *rrs1*, *rps4*, *prx33* and *prx34* 14 days after point inoculation of wounded siliques. The length of the pedicel compared to total pedicel length is shown in (a), with the proportion of pedicel infected is shown in (b). N=6, bar=SE. No significant differences in infection were found between genotypes (ANOVA, $p = >0.05$). Fully infected pedicels are shown in (c) under white light (top) and UV light with a violet (middle) and GFP2 (lower) filter. Bar = 750µm. Blue-green fluorescence under UV light with a violet filter was seen in all genotypes where infection reached the pedicel-stem junction.

7.3.2 Heterologous expression of *Cladosporium fulvum* ECP6 does not increase *Fusarium* susceptibility

Heterologous expression of the *C. fulvum* effector *ECP6* in *Arabidopsis* Col-0 had previously been shown to increase plant susceptibility to pathogens such as *Verticillium dahliae*, by masking chitin recognition (Thomma *et al.*, *pers. comm.*). *Arabidopsis* Col-0 plants constitutively expressing *CfECP6* were therefore screened for altered susceptibility to *F. culmorum*. However, very low levels of floral infection were observed in both wild type and *ECP6* transgenic plants, along with no variation in silique infection and little variation in leaf colonisation at 14 dpi (Table 7.2).

Table 7.2: *Fusarium culmorum* disease formation on the floral, silique and leaf tissue of *Arabidopsis* Col-0 expressing *CfECP6* compared to wild type Col-0. SEM= standard error of the mean.

Genotype	Tissue type					
	Floral FAD		Silique FAD		Infected leaves	
	MEAN	SEM	MEAN	SEM	MEAN	SEM
Col-0	0.36	0.13	5	0	7.14	0.43
ECP6	0.36	0.13	5	0	6.43	0.36

The effect of heterologous *ECP6* expression on *Fusarium* susceptibility was also tested in the *Ler-0* background, which has a more consistent *Fusarium* susceptibility phenotype than Col-0 (Urban *et al.*, 2002). There were no significant differences in recorded disease scores at 7, 10 and 14 dpi (Figure 7.3). Infected plants were observed until senescence but no differences in disease symptoms were evident between genotypes.

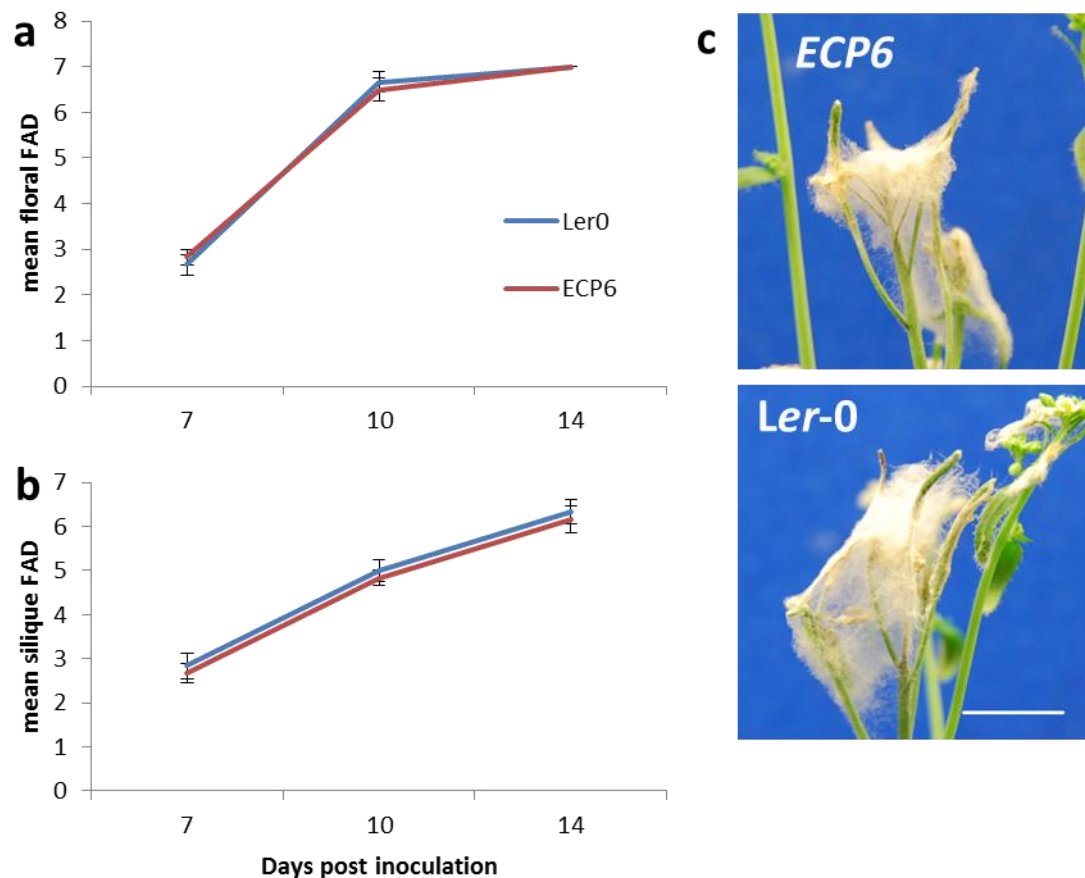


Figure 7.3: Analysis of *F. culmorum* susceptibility in Arabidopsis Ler-0 heterologously expressing *CfECP6*. Disease was assessed in floral (a) and silique (b) tissue at 7, 10 and 14 days post spray inoculation with *F. culmorum* conidia. Error bars = standard error, N=12. Representative images of infection at 10 dpi are shown in c (bar=1cm). This experiment was repeated with equivalent results.

7.3.3 Susceptibility to pedicel infection is not altered in the scopoletin deficient mutant *f6'h1*

The antifungal coumarin scopoletin has previously been found to accumulate at the pedicel-stem junction in response to *F. culmorum* infection of the silique and pedicel (see Chapter 4). Infection is not observed to progress beyond the junction into the main stem. Susceptibility to *F. culmorum* and *F. graminearum* pedicel and main stem infection in the Arabidopsis scopoletin biosynthesis mutant *f6'h1*, which is deficient in scopoletin, was therefore assessed (Fig. 7.4). Infection of *f6'h1* mutant pedicels was equivalent to that of wild type Col-0 at 14 dpi, both in terms of total length of pedicel infected and proportion of pedicel infected (Fig. 7.4a, b). However, no blue-green autofluorescence characteristic of

scopoletin accumulation was seen in the *f6'h1* mutant where infection reached the pedicel-stem junction (Fig7.4c). There was no evidence of main stem colonisation in either genotype after 14 days. The distance that the infection progressed did differ significantly between *F. culmorum* and *F. graminearum* (ANOVA, $p=0.005$), with *F. culmorum* infecting a greater part of the stem than *F. graminearum* (mean infected pedicel = 9.86 and 8.88mm ± 0.36 SED, respectively).

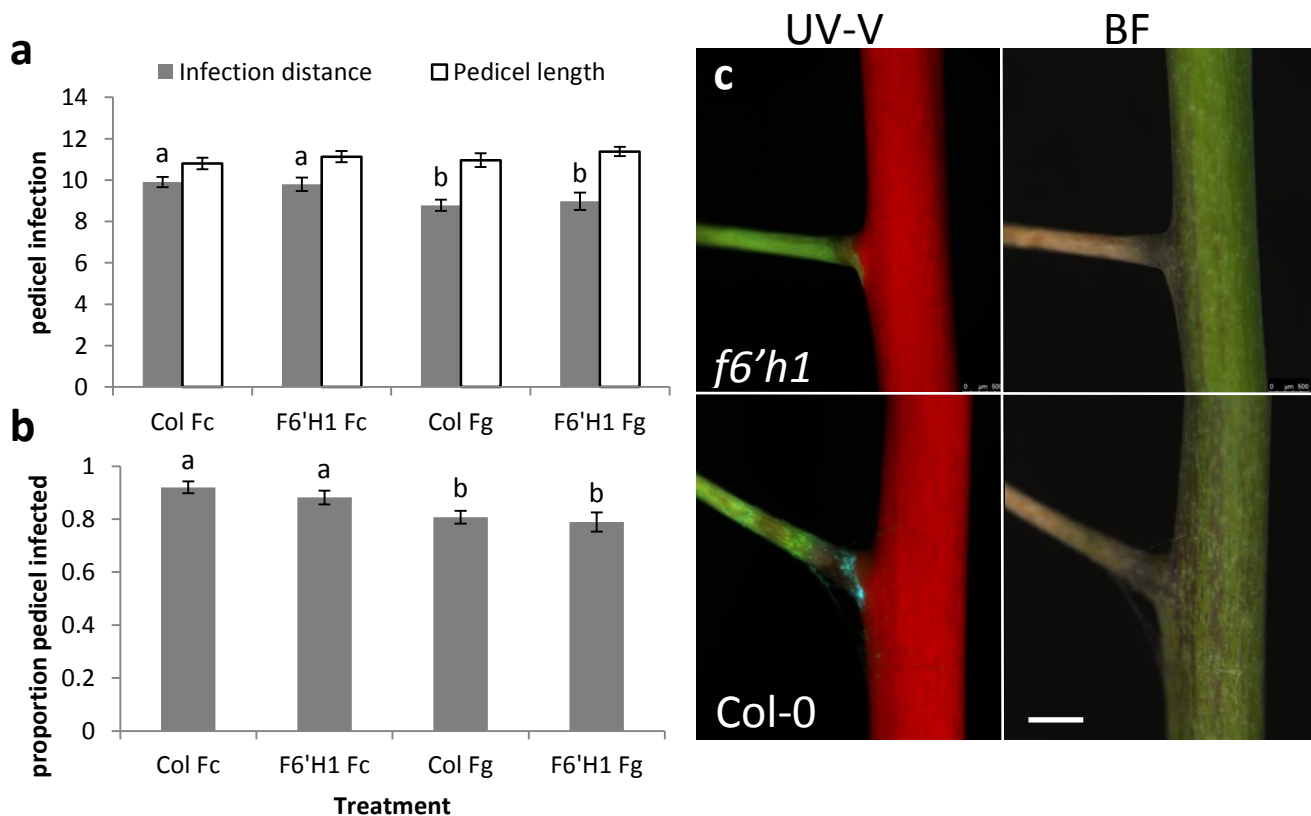


Figure 7.4: *Fusarium culmorum* (Fc) and *F. graminearum* (Fg) infection of *f6'h1* pedicels compared to wild type Col-0. Total length of pedicel (a) and proportion of pedicel infected (b) was assessed 14 days after point inoculation of wounded silique tips with *Fusarium* spores. Different letters denote statistically significant differences between treatments (ANOVA, $p > 0.05$).

Fully *F. culmorum* infected pedicels are shown in (c) under UV light with a violet filter (left) and white light (right). Blue-green auto-fluorescence indicative of scopoletin accumulation at the pedicel-stem junction was observed in wild type Col-0 but not *f6'h1* plants. Bar = 750 μ m.

7.3.4 The role of the *erecta* mutation in *Ler-0* floral susceptibility to *Fusarium culmorum*

Infection of *Ler-0* floral tissue by *Fusarium* is both more consistent and more severe than that seen in *Col-0*, and this was previously attributed to the compact floral architecture and shorter bolt stature in *Ler-0* compared to *Col-0*, caused by the *erecta* mutation (Urban *et al.*, 2002). This was further tested by assessing the floral susceptibility of *Col-0* harbouring the *erecta* mutation (*Col-er*), compared to wild type *Col-0*, following spray inoculation. The *Col-er* line has compact floral morphology equivalent with that of *Ler-0* (Fig. 7.5, top image panel). Comparison of susceptibility between *Col-0* and *Ler-0* was also intended, however the *Ler-0* seed planted for these experiments did not germinate. *Ler-0 eds1* was therefore used, since it has equivalent floral susceptibility to wild type *Ler-0* (Chapter 6, Cuzick *et al.*, 2009).

In these experiments, there was a significant difference in floral infection between genotypes ($F_{15,63} = 150$ $p < 0.001$). No disease symptoms or fungal hyphae were visible on the flowers and buds of *Col-0* plants, at 7 or 11 dpi (Fig. 7.5). By contrast, floral infection was consistently visible in *Col-er* and *Ler-0 eds1* plants. However, infection was slightly but significantly lower in *Col-er* flowers compared to *Ler-0* ($p = 0.05$). *Col-er* plants had a mean FAD score of 3.38 at 7 dpi and 5.5 at 11 dpi, while *Ler-0* plants had a mean FAD score of 4 and 6.75 at 7 and 11 dpi, respectively. The difference was therefore particularly marked at the later time point. This corresponded with increased incidence of constriction of the main stem below the apical inflorescence in *Ler-0* plants at 11 dpi, as indicated by the white arrow in the lower panel of Fig. 7.5. By contrast, stem constriction in *Col-er* was generally limited to within the inflorescence.

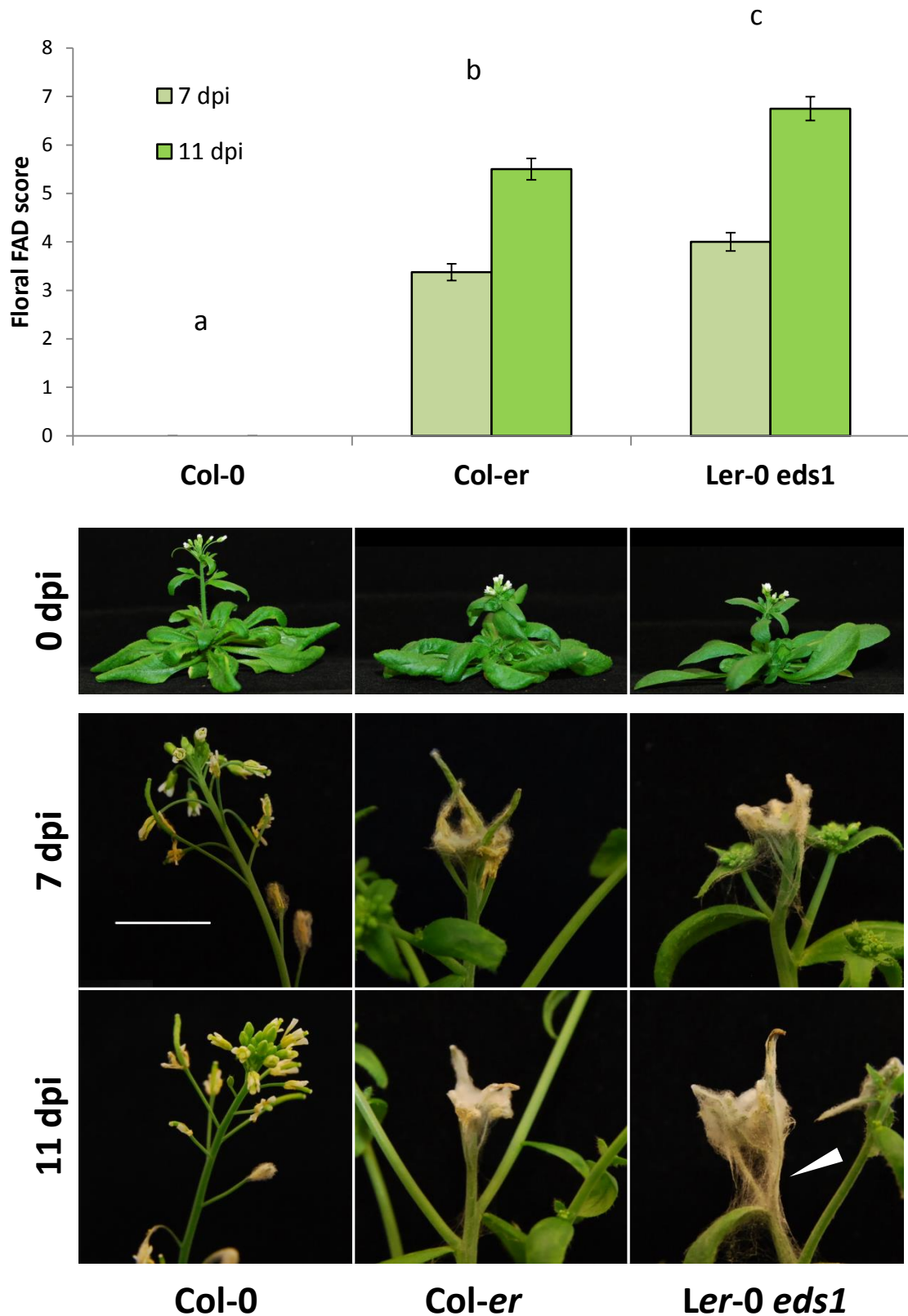


Figure 7.5: Assessment of susceptibility of *Columbia erecta* (Col-er) to *F. culmorum* infection, compared to wild type Col-0 and Landsberg *erecta eds1-2* (Ler-0 *eds1*). Floral disease symptoms were assessed at 7 and 11 days after spray inoculation with *F. culmorum* spores. Letters indicate significant differences in symptoms between genotypes (Regression analysis and calculation of LSDs, $p = <0.05$) Error bars = standard error. Top image panel shows whole plants at the time of inoculation. Lower panels show close-up images of inoculated floral tissue. Bar = 1cm. White arrow = upper stem constriction.

7.4 Discussion

In this chapter, the contribution of a number of components of plant defence signalling to *Fusarium* resistance was assessed using mutant and transgenic *Arabidopsis* lines. These encompassed the oxidative burst (*oxi1*, *prx33*, *prx34*), recognition of effectors by specific *R* genes (*rps4-21*, *rrs1-1*), recognition of the fungal MAMP chitin (*ECP6*), and production of the antifungal phytoalexin scopoletin (*f6'h1*). However, none of these mutants/transgenic lines differed from wild type plants in terms of their susceptibility to *Fusarium* under the infection conditions used. While this could indicate that these aspects of defence are not required for resistance to *Fusarium*, there are clear limitations with the *Arabidopsis-Fusarium* floral pathosystem which make these negative results difficult to interpret:

7.4.1 Lack of consistent floral infection in Ws-0 and Ws-2

Mutants in the Ws-0 and Ws-2 background were selected in the hope that these ecotypes would succumb to *Fusarium* floral infection more consistently than ecotype Col-0, facilitating the identification of mutants with altered infection levels. This was not the case: Very low levels of floral infection were seen in all genotypes in the Ws backgrounds and experiments had to be run for nearly 3 weeks in order to observe floral infection, which was very variable. Silique and pedicel infection following both spray and point inoculation with *Fusarium* was equivalent between all genotypes, indicating that none of the mutations affected progression of this infection. This is consistent with all mutants previously screened in the Col-0 background under the infection conditions used in this study: Cuzick *et al.* (2008a) did not observe alterations in silique and pedicel susceptibility even in mutants *npr1* and *eds11* which show reproducibly high levels of floral infection. This indicates that these tissues may not be well suited

for evaluation of defence signalling against *Fusarium*. However, as illustrated in chapter 6, the potential for identification of mutants conferring increased resistance to silique infection should not be overlooked. Furthermore, assessment of the incidence of infection of the stem-pedicel junction, rather than measurement of disease progression along the pedicel, revealed differences in susceptibility between the genotypes assessed in Chapter 4.

7.4.2 The oxidative burst and *Fusarium* infection

It is not clear whether the lack of an altered defence phenotype shown by oxidative burst related mutants *oxi1*, *prx33* and *prx34* to *F. culmorum* floral and silique infection is due to the previously described limitations of the Arabidopsis pathosystem, as opposed to these genes not having a role in the *Fusarium*-Arabidopsis interaction. This is particularly true when considering the applicability of these results to *Fusarium* infection of natural cereal hosts such as wheat.

The oxidative burst is known to play a major role in plant defence to a number of pathogens, particularly biotrophs, through the induction of HR (Levine *et al.*, 1994). However, ROS mediated HR is known to be exploited by necrotrophic pathogens such as *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Govrin and Levine, 2000, Govrin *et al.*, 2006). The implications of this for the *Fusarium* – wheat interaction are not clear. Wheat infecting *Fusaria* species are considered to have a hemi-biotrophic or switching lifestyle, with a short symptomless phase followed by induction of host cell death (Brown *et al.*, 2010, Scherm *et al.*, 2013, Kazan *et al.*, 2011). Production of DON mycotoxin by *F. graminearum* has been shown to elicit ROS production and HR in wheat, and H₂O₂ has been shown to elicit DON production *in vitro*, suggesting a positive feedback loop of DON and ROS production, but it is not known whether this ROS generation contributes to

fungal virulence or promotes plant defence (Desmond *et al.*, 2008, Ponts *et al.*, 2006). DON production is required for full virulence during colonisation of wheat ears and stems, which implies that the associated oxidative burst may aid colonisation, or at least does not hinder it (Cuzick *et al.*, 2008b, Mudge *et al.*, 2006). However, in *Arabidopsis* DON production is not required for virulence on floral tissues (Cuzick *et al.*, 2008b). It is also possible that *F. culmorum* is adapted to 'ignore' oxidative stress in a similar manner to *B. cinerea* (Temme and Tudzynski, 2009), meaning that mutations that modulate ROS based defence responses *in planta* do not affect the infection outcome. Furthermore, while all three of the mutants investigated have an oxidative burst related role, none result in complete abolition of ROS production and signalling – ROS is presumably still produced via NADPH oxidase in the *prx* mutants, and the *oxi1* mutant, while blocking ROS induced defence signalling, does not block ROS production itself. This could also explain the lack of an altered defence phenotype observed in these mutants.

7.4.3 R gene mediated defence signalling against *Fusarium*

The *R* genes *RPS4* and *RRS1* contribute to resistance to at least three pathogens with different phylogenies and lifestyles. The effect of mutations in these genes on *F. culmorum* susceptibility was therefore assessed, but no significant effect was found. This may be due to the aforementioned limitations of using the Ws-0 ecotype in the *Arabidopsis-Fusarium* pathosystem.

However, the null hypothesis that these genes are not involved in recognition of *F. culmorum* infection is probably more plausible than the hypothesis that they are: Traits conferring resistance to *Fusarium* infection in wheat are limited, complex, and QTL based, rather than being conferred by a single locus. There is no evidence for Effector Triggered Immunity (ETI) based on a gene-for-gene

interaction, and traits conferring resistance to head blight do not correlate with those conferring resistance to crown rot (Jayatilake *et al.*, 2011, Zhou *et al.*, 2010, Li *et al.*, 2010, Buerstmayr *et al.*, 2009, Liu *et al.*, 2007, Bai and Shaner, 2004, Liu and Anderson, 2003). It therefore follows that resistance to *Fusarium* in Arabidopsis is not mediated by a gene-for-gene interaction, and that neither *RRS1* nor *RPS4* are involved in recognition of the fungus or activation of defence responses.

7.4.4 The effect of blocking chitin recognition on *F. culmorum* susceptibility

Heterologous expression of the *C. fulvum* chitin binding effector ECP6 in Arabidopsis has been shown to increase susceptibility to fungal pathogens, presumably by blocking chitin recognition by Arabidopsis chitin receptors such as CERK1 (Thomma *et al.*, unpublished data). Heterologous expression of *ECP6* in *Fusarium oxysporum* also increased this pathogen's virulence on tomato, indicating that chitin recognition is a factor limiting disease progression by this *Fusarium* pathogen. This led to the hypothesis that the same might be true of *F. culmorum* infection of Arabidopsis floral tissue following spray inoculation. Preliminary studies by Hammond-Kosack and colleagues (unpublished data) had revealed systemic *F. culmorum* infection in Col-0 plants heterologously expressing *ECP6*. Furthermore, as shown in chapter 4, infection of the stem-pedicel junction was higher in *ECP6* plants compared to wild type Col-0 following single silique wound inoculations, although the incidence of this level of infection was very low across all genotypes in these experiments.

In this study, heterologous expression of *ECP6* was not found to significantly alter floral infection following spray inoculation, in either Col-0 or Ler-0 plants. There are a number of possible explanations for this. It could be that *F.*

culmorum blocks recognition of chitin fragments released from the fungal cell walls during infection via a process that has not yet been identified.

Alternatively, chitin recognition may occur, but the activation of downstream defence processes might be blocked by *F. culmorum* effectors. Or, lastly, chitin may be perceived and downstream defence responses activated, such as HR, but these might either be ineffective in blocking *F. culmorum* infection, or indeed facilitate infection as described previously for other pathogens with a necrotrophic infection phase (Govrin *et al.*, 2006, Govrin and Levine, 2000).

7.4.5 The role of scopoletin in preventing colonisation of the main stem

Following inoculation of a wounded silique with *F. culmorum* spores, infection progresses through the silique and pedicel, but arrests at the pedicel-stem junction. This is associated with the accumulation of the coumarin compounds scopolin and scopoletin at the infection front, characterised by blue-green autofluorescence (Cuzick *et al.*, 2008a)(Baker *et al.*, unpublished data, Chapter 4). Production of coumarins has also been associated with increased resistance to FEB in wheat and barley (Kumaraswamy *et al.*, 2011, Ravensdale *et al.*, 2014).

The role of these compounds in the prevention of disease progression into the main stem of *Arabidopsis* was therefore assessed using the scopoletin biosynthesis mutant *f6'h1*. There was no difference in disease progression along the pedicel between mutant and wild type plants, and infection did not visibly progress into the main stem in either genotype. This indicates that, despite its *in vitro* antifungal properties and the association of its presence with prevention of disease progression, scopoletin production is not solely responsible for the restriction of *F. culmorum* growth to the silique and pedicel. As seen in chapter 4, a number of other phytoalexins are induced by infection at

the stem-pedicle junction which could render any one defence molecule or pathway functionally redundant.

It is also possible that the absence of scopoletin allows symptomless colonisation of the main stem vasculature, since only disease symptoms were assessed, or that stem infection would eventually occur in the *f6'h1* mutant if the assay were run over a longer time period. However, confirmation of symptomless infection would require detailed microscopic analysis or sensitive molecular tests for fungal presence within the stem (as opposed to epiphytic growth). Running the infection time-course beyond the two week period would have introduced the complication of senescence of the investigated tissues due to the age of the plants required for silique wound point inoculations.

7.4.6 The contribution of *erecta* to susceptibility in ecotype Ler-0

F. culmorum floral infection is more severe and consistent in the Arabidopsis ecotype Ler-0 than it is in Col-0. This was attributed to the more compact floral morphology of Ler-0 owing to mutation in the *erecta* gene, but this was not formally assessed (Urban *et al.*, 2002). Furthermore, a detached leaf assay revealed increased *F. graminearum* susceptibility in Ler-0 compared to Col-0, and the basis of this susceptibility was mapped to two QTLs unrelated to the *ERECTA* gene (Chen *et al.*, 2006). The contribution of the *erecta* mutation to the floral susceptibility phenotype of Ler-0 compared to Col-0 was therefore assessed by examining the effect of *erecta* mutation in the Col-0 ecotype (Col-*er*). The mutation resulted in consistent infection of the floral tissue, compared to wild type plants which displayed no disease symptoms in these experiments. This indicates that mutation of *erecta* contributes considerably to *F. culmorum* floral susceptibility, likely due to its effect on floral morphology. However, floral susceptibility in Col-*er* was slightly but significantly lower than that observed in

Ler-0 eds1-2, a genotype with equivalent susceptibility to wild type *Ler-0*. It could be that this is due to other polymorphisms between *Col-0* and *Ler-0* which contribute to *Ler-0* susceptibility, such as those identified by Chen *et al.* It is possible that *erecta* facilitates initial colonisation by *F. culmorum* hyphae by compacting the inflorescence, but other genetic factors then facilitate development of this infection and constriction of the main stem below the inflorescence.

Whether the mutant *erecta* allele in the *Col-er* line is the same as that in *Ler-0* is not known. The exact identity of the *Col-er* accession used in this study cannot be traced. While both lines show equivalent floral morphology there could be subtle differences in the mutant alleles which result in the observed differences in susceptibility between *Col-er* and *Ler-0*, and this would need to be verified before attributing these differences to other loci.

7.4.7 Conclusions

This chapter has identified a number of *Arabidopsis* mutants which do not appear to have altered *F. culmorum* susceptibility. While this may indicate that the corresponding genes are not key to defence signalling against *Fusarium*, there are limitations with the floral pathosystem which may complicate interpretation of the results. Furthermore, functional redundancy between different defence responses always has implications for the suitability of using a reverse genetics approach to identify key components in any host-pathogen interaction.

This study has confirmed the contribution of the *erecta* mutation to the susceptibility phenotype of *Ler-0*. However, further work would be required to determine whether this accounts fully for the differences in floral *F. culmorum*

susceptibility observed between ecotypes, particularly in the light of *Ler-0* leaf susceptibility to *F. graminearum* being attributed to other QTLs.

8 Discussion

8.1 Key findings

This thesis has examined several aspects of the Arabidopsis-Fusarium interaction, from the metabolic fingerprint of Fusarium susceptible mutants to the similarities and differences between resistance to Fusarium and to other plant pathogens. The most important finding from these studies is arguably the reduction in Fusarium susceptibility afforded by accumulation of homoserine, either via exogenous application or mutation of the homoserine kinase *DMR1*. This highlights a possible common mechanism of susceptibility occurring in plants to Fusarium, a hemibiotrophic fungal pathogen, and the obligate biotroph oomycete and fungal pathogens *H. arabidopsidis* and *O. neolycopersici*. This mechanism could potentially be manipulated for crop protection purposes. In addition, the finding that mutation of *ERECTA* in Col-0 results in enhanced, consistent Fusarium susceptibility confirms the previous hypothesis by Urban *et al.* (2002), and could be exploited in a forward genetic screen (as discussed in section 8.6.3). It also complements other studies which have identified a role for *ERECTA* in plant defence against the bacterial wilt pathogen *Ralstonia solanacearum*, the hemibiotrophic fungal wilt *Verticillium longisporum* and the necrotrophic fungus *Plectosphaerella cucumerina* (Godiard *et al.*, 2003, Haeffner *et al.*, 2014, Llorente *et al.*, 2005).

Conversely, other findings presented in this thesis may be informative in highlighting distinct differences between pathosystems. The new reverse genetics insights presented in Chapter 7 highlight a number of genes and defence signalling processes which do not appear to be involved in resistance or susceptibility to Fusarium, namely peroxidase mediated ROS generation in the apoplast, and recruitment of the *R* gene pair *RRS1* and *RPS4*.

A central theme in this thesis is the identity and function of the *EDS11* locus, which is involved in resistance to *F. culmorum* and *P. syringae* species via an unidentified pathway(s). Metabolomic analysis of *eds11* mutant plants reveals alterations in primary sugar metabolism and flavonoid accumulation, both in whole flowering plants and pedicels. While the precise genomic location of the *eds11* mutation remains unknown, a mapping by sequencing approach has identified a manageable list of potential candidates, particularly the four genes on the short arm of chromosome four which contain non-synonymous SNPs in coding regions (Chapter 5).

While care must be taken when interpreting the results of the metabolome analyses presented in Chapters 3 and 4 (see section 8.3), these studies have highlighted some interesting trends. For example, in addition to the defence associated secondary metabolites previously identified (Chapter 4, Figure 4.1) the study described in Chapter 4 revealed that many primary metabolites are induced in *Arabidopsis* pedicels infected with *Fusarium*. However, changes in abundance of some of these metabolites were highly dependent on genotype, with possible correlations with susceptibility found for some compounds, such as citrate and glutamine. In addition, several differences in metabolite induction were seen between ecotypes Col-0 and Ler-0, such as a less pronounced accumulation of proline betaine in Ler-0. Therefore, a comparative metabolomics analysis between genotypes Col-0, Col-*er* and Ler-0 in flowering plants and pedicels could be used to identify whether these differences in compound induction are due to the *erecta* mutation.

In addition to differences in metabolite induction between genotypes, the analyses presented in Chapter 4 also included assessment of differences in

susceptibility between genotypes to *F. culmorum* pedicel-stem junction infection, and also to floral infection by *P. olsonii*. This has expanded the phenotypic profile of the various *eds* and other mutants or overexpression lines assessed in terms of their variant defence responses to a number of plant pathogens (Table 8.1).

Table 8.1: Defence response phenotypes of mutants and overexpression lines investigated in Chapters 3 and 4. wt = wild type phenotype, S = increased susceptibility, R = increased resistance, SAR+ = wild type systemic acquired resistance, SAR- = reduced systemic acquire resistance. ISR+ = wild type induced systemic resistance, ISR- = reduced induced systemic resistance. Information collated from Glazebrook *et al.* (1996), Volko *et al.* (1998), Rogers and Ausubel (1997), Ton *et al.* (2002), Cuzick *et al.* (2008a) Cuzick *et al.* (unpublished) and Chapter 4.

Genotypes	Pathogens / treatments							
	<i>Pseudomonas syringae</i> leaves	<i>Erysiphe orontii</i> leaves	<i>Xanthomonas campestris</i> leaves	<i>Fusarium culmorum</i> floral	<i>F. culmorum</i> pedicel/upper stem	<i>Penicillium olsonii</i> floral	systemic acquired resistance	induced systemic resistance
Col-0	wt	wt		wt	wt	wt	SAR+	ISR+
Col-0 <i>npr1-1</i>	S	S	S	S	wt	S	SAR-	ISR+
Col-0 <i>eds4</i>	S			wt	wt	S	SAR+	ISR-
Col-0 <i>eds5-2 / sid1</i>	S	S	S	wt	wt	wt	SAR-	ISR+
Col-0 <i>eds7</i>	S			S	S	S	SAR+	ISR+
Col-0 <i>eds8</i>	S			wt	S	wt	SAR+	ISR-
Col-0 <i>eds10-1</i>	S	S	S	wt	S	wt	SAR+	ISR-
Col-0 <i>eds11-1</i>	S	R	wt	S	S	wt	SAR+	ISR+
Col-0 <i>eds12-1</i>	S	wt	wt	S	S	S	SAR-	ISR+
Col-0 <i>eds13-1</i>	S	S	S	wt	S	wt	SAR+	ISR+
Col-0 <i>ERF1</i>	S			wt	wt	wt		
Col-0 <i>ECP6</i>				S/wt	S	wt		
Ler-0	wt			wt	wt	wt		
Ler-0 <i>sgt1b</i>	wt			R	R	wt		
Ler-0 <i>rar1</i>	wt			wt	wt	S		

8.2 Is Arabidopsis a suitable model host for a cereal disease?

Fusarium infection of Arabidopsis was originally put forward as a suitable model for FEB disease of wheat, due to the comparable disease progression and tissue specificity between the two plant hosts (Urban *et al.*, 2002). Susceptibility is predominantly limited to the floral organs in both species, with infection of healthy leaf tissue requiring experimental manipulation (Chen *et al.*, 2006, Makandar *et al.*, 2010, Daudi and Hammond-Kosack, unpublished).

However, the long term usefulness of the model Arabidopsis-Fusarium pathosystem is potentially questionable, especially with the emergence of the model monocotyledonous plant species *Brachypodium distachyon* as an experimental host for FEB and other cereal diseases. While it has been argued that findings from *Brachypodium* might be more directly translatable than Arabidopsis into monocotyledonous crop species such as wheat and barley, this has not yet been demonstrated. Furthermore, in contrast with cereal crop hosts and Arabidopsis, non-manipulated foliar tissue of *Brachypodium* is readily susceptible to Fusarium infection (Draper *et al.*, 2001, Mur *et al.*, 2004, Parker *et al.*, 2008, Peraldi *et al.*, 2011, Routledge *et al.*, 2004, Thole *et al.*, 2012).

One issue with using Arabidopsis as an FEB model is that several inoculation methods into different tissues have been used, sometimes yielding dissimilar results. Equivalent effects of various defence signalling mutations on *F. graminearum* susceptibility were observed between leaves and floral tissues (Makandar *et al.*, 2010). In contrast, the role of several Arabidopsis defence related genes does not appear to be equivalent in floral tissue following spray inoculation and pedicel/upper stem tissues following single silique wound inoculation with *F. culmorum*, as shown in Table 8.1. It is therefore not clear which inoculation method or tissue type yields

results most analogous to FEB in cereals. However, results from both seedling and floral assays have been shown to be translatable to wheat (Makandar *et al.*, 2006, Schreiber *et al.*, 2011).

A conserved pattern is the role of *NPR1* and its wheat orthologues in defence signalling against *Fusarium*; Mutation of *npr1* enhances leaf and floral susceptibility in *Arabidopsis*, transgenic expression of *Arabidopsis NPR1* in wheat increases FEB resistance, and increased expression of wheat orthologues of *NPR1* is associated with naturally occurring resistance (Yang *et al.*, 2013, Cuzick *et al.*, 2008a, Makandar *et al.*, 2006, Makandar *et al.*, 2010, Makandar *et al.*, 2011). An obvious exception to this pattern is the wild type level of pedicel-stem junction infection seen in *npr1* mutants in Chapter 4, following single silique wound point inoculations. This raises the question of whether this assay, and therefore the findings presented in Chapter 4, is representative of FEB disease in wheat. However, the susceptibility of *eds7*, *eds11* and *eds12*, and resistance of *sgt1b*, seen in both floral and pedicel infection assays suggests shared components in the defence response against *Fusarium* in both these tissue types. Therefore, the apparent lack of a function of *NPR1* in pedicel-stem junction resistance is intriguing and warrants further investigation. It would also be interesting to see whether the SA binding proteins *NPR3* and *NPR4* have a role in pedicel-stem junction resistance (Fu *et al.*, 2012, Yan and Dong, 2014, Moreau *et al.*, 2012).

As discussed in Section 8.4, another limitation of the pathosystem is that, under the conditions used in these studies, consistent *Arabidopsis* floral susceptibility following spray inoculation is dependent on the *erecta* mutation. The apical inflorescence of wild type *ERECTA* genotypes is not readily susceptible to *Fusarium*, resulting in low or inconsistent infection levels. It would be interesting to assess the effect of

silencing of the two known wheat *ERECTA* orthologues on FEB susceptibility (Huang *et al.*, 2013).

Broadly speaking, defence responses against *Fusarium* floral infection in *Arabidopsis* and wheat are comparable. Both species are dependent on components of SA and JA signalling, but with a potential bias towards SA, at least in the case of *F. graminearum* (Makandar *et al.*, 2011, Makandar *et al.*, 2010). This may be linked to the symptomless infection phase observed in wheat (Brown *et al.*, 2010), although a comparable phase has not been observed in *Arabidopsis*. Synthesis of phenylpropanoid derived secondary metabolites such as cinnamic acid and scopoletin also appears to be associated with limitation of infection spread in both species, along with maize and barley, although susceptibility was found not to be visibly enhanced in the *Arabidopsis* scopoletin biosynthesis mutant *f6'h1* (Chapter 7). Furthermore, in both species, host susceptibility is predominantly confined to the floral tissues.

It is unclear whether the findings presented in this thesis represent further similarities between *Arabidopsis* and wheat. The metabolites induced by pedicel infection as described in Chapter 4 do not appear to correlate strongly with metabolites induced during wheat infection (Gunnaiah *et al.*, 2012, Paranidharan *et al.*, 2008, Hamzehzarghani *et al.*, 2005). It is also still not known whether wheat has an *EDS11* ortholog, or whether silencing of the wheat *homoserine kinase* gene has equivalent effects to those seen for *DMR1* in *Arabidopsis*.

However, on balance the *Arabidopsis*-*Fusarium* is arguably still a useful working model for understanding FEB disease in wheat, at least while the wheat genome is refined and methods of genetic manipulation optimised. For example a forward

genetic screen of *Arabidopsis* might reveal additional susceptibility genes with orthologues in wheat, as described in section 8.6.3.

Furthermore, unsuitable host species can in themselves provide useful starting points from which to study the determinants of non-host resistance (the mechanism by which all variants of a plant species are resistant to all variants of a pathogen species). For example, screens for *Arabidopsis* mutants which are susceptible to the cereal powdery mildews *Blumeria graminis* f. sp. *tritici* and *B. graminis* f. sp. *hordei* has identified several genes required for non-host resistance to these pathogens (Stein *et al.*, 2006, Lipka *et al.*, 2005). The leaf tissues of both *Arabidopsis* and wheat can be argued as non-host for *Fusarium*. Similar screens to those used for cereal powdery mildews could therefore be used to elucidate the basis of non-host resistance to *Fusarium graminearum* and *F. culmorum* in *Arabidopsis* leaves, potentially identifying the determinants of tissue specificity during FEB infection of wheat.

8.3 Use of metabolomics to study plant-pathogen interactions

This thesis incorporates two large scale metabolomics datasets generated by MeT-RO using tissue samples generated and prepared by the author, as presented in Chapters 3 and 4. These datasets contain large amounts of information and highlight compounds and pathways that might have a role in determining the outcome of the *Fusarium*-*Arabidopsis* interaction. However, drawing conclusions from these datasets is difficult for a number of reasons. For example, many of the compounds identified in these analyses are of unknown identity. Significant expertise and further work will be required to identify them. Linked to this is the issue that expertise in metabolomic analyses is also required to interpret and refine raw data relating to known compounds. This can lead to substantial bottlenecks in the knowledge

production pipeline, as seen in Chapter 4. Furthermore, the association between a compound and a process or plant phenotype does not necessarily represent causality. For the majority of the metabolites identified in Chapter 4, there was no clear relationship between the degree of infection incidence and changes in compound abundance. The previously described wheat metabolome analyses compared resistant and susceptible lines and/or linked to transcriptome analyses (Gunnaiah *et al.*, 2012, Paranidharan *et al.*, 2008, Hamzehzarghani *et al.*, 2005). Even so, it is not clear whether compounds associated with resistance contribute to the observed phenotype.

In addition, successful interpretation of results from analyses such as those presented in this thesis requires substantial knowledge of plant metabolic processes and/or the ability to compare and contrast datasets generated in different studies. This requires that studies be comparable, highlighting a need for international community standardisation of both methods and data annotation and interpretation, as well as statistical analysis (Saito and Matsuda, 2010).

8.4 Experimental difficulties encountered and possible solutions

There were a number of experimental challenges encountered during the development of this thesis. The principal difficulty was the lack of floral infection generally observed when studying genotypes in the Col-0 background. Those mutants which did show enhanced susceptibility generally still had variable infection levels. This prevented the intended metabolome analysis comparing healthy and *Fusarium* infection floral tissue in a collection of Col-0 genotypes, as outlined in Chapter 4. Due to this difficulty, subsequent selection of mutants to screen for altered *Fusarium* susceptibility was limited to non Col-0 genotypes (Chapters 6 and 7). Indeed, clear and consistent results were chiefly obtained for genotypes in the

Ler-0 background, such as the *dmr1* mutants. Since most existing Arabidopsis mutant lines have been generated in the Col-0 background, this limited the extent to which various genes and defence signalling pathways could be explored using a reverse genetics approach. However, as described in Section 8.6.3, there is the potential for a forward genetic screen via mutagenesis of an *erecta* line.

A principal aim of the project was to map the *EDS11* locus to a genomic location. However, this was made difficult by high levels of variability in the susceptibility phenotype of mutant plants. Susceptibility to *F. culmorum* floral infection sometimes overlapped with wild type plants. In addition, *P. syringae* disease symptoms were not consistently different between *eds11* and wild type plants, though bacterial counts were generally higher in *eds11*. *F. culmorum* susceptibility was used to screen the F₂ population, but this may have resulted in contamination of the mapping population with wild type plants and, furthermore, it is not clear whether the mutation conferring *F. culmorum* susceptibility is the same mutation conferring *P. syringae* susceptibility. In hindsight, it would have been beneficial to visit the laboratory of a collaborator who already has optimised conditions and protocols for identifying discriminatory *P. syringae* susceptibility phenotypes. This would potentially have allowed for the rapid identification of an inoculation method which results in a clearly visible *P. syringae* susceptibility phenotype in *eds11* plants. Parallel screens for both *P. syringae* and *F. culmorum* susceptibility could then have been done.

8.5 The working model

Prior to the commencement of this project in 2010, knowledge of defence against Fusarium in Arabidopsis floral tissue was limited to the likely contributions of the SA, JA and ET signalling pathways to resistance and/or susceptibility, the importance of *NPR1* and the unmapped genes *EDS11* and *ESA1* for resistance, as well as the

contribution of SGT1b to susceptibility (Cuzick *et al.*, 2008a, Cuzick *et al.*, 2009, Makandar *et al.*, 2010, Van Hemelrijck *et al.*, 2006). Other loci such as the GLK1 regulon had been implicated in leaf resistance (Savitch *et al.*, 2007).

The studies presented in this thesis, combined with other research which has been published in the same time period, create a more complex picture of the genes, proteins, compounds, pathways and processes which influence the interaction outcome, some of which are analogous to findings from wheat studies. These findings have been incorporated into a new working model, shown in Figure 8.1. This model highlights possible differences between defence components acting in *Arabidopsis* floral and new silique tissue following spray inoculation, and those acting in the pedicel and pedicel stem junction following silique point inoculations. The role of NPR1 in floral, but not pedicel defence, combined with recruitment of genes associated with JA mediated defence signalling in the pedicel, suggests there may be a biotrophic phase of infection occurring in flowers which is not present in the pedicel. The floral infection data certainly suggests that the defence response to *Fusarium* has features in common with defence against both biotrophic and necrotrophic pathogens. There is currently no evidence of a symptomless phase of infection in either tissue type. However, there is an increasing body of evidence that even pathogens previously defined as ‘classical’ necrotrophs, such as *S. sclerotiorum* and *B. cinerea*, may have an asymptomatic growth phase, or grow endophytically on some plant species without causing disease, via complex interactions with the host immune system (Williams *et al.*, 2011, van Kan *et al.*, 2014). It would therefore seem likely that *Fusarium* might have a symptomless growth phase in *Arabidopsis*, and this warrants further investigation.

Similarities between *Arabidopsis* and wheat include the importance of NPR1 and its wheat orthologues, (Yang *et al.*, 2013, Makandar *et al.*, 2006, Makandar *et al.*, 2010, Cuzick *et al.*, 2008a), the likely role of SA signalling (Makandar *et al.*, 2011, Ding *et al.*, 2011), the accumulation of cinnamic acids (Gunnaiah *et al.*, 2012, Paranidharan *et al.*, 2008, Hamzehzarghani *et al.*, 2005) and a supporting role for JA signalling (Ding *et al.*, 2011, Makandar *et al.*, 2010). The role of ET signalling is still not clear in either species. There are also similarities in the division between host and non-host tissue in each species. Infection occurs via floral tissues at anthesis in both species, and is limited to the spikelets and rachis in wheat, and to the flowers/silques and adjoining pedicel in *Arabidopsis*. This suggests that the pedicel tissue may be analogous to the rachis, with further disease progression limited at the main stem.

In addition to using knockout mutants to highlight existing components of resistance to infection (solid lines), several studies by other researchers have highlighted potential transgenic approaches to increasing resistance (dashed lines), such as overexpression of CWDE inhibiting proteins and thionins (Asano *et al.*, 2013, Ferrari *et al.*, 2012). *Arabidopsis* has also been used to identify chemistries acting against *Fusarium* (Schreiber *et al.*, 2011). The potential to exploit these findings for enhancement of FEB resistance in wheat is discussed in Section 8.7.

In addition to the possible existence of a symptomless phase, several other aspects of the *Arabidopsis*-*Fusarium* interaction are still unclear. These include the mechanism by which SGT1b contributes to susceptibility. Since SGT1b is known to be involved in *R* gene mediated signalling, it could be that *Fusarium* susceptibility is dependent on the activity of an R-like protein, as seen for the necrotrophic fungi *P. nodorum* and *C. victoriae* (Lorang *et al.*, 2007). Mutation of *SGT1b* might block this activity. However, it is not clear why mutants of *RAR1*, which forms a cytosolic

defence signalling complex with SGT1b and HSP90, do not show the equivalent susceptibility phenotype, or why resistance is limited to the bud tissue (Azevedo *et al.*, 2002, Cuzick *et al.*, 2009). Extending the previous hypothesis, it is possible that the targeted R-like protein might only be expressed in the buds (see section 8.6.4). The identities of the majority of the *EDS* loci are also still unknown.

RNA sequencing has recently been used to study transcriptional changes in *F. graminearum* during the symptomless growth phase and symptomatic disease development in wheat ears (Brown, 2011). This dataset could also be used to identify differences in transcription of wheat genes during these two phases of infection, compared to uninfected tissue. This may provide additional information on defence signalling in wheat during these two contrasting infection phases. RNA sequencing of Arabidopsis bud tissue between inoculated and non-inoculated *Ler-0*, *Ler-0 sgtb* and *Ler-0 rar1* might also be useful in understanding why these mutants do not show equivalent phenotypes, despite the corresponding genes being known to have a coupled role.

Figure 8.1 represents those studies where genes with a role in the Arabidopsis-Fusarium interaction have been identified based on an altered phenotype in mutant plants. However, several mutants have been screened which do not show significant alterations in *F. culmorum* susceptibility, namely those presented in Chapter 7 and reported by Cuzick *et al.* (2008 and 2009). These mutants are summarised in Table 8.2. For example, three ET signalling mutants were found by Cuzick *et al.* (2008) to have wild type *F. culmorum* susceptibility levels. This adds to the lack of certainty over the role of ET signalling in defence against Fusarium.

Table 8.2: Arabidopsis mutant and transgenic lines which do not show altered floral or silique susceptibility to *F. culmorum* infection.

Genotype	Gene function	Reference
Col-0 35S:ERF1	JA/ET signalling	Cuzick <i>et al.</i> (2008)
Col-0 eto1	ET regulation	Cuzick <i>et al.</i> (2008)
Col-0 etr1	ET signalling	Cuzick <i>et al.</i> (2008)
Col-0 f6'h1	Scopoletin biosynthesis	Chapter 7
Ler-0 eds1	Basal and <i>R</i> gene mediated defence signalling	Cuzick <i>et al.</i> (2009)
Ler-0 lms1	Resistance to <i>Leptosphaeria maculans</i>	Cuzick <i>et al.</i> (2009)
Ler-0 rar1	<i>R</i> gene signalling	Cuzick <i>et al.</i> (2009)
Ws-0 prx33	Peroxidase mediated ROS production	Chapter 7
Ws-0 prx34	Peroxidase mediated ROS production	Chapter 7
Ws-0 rps4	<i>R</i> genes effective against <i>R. solanacearum</i> , <i>P. syringae</i> and <i>C. higginsianum</i>	Chapter 7
Ws-0 rrs1		
Ws-2 oxi1	Oxidative burst response	Chapter 7

The current working model focuses exclusively on the defence response of Arabidopsis to Fusarium infection compared to wheat. However, the fungal virulence mechanisms also appear to differ during infection of the two plant species. For example, it has previously been shown that DON mycotoxin is produced by the fungus during Arabidopsis infection, but is not required for full virulence on flowers, which contrasts with the requirement for DON in Fusarium colonisation of wheat ears (Cuzick *et al.*, 2008b, Urban *et al.*, 2002). However, DON may still be eliciting defence responses in Arabidopsis, which could be analysed by comparing transcriptome and/or metabolome data between plants infected by wild type Fusarium and a DON biosynthesis mutant.

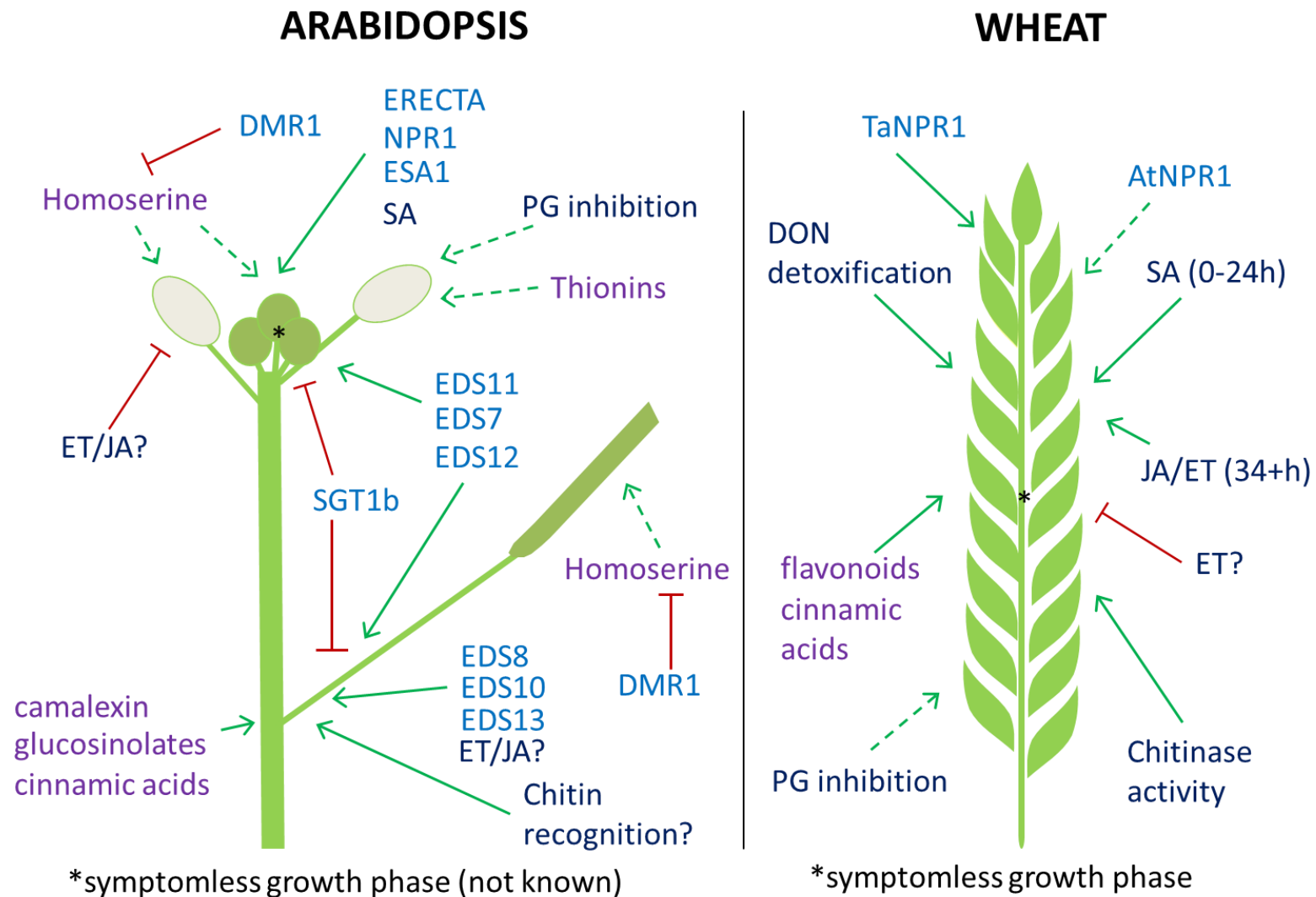


Figure 8.1: The 2014-2015 working model: Proteins/Genes (pale blue), compounds (purple) pathways and processes (dark blue) associated with susceptibility (red lines) and resistance (green arrows) to Fusarium infection in Arabidopsis buds, flowers, siliques and pedicels (left), compared to plant responses to FEB disease in wheat (right). Solid lines – existing mechanisms, dashed lines – novel mechanisms resulting from gene silencing or overexpression. Note that wheat data relates exclusively to *F. graminearum*, while Arabidopsis data predominantly relates to *F. culmorum*. Arabidopsis leaf data is not presented here.

8.6 Further work

There are many opportunities for further work arising from and/or complimenting the findings presented in this thesis. These include novel genetic screens and translational research in monocotyledonous hosts of *Fusarium*, as outlined below.

8.6.1 The future of *EDS11*

As explained in Chapter 5, it has not yet been possible to pinpoint the genomic location of *EDS11*. However, the list of candidates is modest and will hopefully soon be narrowed down to a single gene, assuming that the susceptibility phenotype results from a single mutation. This will allow analysis of *EDS11* expression in distinct Arabidopsis tissues and the effect of *Fusarium* infection on expression, as well as the study of the interaction between *EDS11* and other proteins. A transgenic Ler-0 line overexpressing *EDS11* could also be generated, to assess whether this would lead to increased resistance in this susceptible ecotype. Likewise, a reporter line expressing a *GFP:EDS11* fusion construct could be used to study subcellular localisation.

The potential translation of findings pertaining to *EDS11* into crop species would depend whether the gene is ubiquitous in higher plants or limited to Arabidopsis and related species, and knowledge of this could be achieved by a search for orthologues in wheat and other *Fusarium* hosts. If a wheat ortholog exists, transient silencing could be used to study its role in FEB resistance.

Alternatively, if the gene is absent from wheat, the susceptibility of transgenic wheat lines constitutively expressing Arabidopsis *EDS11* could be assessed, as was previously done for Arabidopsis *NPR1* (Makandar *et al.*, 2006).

8.6.2 The effect of homoserine accumulation on pathogenesis

As shown in Chapter 6, mutation of the Arabidopsis homoserine kinase gene *DMR1* leads to accumulation of homoserine in siliques and resistance to *Fusarium*, along with the mildew pathogens *H. arabidopsidis* and *O. neolycopersici*. Senescence is also delayed in mutant plants (Huibers *et al.*, 2013, van Damme *et al.*, 2009, Brewer *et al.*, 2014). Exogenous application of homoserine onto wheat ears did not result in a substantial reduction in FEB disease symptoms. However, transient silencing of the wheat *DMR1* ortholog is underway, and may result in a more significant reduction in FEB either alone or combined with exogenous homoserine application. Furthermore, it will be interesting to see whether homoserine kinase silenced wheat plants show the reduced size or delayed senescence phenotypes observed in Arabidopsis *dmr1* plants. The utility of reduced plant size as a phenotype would depend on the nature of the reduction – while reduced grain size would be undesirable, reduced stature, pedicel length, leaf length or tillering could be beneficial in wheat breeding programmes. Similarly, delayed senescence might translate into a longer grain filling period.

The mechanism by which chloroplastic homoserine accumulation confers resistance to three distinct pathogens still remains to be determined (van Damme *et al.*, 2009, Huibers *et al.*, 2013, Brewer *et al.*, 2014). Future research might investigate the effect of homoserine application on *Fusarium* gene expression *in vitro*, as well as on Arabidopsis gene expression. Furthermore, since mutation of homoserine kinase reduces viability and virulence of human pathogens, it would be interesting to explore the effect of silencing the *Fusarium* homoserine kinase gene on fungal growth and pathogenicity (Kingsbury and

McCusker, 2010a, Kingsbury and McCusker, 2010b, Kingsbury and McCusker, 2008).

8.6.3 A forward genetic screen for enhanced resistance

The mutants screened for altered *Fusarium* susceptibility in this and similar studies were either isolated from forward genetic screens for altered resistance to other pathogens, or from reverse genetics analysis of genes involved in specific aspects of plant defence. A forward genetic screen would allow for isolation of additional mutants with significantly altered resistance to *Fusarium* floral or silique infection. Since mutation of *ERECTA* results in consistent and reliable floral infection levels in both Columbia and Landsberg ecotypes, it would be most advantageous to carry out such a screen in an *erecta* background. Indeed, this background has already yielded two mutant genotypes, *dmr1* and *sgt1b*, which have reduced resistance to *Fusarium* in reproductive tissues (Brewer *et al.*, 2014, Cuzick *et al.*, 2009). A screen for resistant mutants in an EMS mutagenised *Ler-0* population would help to identify more genes which contribute to *Fusarium* susceptibility, and potentially susceptibility to other pathogens. Since a Col-0 *erecta* (Col-*er*) line is also available, isolated mutants could be outcrossed to this genotype and mapped in the F₂ using a conventional marker based approach or whole genome re-sequencing (Schneeberger *et al.*, 2009, Konieczny and Ausubel, 1993, Neff *et al.*, 1998). Alternatively, mutations could be mapped by backcrossing to *Ler-0*, using the pipeline demonstrated in Chapter 5. Doing forward genetic screens in both backgrounds could also be highly informative.

8.6.4 Analysis of transcriptome data from distinct *Arabidopsis* tissue types

Altered susceptibility phenotypes in many of the mutants assessed appears to be limited to one or two tissue types. For example, *sgt1b* has enhanced bud resistance and a lower incidence of stem-pedicel junction infection compared to wild-type plants, but shows wild type infection of new siliques. Mutants *npr1* and *eds11* both show enhanced floral susceptibility, but infection does not extend into the main stem and become systemic. These disparities could be due to the effects of resistance or susceptibility genes acting in a tissue specific manner. Analyses of transcription patterns for identified *R* genes and defence related genes in bud, silique and pedicel tissues could be used to investigate tissue specific gene expression patterns which might account for restriction of observed mutant phenotypes to specific tissues. This could be done using the Genevestigator tool presented in Chapter 6 and by Cuzick *et al.* (2008a), or the expression visualisation tool available via the new Arabidopsis Information Portal (Baerenfaller *et al.*, 2012, Krishnakumar *et al.*, 2014). This might highlight potential interacting partners contributing to disease or susceptibility.

8.7 Current and future perspectives for FEB management

As described in Chapter 1, the control strategies for FEB on wheat are currently very limited. Effective use of fungicides is a major challenge. This is due to intrinsic and developing resistance, along with the need for accurate spray timing and application which can be hard to predict. Furthermore, some fungicides have been associated with elevated DON production, there are few new antifungal chemistries being developed, and those already in use may face bans under new EU legislation. With FEB disease incidence likely to increase in the future due to climate change associated factors, along with increased maize

cultivation in predominantly wheat growing regions of the UK and elsewhere, improved control measures are needed (West *et al.*, 2012).

These measures include improving accuracy of existing risk assessments, by combining advanced forecasting models with the use of biosensors to detect regions with high spore loads (Shah *et al.*, 2013, Moshou *et al.*, 2011).

Monitoring could be used to guide crop protection decisions in the field. In addition, novel strategies for detecting *Fusarium* infection in grains pre- and post-harvest, such as screens for associated plant and fungal metabolites, could be used to reduce the risks of food chain contamination and the costs associated with DON mycotoxin testing.

There is also the potential for improved chemical control, particularly that which enhances plant resistance rather than targeting the fungus. For example, there could be applications for the resistance inducing compounds found in the high throughput screen carried out by Schreiber *et al.* (2011). The potential applications of homoserine to improve resistance presented in this thesis also warrant further exploration.

8.7.1 Genetic improvement of FEB resistance

Targeted genetic improvement of wheat has historically presented a challenge due to the large, polyploid nature of the genome and limitations of transformation techniques. However, the gene coding portions of the wheat genome are now well sequenced, and novel precision genome editing techniques could be used to modify multiple copies of genes in order to enhance disease resistance. Proof of this concept has recently been shown by Wang *et al.* (2014b), who induced targeted mutations in all alleles of the wheat *MLO* (mildew resistance locus), resulting in broad spectrum powdery mildew

resistance. If transient VIGS based disruption of the wheat *DMR1* or *SGT1b* orthologues is shown to enhance FEB resistance in wheat, these loci could also be durably modified using a similar genome editing approach.

Studies in *Arabidopsis* and wheat have also highlighted other potential mechanisms of improving FEB resistance via transgenic approaches. As previously described, these include overexpression of native or heterologous genes encoding defence related metabolites and proteins inhibiting fungal virulence mechanisms such as cell wall degradation, along with RNA interference constructs which target *Fusarium* genes (Koch *et al.*, 2012, Asano *et al.*, 2013, Kaur *et al.*, 2012, Ferrari *et al.*, 2012, Koch *et al.*, 2013). However, it is not known whether these approaches would provide durable resistance in the field, or what the impacts on yield or quality might be. Furthermore, the adoption of genetic modification (GM) based crop improvement approaches by farmers and consumers depends highly on public attitudes towards GM along with national and international regulation policies (Border, 2014, Government Office for Science, 2011). However, there is some evidence that existing commercially grown transgenic maize expressing the insecticidal *Bacillus thuringiensis* (Bt) toxin supports lower levels of *Fusarium* mycotoxin contamination than non GM maize, supporting the use of GM crops for FEB control (Ostry *et al.*, 2010).

8.7.2 Broad spectrum resistance strategies and limitations

While *Fusarium* is an incredibly important global pathogen of small grain cereals, it is one of many. Development and implementation of broad spectrum resistance strategies against multiple diseases and pathogens, and potentially other biotic and abiotic stresses is therefore desirable.

However, many of the 'broad spectrum' resistance loci identified are specific to pathogens with shared infection lifestyles, such as biotrophic rusts and mildews (Campbell *et al.*, 2012, Wang *et al.*, 2014b, Spielmeyer *et al.*, 2005), or hemibiotrophic/necrotrophic pathogens such as *Z. tritici*, *P. stagonospora* and *Fusarium* (Miedaner *et al.*, 2012). Indeed, as previously described, defence against pathogens of contrasting lifestyles may require opposing defence pathways. This is evidenced by the recent finding that the *MLO* allele conferring resistance to biotrophic cereal pathogens in barley is required for susceptibility to the emerging necrotrophic leaf pathogen *Ramularia collo-cygni* (McGrann *et al.*, 2014). This may have implications for the use of mutated *MLO* loci in wheat (Wang *et al.*, 2014b). Even where two distinct diseases are caused by one pathogen, requirements for resistance may differ, as demonstrated by the contrasting effects of *Arabidopsis NPR1* expression on FEB and *Fusarium* seedling blight in wheat (Gao *et al.*, 2013).

However, while fungal pathogens of cereals may be divided by infection lifestyle, many may still share similar weaknesses which can be exploited for disease control. For example, as demonstrated in this thesis, homoserine accumulation induces resistance not only to obligate biotrophs but also to the hemibiotroph *Fusarium*. Likewise, HIGS might prove useful for silencing conserved genes required for survival of multiple fungal pathogens with divergent lifestyles. There is also the possibility of using non-pathogenic organisms as biocontrol agents which afford protection against multiple pathogen types, either via antagonism in the soil or on the epidermis, or by inducing plant defences. One such organism is *Piriformospora indica* (NCBI taxonomy ID 1109443), which has been shown to enhance resistance to multiple fungal pathogens of cereal crops (Waller *et al.*, 2005).

8.8 Conclusions

The requirement for global food security is a multifaceted problem which no single solution or technology is likely to overcome. While crop diseases pose a major threat to meeting the increasing global demand for food, issues such as distribution and domestic wastage also need to be addressed, requiring overall improved governance of the global food system (Godfray *et al.*, 2010, Government Office for Science, 2011). Furthermore, while it may be possible to find new ways to control existing pathogens in their current host ranges, it is to be expected that new pathogens will continue to emerge and spread (Fisher *et al.*, 2012, Bebber *et al.*, 2014). In addition, the durability of control strategies is likely to always be threatened by evolution of pathogens to overcome them.

However, in order to meet the challenge of sustainably feeding the world's growing population, while meeting nutritional requirements and cultural demands, agricultural processes need to be intensified (The Royal Society, 2009). This sustainable intensification will only be possible if the potential yields of crops can be adequately protected against pests and pathogens, without the need for energy-intensive chemical inputs which may also cause environmental pollution. This must also be done in the face of volatile and changing climate patterns, which could hamper forecasting of outbreaks of weather dependent diseases such as FEB. Therefore while no magic bullet exists for sustainable intensification, novel crop protection strategies have a key part to play, and the findings presented in this thesis may be a useful piece in the (albeit rather large) puzzle that is global food security.

Appendices

Appendix 1: Arabidopsis lines used and sources of seed

Name	Gene locus	Type	Source	Background
35S:ERF1	AT3G23240	transgene	NASC line N6142	Col-0
BGL2:GUS	AT3G57260	transgene	Xinnian Dong	Col-0
Col-0	N/A	N/A	Jing	Col-0
dmr1-1	AT2G17265	EMS	Guido van den Ackervekken	Ler-0 <i>eds1-2</i>
dmr1-2	AT2G17265	EMS	Guido van den Ackervekken	Ler-0 <i>eds1-2</i>
dmr1-3	AT2G17265	EMS	Guido van den Ackervekken	Ler-0 <i>eds1-2</i>
dmr1-4	AT2G17265	EMS	Guido van den Ackervekken	Ler-0 <i>eds1-2</i>
dmr1-6	AT2G17265	EMS	Guido van den Ackervekken	Ler-0 <i>eds1-2</i>
dmr5	unknown	EMS	Guido van den Ackervekken	Ler-0 <i>eds1-2</i>
dmr6	AT5G24530	EMS	Guido van den Ackervekken	Ler-0 <i>eds1-2</i>
ECP6	N/A	transgene	Bart Thomma	Col-0
ECP6	N/A	transgene	Bart Thomma	Ler-0
eds10	unknown	EMS	Jane Glazebrook	Col-0 <i>BGL2:GUS</i>
eds11	unknown	EMS	Jane Glazebrook	Col-0 <i>BGL2:GUS</i>
eds12	unknown	EMS	Jane Glazebrook	Col-0 <i>BGL2:GUS</i>
eds1-2	AT3G48090	fast neutron	Guido van den Ackervekken	Ler-0
eds13	unknown	EMS	Jane Glazebrook	Col-0 <i>BGL2:GUS</i>
eds4	unknown	EMS	Jane Glazebrook	Col-0
eds5-1 (sid1)	AT4G39030	EMS	Jane Glazebrook	Col-0
eds7	unknown	EMS	Jane Glazebrook	Col-0
eds8	unknown	EMS	Jane Glazebrook	Col-0 <i>fah1-2</i>
erecta	AT2G26330	?	Guido van den Ackervekken	Col-0
f6'h1	At3g13610	T-DNA	Bun Shimizu	Col-0
Ler-0	N/A	N/A	Graham McGrann	Ler-0
npr1-1	AT1G64280	EMS	Xinnian Dong	Col-0 <i>BGL2:GUS</i>
oxi1	AT3G25250	t-DNA	NASC line N9423	Ws-2
prx33	AT3G49110	t-DNA	Arslan Daudi	Ws-0
prx33:prx34	AT3G49120	t-DNA	Arslan Daudi	Ws-0
rar1	AT5G51700	fast neutron	Jane Parker	Ler-0
rps4	AT5G45250	t-DNA	Yoshihiru Narusaka	Ws-0
rrs1	AT5G45260	t-DNA	Yoshihiru Narusaka	Ws-0
sgt1b	AT4G11260	EMS	Jane Parker	Ler-0
Ws-0	N/A	N/A	Yoshihiru Narusaka	Ws-0
Ws-2	N/A	N/A	NASC line N1601	Ws-2

Appendix 2: Excerpt from the unrefined SHOREmap SNP call

Output from the SHOREmap BACKCROSS function.

	Column	Description
1	Chromosome	Chromosome identifier
2	Position	Position in the reference sequence
3	Reference allele	Either A, C, G or T, describing the allele of the wild-type parent.
4	Mutant allele	Either A, C, G or T, describing the mutant allele.
5	Support	Total number of reads aligned to this mutation site.
6	Frequency	Frequency of the mutation
7	Quality score	Base calling quality score (from a resequencing program)
8	Type	Either this is a NEWSNP or a known REFERROR
9	Sequence feature	Type of DNA that is affected.
10	ID	Gene identifier (if mutation resides in gene)
11	Isoform	Isoform of the gene
12	Mutation position	Coding sequence position of the change.
13	Codon position	Codon position of the change
14	Type of change	Either syn or nonsyn.
15	Reference amino acid	
16	Mutation-induced amino acid	

1	4556877	G	T	9	1.00	144	NEWSNP	intergenic
1	5819658	C	A	17	1.00	222	NEWSNP	intergenic
1	7960976	T	A	43	0.30	62	NEWSNP	intergenic
1	9958155	T	A	50	1.00	222	NEWSNP	intergenic
1	11148735		G	T	37	1.00	120	NEWSNP intergenic
1	11719168		C	A	116	1.00	222	NEWSNP intronic/noncoding
	AT1G32450.1			11719167				
1	11895819		G	T	35	1.00	220	NEWSNP intergenic
1	12823594		A	G	48	0.36	75	NEWSNP intergenic
1	13728131		C	T	45	0.48	225	NEWSNP intergenic
1	13841889		C	A	31	0.26	112	NEWSNP intergenic
1	13841896		G	A	32	0.27	122	NEWSNP intergenic
1	14237613		A	G	30	0.25	144	NEWSNP intergenic
1	14237622		C	T	29	0.24	124	NEWSNP intergenic
1	14238773		A	T	83	0.45	106	NEWSNP intergenic
1	14309238		G	T	23	0.33	112	NEWSNP intergenic
1	14309242		G	A	23	0.33	111	NEWSNP intergenic
1	14309249		T	A	21	0.32	119	NEWSNP intergenic
1	14309586		C	A	15	0.27	162	NEWSNP intergenic
1	14309591		T	C	16	0.28	152	NEWSNP intergenic
1	14309592		T	C	16	0.28	147	NEWSNP intergenic
1	14453537		A	C	26	0.27	67	NEWSNP intergenic
1	14453557		C	G	25	0.28	72	NEWSNP intergenic
1	14453734		C	T	26	0.26	80	NEWSNP intergenic
1	14508824		C	A	26	0.23	71	NEWSNP intergenic
1	14508877		T	G	23	0.23	118	NEWSNP intergenic
1	14508890		T	C	18	0.20	100	NEWSNP intergenic
1	14509027		C	T	29	0.25	125	NEWSNP intergenic
1	14509123		T	A	20	1.00	209	NEWSNP intergenic

1	14509131	C	G	14	1.00	172	NEWSNP	intergenic
1	14509140	T	C	12	1.00	158	NEWSNP	intergenic
1	14509657	T	G	21	0.30	132	NEWSNP	intergenic
1	14510406	C	A	40	0.27	203	NEWSNP	intergenic
1	14545583	T	C	11	0.65	181	NEWSNP	intergenic
1	14545584	T	C	11	0.65	181	NEWSNP	intergenic
1	14545588	G	T	11	0.61	176	NEWSNP	intergenic
1	14545589	C	T	11	0.61	174	NEWSNP	intergenic
1	14545590	C	G	11	0.61	180	NEWSNP	intergenic
1	14545592	A	G	11	0.61	179	NEWSNP	intergenic
1	14545598	G	A	11	0.61	175	NEWSNP	intergenic
1	14545610	G	C	11	0.61	171	NEWSNP	intergenic
1	14592401	G	A	36	0.23	144	NEWSNP	intergenic
1	14592492	C	A	82	0.37	225	NEWSNP	intergenic
1	14592654	G	A	36	0.23	131	NEWSNP	intergenic
1	14607910	A	T	15	1.00	222	NEWSNP	intergenic
1	14607913	G	A	15	1.00	221	NEWSNP	intergenic
1	14608172	A	G	13	1.00	135	NEWSNP	intergenic
1	14608180	A	T	11	1.00	114	NEWSNP	intergenic
1	14608181	T	C	10	0.91	97	NEWSNP	intergenic
1	14608281	C	G	17	1.00	149	NEWSNP	intergenic
1	14608287	T	A	21	1.00	160	NEWSNP	intergenic
1	14608339	C	A	21	1.00	157	NEWSNP	intergenic
1	14608366	T	C	12	1.00	126	NEWSNP	intergenic
1	14609970	C	A	34	0.30	140	NEWSNP	intergenic
1	14609971	C	T	34	0.30	141	NEWSNP	intergenic
1	14610148	C	T	29	0.23	135	NEWSNP	intergenic
1	14610401	A	C	30	1.00	222	NEWSNP	intergenic
1	14610457	C	A	7	1.00	92	NEWSNP	intergenic
1	14610472	C	A	20	0.95	150	NEWSNP	intergenic
1	14656926	G	A	16	1.00	78	NEWSNP	intergenic
1	14657448	A	T	9	1.00	137	NEWSNP	intergenic
1	14660225	A	G	18	0.95	126	NEWSNP	intergenic
1	14660244	G	A	12	0.92	103	NEWSNP	intergenic
1	14660245	C	T	12	0.92	103	NEWSNP	intergenic
1	14685504	C	G	15	0.25	60	NEWSNP	intergenic
1	14685550	T	G	14	0.26	71	NEWSNP	intergenic
1	14691955	A	T	20	0.29	96	NEWSNP	intergenic
1	14722038	T	C	164	0.61	225	NEWSNP	intergenic
1	14722181	G	A	48	0.45	117	NEWSNP	intergenic
1	14722182	C	T	48	0.46	117	NEWSNP	intergenic
1	14722183	A	T	48	0.45	115	NEWSNP	intergenic
1	14722201	T	C	35	0.36	96	NEWSNP	intergenic
1	14922428	T	A	24	0.22	137	NEWSNP	CDS AT1G40083.1
	14922427	517	1	Nonsyn	T	S		
1	14922454	T	C	23	0.22	113	NEWSNP	CDS AT1G40083.1
	14922453	491	2	Nonsyn	Y	C		
1	14922478	C	T	28	0.25	133	NEWSNP	CDS AT1G40083.1
	14922477	467	2	Nonsyn	R	Q		
1	14922486	G	A	29	0.28	130	NEWSNP	CDS AT1G40083.1
	14922485	459	3	Syn	I	I		
1	14922497	C	T	26	0.26	89	NEWSNP	CDS AT1G40083.1
	14922496	448	1	Nonsyn	G	R		
1	14922498	G	C	26	0.26	92	NEWSNP	CDS AT1G40083.1
	14922497	447	3	Syn	G	G		
1	15043615	T	C	34	0.34	82	NEWSNP	intergenic
1	15061574	C	T	29	0.30	95	NEWSNP	intergenic
1	15061611	C	G	28	0.26	74	NEWSNP	intergenic
1	15085691	A	C	565	0.53	225	NEWSNP	CDS AT1G40104.1
	15085690	1760	2	Nonsyn	D	A		
1	15085911	A	T	502	0.88	80	NEWSNP	intergenic
1	15089556	A	T	83	0.38	76	NEWSNP	intergenic
1	15092057	T	G	76	0.40	114	NEWSNP	intergenic
1	15096676	T	C	48	0.27	64	NEWSNP	intergenic
1	15096849	G	A	34	0.20	71	NEWSNP	intergenic
1	15096954	C	T	34	0.33	161	NEWSNP	intergenic
1	15096958	A	T	33	0.32	155	NEWSNP	intergenic
1	15096974	A	C	38	0.35	108	NEWSNP	intergenic
1	15097006	T	A	52	0.39	82	NEWSNP	intergenic
1	15098775	T	C	51	0.33	68	NEWSNP	intergenic
1	15099912	C	T	166	0.27	107	NEWSNP	intergenic
1	15100046	T	C	96	0.34	156	NEWSNP	intergenic
1	15100164	A	T	73	0.32	100	NEWSNP	intergenic
1	15105792	A	G	72	0.45	120	NEWSNP	intergenic
1	15106762	A	G	82	0.31	82	NEWSNP	intergenic
1	15107681	G	A	32	0.24	90	NEWSNP	intergenic
1	15108165	T	G	64	0.42	95	NEWSNP	intergenic

Appendix 3: Revised SNP call

Chromosome	Location	Reference	SNP	Quality	Supporting Reads	Frequency
1	12823594	A	G	75	48	0.3636
1	18544646	G	A	225	50	0.431
1	18558731	C	T	225	49	0.3828
1	18671409	C	T	225	45	0.4412
1	18708105	C	T	225	60	0.5607
1	18761334	C	T	225	44	0.4583
1	18970208	C	T	225	59	0.513
1	18995641	G	C	225	43	0.5119
1	19015465	C	T	225	58	0.4296
1	19138576	C	T	225	35	0.4795
1	19392208	C	T	225	31	0.4493
1	19577128	C	T	225	39	0.4105
1	20243307	C	T	225	25	0.4808
1	20458572	C	T	225	46	0.4646
1	27801552	C	T	225	45	0.4545
1	27982216	C	T	225	46	0.5111
1	28379684	C	T	225	38	0.4419
1	29084873	C	T	225	48	0.4948
1	29338984	C	T	225	44	0.4835
1	29665377	G	A	225	43	0.4433
1	29898748	G	A	225	28	0.3457
1	30006622	G	A	225	58	0.5225
1	30165580	G	A	225	51	0.4679
1	30284797	G	A	225	69	0.552
1	30292182	G	A	225	50	0.4902
1	30389418	G	A	225	45	0.3814
2	407623	T	A	225	35	0.4167
2	6269078	C	T	225	27	0.4286
2	6874676	A	C	225	49	0.5158
2	7338597	G	A	225	43	0.5
2	7430744	G	A	225	66	0.5546
2	7453624	G	A	225	46	0.4381
2	7495430	G	A	225	48	0.5161
2	7506870	G	A	225	37	0.4805
2	7541810	G	A	225	71	0.5035
2	7550419	G	A	225	56	0.549
2	7571697	G	A	225	56	0.6022
2	7657571	C	T	225	38	0.4419
2	7726055	G	A	225	60	0.5217
2	7882885	G	A	225	52	0.5843
2	8551977	G	A	225	52	0.4685
2	8639378	G	A	225	56	0.5773
2	8758102	G	A	225	56	0.5091
2	9065147	G	A	225	56	0.5773
2	9124286	G	A	225	73	0.5407

2	9188319	G	A	225	63	0.6
2	9206341	G	A	225	45	0.5294
2	9328658	G	A	225	44	0.4632
2	9396582	G	A	225	53	0.5
2	9402923	G	A	225	66	0.5455
2	9427270	G	A	225	48	0.5581
2	15323688	C	T	225	74	0.5968
2	15350824	C	T	225	90	0.6338
2	15365352	C	T	225	64	0.6465
2	15425788	C	T	225	68	0.6018
2	15450817	C	T	225	51	0.5368
2	15698225	C	T	225	60	0.5769
2	15734711	C	T	225	55	0.5789
2	15776849	C	T	225	79	0.594
2	15944466	C	T	225	56	0.5545
2	16025774	G	A	225	43	0.5513
2	16089603	C	T	225	53	0.5955
2	16153486	C	T	225	58	0.5631
2	16232126	C	T	225	58	0.5088
2	16308451	C	T	225	60	0.566
2	16431468	C	T	225	76	0.608
2	16498763	C	T	225	58	0.5631
2	16645720	C	T	225	72	0.5294
2	16897217	C	T	225	60	0.5
2	17015117	C	T	225	63	0.5
2	17205732	C	T	225	49	0.5326
2	17385385	C	T	225	65	0.5603
2	17641279	C	T	225	81	0.609
2	17946987	C	T	225	63	0.4632
2	18216969	C	T	225	58	0.5088
2	18266869	G	A	225	56	0.5333
2	18587656	C	T	225	40	0.3478
2	18626528	C	T	225	44	0.4835
2	18695013	C	T	225	55	0.5556
2	18800761	C	T	225	47	0.5109
2	19097520	C	T	225	46	0.4423
3	905101	C	T	225	46	0.4842
3	1247186	C	T	225	81	0.5364
3	1331640	C	T	225	64	0.5517
3	1392085	C	T	225	63	0.5207
3	1895423	C	T	225	58	0.5133
3	2081411	C	T	225	66	0.5641
3	2175696	C	T	225	44	0.5238
3	2279468	C	T	225	58	0.5918
3	2374403	C	T	225	54	0.5806
3	2488484	C	T	225	68	0.5037
3	2644451	C	T	225	60	0.5217
3	2797284	C	T	225	52	0.5714

3	2892189	C	T	225	61	0.5398
3	2904659	C	T	225	45	0.5
3	3410416	C	T	225	52	0.52
3	3561581	C	T	225	54	0.5455
3	3899478	C	T	225	50	0.463
3	4134397	C	T	225	48	0.5161
3	4389509	C	T	225	54	0.432
3	4468132	C	T	225	23	0.5476
3	4556108	C	T	225	52	0.5532
3	4686826	C	T	225	66	0.5323
3	13326596	G	C	225	48	0.5217
3	19578944	C	T	225	53	0.5096
3	19828584	C	T	225	52	0.5306
3	20337052	C	T	225	68	0.5714
3	21447552	C	T	225	56	0.5045
3	21579167	C	T	225	52	0.5049
3	21900990	G	A	225	53	0.4569
3	21924614	C	T	225	55	0.5612
3	22275578	C	T	225	58	0.5
3	22742511	C	T	225	39	0.4333
3	23250660	C	T	225	50	0.5556
4	13187	G	A	225	91	0.7222
4	205368	G	A	225	65	0.6701
4	284677	G	A	225	71	0.6893
4	432043	G	A	225	70	0.6542
4	675948	G	A	225	71	0.7245
4	716655	G	A	225	65	0.6633
4	792979	G	A	225	36	0.7347
4	850162	G	A	225	67	0.6907
4	965551	G	A	225	62	0.6889
4	1055622	G	A	225	65	0.7303
4	1082514	G	A	225	74	0.7255
4	1098091	G	A	225	72	0.766
4	1276833	G	A	225	63	0.7683
4	1302926	G	A	225	61	0.7176
4	1404505	G	A	225	63	0.6702
4	1600758	G	A	225	66	0.7097
4	1654258	G	A	225	63	0.7159
4	1655653	G	A	225	77	0.7549
4	1794927	G	A	225	70	0.6863
4	1981110	G	A	225	76	0.6909
4	1999123	G	A	225	82	0.6891
4	2004196	G	A	225	68	0.6415
4	2068244	G	A	225	77	0.713
4	2194038	G	A	225	61	0.6289
4	2655499	G	A	225	74	0.6852
4	2730467	G	A	225	75	0.6579
4	2730678	G	A	225	59	0.7108

4	2853251	G	A	225	71	0.6961
4	2892514	G	A	225	66	0.6667
4	3164420	G	A	225	71	0.7245
4	3186625	G	A	225	57	0.6786
4	3263975	G	A	225	68	0.7556
4	3428470	G	A	225	67	0.6768
4	3597373	G	A	225	64	0.6957
4	3619277	G	A	225	60	0.6452
4	3678331	G	A	225	71	0.7172
4	3680222	G	A	225	69	0.7188
4	3714724	G	A	225	81	0.75
4	3773854	G	A	225	46	0.6866
4	3806462	G	A	225	72	0.6923
4	3845762	G	A	225	58	0.6905
4	3898127	G	A	225	52	0.7647
4	4080947	G	A	225	68	0.6667
4	4126012	G	A	225	58	0.7073
4	4171716	G	A	225	64	0.6667
4	4544258	G	A	225	68	0.7083
4	4552978	G	A	225	74	0.7048
4	4722251	T	C	76	20	0.1527
4	5197914	G	A	225	64	0.7273
4	5276516	G	A	225	99	0.7984
4	5346521	G	A	137	32	0.8205
4	5368413	G	A	225	87	0.685
4	6161576	G	A	225	75	0.6579
4	6398620	G	A	225	91	0.7459
4	6417927	G	A	225	45	0.625
4	6643446	G	A	225	58	0.6667
4	6738672	G	A	225	68	0.6667
4	6863387	G	A	225	76	0.6441
4	9499819	T	G	225	33	0.3367
4	9499825	T	C	187	27	0.3034
4	15637425	C	T	225	38	0.3958
5	3131345	G	C	225	59	0.4876
5	14809767	C	T	225	25	0.3788
5	15089919	A	T	225	38	0.4222
5	19789468	C	T	225	42	0.4516
5	25936238	C	T	225	37	0.4458

Appendix 4: SNPs resulting in non-synonymous changes in genes

Chr	Location	Ref	SNP	Quality	Supporting Reads	Frequency	Gene
1	19138576	C	T	225	35	0.4795	AT1G51610.1
1	20243307	C	T	225	25	0.4808	AT1G54215.1
1	27982216	C	T	225	46	0.5111	AT1G74448.1
1	29665377	G	A	225	43	0.4433	AT1G78910.1
1	29898748	G	A	225	28	0.3457	AT1G79480.1
1	30006622	G	A	225	58	0.5225	AT1G79740.1
1	30165580	G	A	225	51	0.4679	AT1G80210.1
1	30292182	G	A	225	50	0.4902	AT1G80570.1
2	6874676	A	C	225	49	0.5158	AT2G15780.1
2	7495430	G	A	225	48	0.5161	AT2G17230.1
2	7506870	G	A	225	37	0.4805	AT2G17260.1
2	7550419	G	A	225	56	0.549	AT2G17370.1
2	8639378	G	A	225	56	0.5773	AT2G20010.1
2	8758102	G	A	225	56	0.5091	AT2G20300.1
2	9427270	G	A	225	48	0.5581	AT2G22170.1
2	15350824	C	T	225	90	0.6338	AT2G36620.1
2	15365352	C	T	225	64	0.6465	AT2G36670.1
2	15425788	C	T	225	68	0.6018	AT2G36810.1
2	15698225	C	T	225	60	0.5769	AT2G37410.1
2	16431468	C	T	225	76	0.608	AT2G39350.1
2	16897217	C	T	225	60	0.5	AT2G40460.1
2	17641279	C	T	225	81	0.609	AT2G42360.1
2	18266869	G	A	225	56	0.5333	AT2G44010.1
2	18626528	C	T	225	44	0.4835	AT2G45180.1
3	2488484	C	T	225	68	0.5037	AT3G07790.1
3	2644451	C	T	225	60	0.5217	AT3G08700.1
3	2892189	C	T	225	61	0.5398	AT3G09400.2
3	2904659	C	T	225	45	0.5	AT3G09440.2
3	3410416	C	T	225	52	0.52	AT3G10900.1
3	3899478	C	T	225	50	0.463	AT3G12230.1
3	4686826	C	T	225	66	0.5323	AT3G14130.1
3	19828584	C	T	225	52	0.5306	AT3G53480.1
3	21447552	C	T	225	56	0.5045	AT3G57930.1
3	21579167	C	T	225	52	0.5049	AT3G58280.1
3	23250660	C	T	225	50	0.5556	AT3G62900.1
4	716655	G	A	225	65	0.6633	AT4G01680.1
4	1055622	G	A	225	65	0.7303	AT4G02400.1
4	1082514	G	A	225	74	0.7255	AT4G02480.1
4	1404505	G	A	225	63	0.6702	AT4G03180.1

Appendix 5: Statistical analysis outputs and LSD tables

Figure 4.2a – contamination by *P. olsonii*

Regression analysis

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Binomial totals: N

Distribution: Binomial

Link function: Logit

Fitted terms: Constant + Rep + Genotype + Treatment + Genotype.Treatment

Predictions from regression model

	Prediction	s.e.
Genotype		
Col0	0.3618	0.10751
ECP6	0.1630	0.07663
eds10	0.2972	0.09573
eds11	0.3926	0.10456
eds12	0.9267	0.06298
eds13	0.5175	0.10860
eds4	0.7084	0.10379
eds5-2	0.4726	0.10981
eds7	0.6809	0.10595
eds8	0.4622	0.09704
erf1	0.3569	0.10144
ler	0.0397	0.05228
nrp1-1	0.9005	0.07095
rar1	0.3094	0.10862
sgt1b	0.0001	0.00130

Least significant differences of predictions (5% level)

Genotype Col0	1	*			
Genotype ECP6	2	0.2623	*		
Genotype eds10	3	0.2860	0.2436	*	
Genotype eds11	4	0.2979	0.2574	0.2816	*
Genotype eds12	5	0.2478	0.1973	0.2279	0.2428
Genotype eds13	6	0.3038	0.2641	0.2877	0.2994
Genotype eds4	7	0.2974	0.2566	0.2808	0.2930
Genotype eds5-2	8	0.3057	0.2663	0.2897	0.3014
Genotype eds7	9	0.3003	0.2600	0.2839	0.2959
Genotype eds8	10	0.2881	0.2461	0.2714	0.2840
Genotype erf1	11	0.2938	0.2526	0.2771	0.2893
Genotype ler	12	0.2384	0.1851	0.2175	0.2332
Genotype nrp1-1	13	0.2562	0.2077	0.2370	0.2512
Genotype rar1	14	0.3055	0.2658	0.2894	0.3018
Genotype sgt1b	15	0.2139	0.1525	0.1905	0.2081
		1	2	3	4
Genotype eds12	5	*			
Genotype eds13	6	0.2497	*		
Genotype eds4	7	0.2415	0.2987	*	
Genotype eds5-2	8	0.2518	0.3070	0.3004	*
Genotype eds7	9	0.2452	0.3016	0.2949	0.3034
Genotype eds8	10	0.2303	0.2901	0.2831	0.2919
Genotype erf1	11	0.2375	0.2953	0.2886	0.2972

Genotype ler	12	0.1630	0.2403	0.2313	0.2422
Genotype npr1-1	13	0.1887	0.2580	0.2500	0.2600
Genotype rar1	14	0.2501	0.3070	0.2993	0.3080
Genotype sgt1b	15	0.1253	0.2161	0.2065	0.2185
		5	6	7	8
Genotype eds7	9	*			
Genotype eds8	10	0.2862	*		
Genotype erf1	11	0.2916	0.2796	*	
Genotype ler	12	0.2352	0.2193	0.2275	*
Genotype npr1-1	13	0.2536	0.2394	0.2462	0.1755
Genotype rar1	14	0.3026	0.2893	0.2971	0.2381
Genotype sgt1b	15	0.2108	0.1931	0.2019	0.1041
		9	10	11	12
Genotype npr1-1	13	*			
Genotype rar1	14	0.2586	*		
Genotype sgt1b	15	0.1412	0.2161	*	
		13	14	15	

Figure 4.2b – Incidence of infection at the stem-pedicel junction

Regression analysis

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Binomial totals: BinN
Distribution: Binomial
Link function: Logit
Fitted terms: Constant, Rep, Genotype

Predictions from regression model

Genotype	Prediction	s.e.
185	0.01782	0.01060
116	0.18223	0.03076
138	0.06003	0.01931
175	0.15022	0.02865
177	0.02636	0.01273
192	0.04603	0.01702
194	0.00442	0.00511
232	0.12463	0.02619
243	0.08042	0.02169
352	0.15644	0.02735
368	0.06154	0.01903
442	0.33106	0.03576
535	0.03039	0.01356
895	0.04717	0.01661
935	0.10452	0.02409

Least significant differences of predictions (5% level)

Genotype 185	1	*			
Genotype 116	2	0.06390	*		
Genotype 138	3	0.04326	0.07135	*	
Genotype 175	4	0.06000	0.08259	0.06786	*

Genotype 177	5	0.03253	0.06538	0.04543	0.06158	*
Genotype 192	6	0.03938	0.06905	0.05056	0.06546	0.04174
Genotype 194	7	0.02310	0.06124	0.03923	0.05716	0.02694
Genotype 232	8	0.05549	0.07932	0.06392	0.07625	0.05719
Genotype 243	9	0.04742	0.07394	0.05705	0.07059	0.04941
Genotype 352	10	0.05762	0.08086	0.06576	0.07780	0.05926
Genotype 368	11	0.04279	0.07103	0.05327	0.06757	0.04498
Genotype 442	12	0.07325	0.09264	0.07983	0.09001	0.07456
Genotype 535	13	0.03380	0.06603	0.04635	0.06226	0.03653
Genotype 895	14	0.03871	0.06865	0.05004	0.06506	0.04111
Genotype 935	15	0.05170	0.07678	0.06064	0.07352	0.05352
		1	2	3	4	5

Genotype 192	6	*				
Genotype 194	7	0.03490	*			
Genotype 232	8	0.06134	0.05241	*		
Genotype 243	9	0.05416	0.04378	0.06680	*	
Genotype 352	10	0.06327	0.05465	0.07438	0.06858	*
Genotype 368	11	0.05015	0.03871	0.06358	0.05669	0.06544
Genotype 442	12	0.07779	0.07096	0.08706	0.08216	0.08846
Genotype 535	13	0.04274	0.02846	0.05792	0.05025	0.05996
Genotype 895	14	0.04672	0.03414	0.06092	0.05368	0.06285
Genotype 935	15	0.05794	0.04838	0.06990	0.06368	0.07159
		6	7	8	9	10

Genotype 368	11	*				
Genotype 442	12	0.07958	*			
Genotype 535	13	0.04590	0.07513	*		
Genotype 895	14	0.04961	0.07746	0.04212	*	
Genotype 935	15	0.06032	0.08470	0.05430	0.05750	*
		11	12	13	14	

15

Figure 6.1 – Fc infection of *dmr* mutants

Regression analysis

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Distribution: Poisson
Link function: Log
Fitted terms: Constant + Genotype + Time + Genotype.Time

Summary of analysis - Siliques

Predictions from regression model

Time	7		11	
	Prediction	s.e.	Prediction	s.e.
Genotype				
eds1	4.533	0.2971	5.867	0.3382
dmr1-1	4.375	0.3999	4.875	0.4221
dmr1-2	1.750	0.2521	2.750	0.3170
dmr5	2.714	0.3360	5.625	0.4535
dmr6	3.250	0.3444	4.500	0.4055
Ler	4.250	0.5572	5.750	0.6484

Time	14
Prediction	s.e.

Genotype		
eds1	6.400	0.3533
dmr1-1	6.000	0.4683
dmr1-2	4.750	0.4167
dmr5	6.875	0.5014
dmr6	5.625	0.4535
Ler	7.000	0.7154

Least significant differences of predictions (5% level)

Genotype eds1 Time 7	1	*			
Genotype eds1 Time 11	2	0.890	*		
Genotype eds1 Time 14	3	0.913		*	
Genotype dmr1-1 Time 7	4	0.985	1.036	1.055	*
Genotype dmr1-1 Time 11	5	1.021	1.070	1.089	1.150
Genotype dmr1-1 Time 14	6	1.097	1.143	1.160	1.218
Genotype dmr1-2 Time 7	7	0.771	0.834	0.858	0.935
Genotype dmr1-2 Time 11	8	0.859	0.917	0.939	1.009
Genotype dmr1-2 Time 14	9	1.012	1.061	1.080	1.142
Genotype dmr5 Time 7	10	0.887	0.943	0.964	1.033
Genotype dmr5 Time 11	11	1.072	1.119	1.137	1.196
Genotype dmr5 Time 14	12	1.153	1.196	1.213	1.268
Genotype dmr6 Time 7	13	0.899	0.955	0.976	1.044
Genotype dmr6 Time 11	14	0.994	1.044	1.064	1.126
Genotype dmr6 Time 14	15	1.072	1.119	1.137	1.196
Genotype Ler Time 7	16	1.249	1.289	1.305	1.357
Genotype Ler Time 11	17	1.411	1.446	1.460	1.507
Genotype Ler Time 14	18	1.532	1.565	1.578	1.621
		1	2	3	4

Genotype dmr1-1 Time 11	5	*			
Genotype dmr1-1 Time 14	6	1.247	*		
Genotype dmr1-2 Time 7	7	0.972	1.052	*	
Genotype dmr1-2 Time 11	8	1.044	1.119	0.801	*
Genotype dmr1-2 Time 14	9	1.173	1.240	0.963	1.035
Genotype dmr5 Time 7	10	1.067	1.140	0.831	0.914
Genotype dmr5 Time 11	11	1.225	1.289	1.026	1.094
Genotype dmr5 Time 14	12	1.296	1.357	1.110	1.173
Genotype dmr6 Time 7	13	1.077	1.150	0.844	0.926
Genotype dmr6 Time 11	14	1.158	1.225	0.944	1.018
Genotype dmr6 Time 14	15	1.225	1.289	1.026	1.094
Genotype Ler Time 7	16	1.383	1.440	1.210	1.268
Genotype Ler Time 11	17	1.530	1.582	1.376	1.428
Genotype Ler Time 14	18	1.643	1.691	1.500	1.548
		5	6	7	8

Genotype dmr1-2 Time 14	9	*			
Genotype dmr5 Time 7	10	1.059	*		
Genotype dmr5 Time 11	11	1.218	1.116	*	
Genotype dmr5 Time 14	12	1.289	1.194	1.337	*
Genotype dmr6 Time 7	13	1.069	0.952	1.126	1.203
Genotype dmr6 Time 11	14	1.150	1.042	1.203	1.275
Genotype dmr6 Time 14	15	1.218	1.116	1.268	1.337
Genotype Ler Time 7	16	1.376	1.287	1.421	1.483
Genotype Ler Time 11	17	1.524	1.444	1.565	1.621
Genotype Ler Time 14	18	1.638	1.563	1.675	1.728
		9	10	11	12

Genotype dmr6 Time 7	13	*			
Genotype dmr6 Time 11	14	1.052	*		
Genotype dmr6 Time 14	15	1.126	1.203	*	
Genotype Ler Time 7	16	1.296	1.363	1.421	*

Genotype Ler	Time 11	17	1.452	1.513	1.565	1.691
Genotype Ler	Time 14	18	1.570	1.626	1.675	1.794
			13	14	15	16

Genotype Ler	Time 11	17	*
Genotype Ler	Time 14	18	1.910
			17
			18

Summary of analysis - Green Siliques

Predictions from regression model

Time	7		11	
	Prediction	s.e.	Prediction	s.e.
Genotype				
eds1	0.6250	0.1561	0.0667	0.0526
dmr1-1	0.7500	0.2418	0.5000	0.1974
dmr1-2	1.6250	0.3559	1.5000	0.3419
dmr5	0.8750	0.2612	0.1250	0.0987
dmr6	3.6250	0.5316	1.5000	0.3419
Ler	0.7500	0.3419	0.5000	0.2792

Time	14	
	Prediction	s.e.
Genotype		
eds1	0.0001	0.0010
dmr1-1	0.0001	0.0013
dmr1-2	0.6250	0.2207
dmr5	0.0001	0.0013
dmr6	0.2500	0.1396
Ler	0.0001	0.0019

Least significant differences of predictions (5% level)

Genotype eds1	Time 7	1	*		
Genotype eds1	Time 11	2	0.3257	*	
Genotype eds1	Time 14	3	0.3087	0.1041	*
Genotype dmr1-1	Time 7	4	0.5691	0.4894	0.4782
Genotype dmr1-1	Time 11	5	0.4977	0.4041	0.3904
Genotype dmr1-1	Time 14	6	0.3087	0.1041	0.0033
Genotype dmr1-2	Time 7	7	0.7685	0.7115	0.7038
Genotype dmr1-2	Time 11	8	0.7433	0.6842	0.6762
Genotype dmr1-2	Time 14	9	0.5346	0.4487	0.4365
Genotype dmr5	Time 7	10	0.6017	0.5269	0.5165
Genotype dmr5	Time 11	11	0.3652	0.2212	0.1952
Genotype dmr5	Time 14	12	0.3087	0.1041	0.0033
Genotype dmr6	Time 7	13	1.0956	1.0564	1.0512
Genotype dmr6	Time 11	14	0.7433	0.6842	0.6762
Genotype dmr6	Time 14	15	0.4141	0.2950	0.2761
Genotype Ler	Time 7	16	0.7433	0.6842	0.6762
Genotype Ler	Time 11	17	0.6325	0.5619	0.5521
Genotype Ler	Time 14	18	0.3087	0.1042	0.0042
			1	2	3

Genotype dmr1-1	Time 11	5	*		
Genotype dmr1-1	Time 14	6	0.3904	*	
Genotype dmr1-2	Time 7	7	0.8049	0.7038	*
Genotype dmr1-2	Time 11	8	0.7808	0.6762	0.9760
Genotype dmr1-2	Time 14	9	0.5856	0.4365	0.8282
Genotype dmr5	Time 7	10	0.6474	0.5165	0.8730
Genotype dmr5	Time 11	11	0.4365	0.1952	0.7304
Genotype dmr5	Time 14	12	0.3904	0.0037	0.7038
Genotype dmr6	Time 7	13	1.1214	1.0512	1.2651
					1.2499

Genotype dmr6 Time 11	14	0.7808	0.6762	0.9760	0.9563
Genotype dmr6 Time 14	15	0.4782	0.2761	0.7560	0.7304
Genotype Ler Time 7	16	0.7808	0.6762	0.9760	0.9563
Genotype Ler Time 11	17	0.6762	0.5521	0.8945	0.8730
Genotype Ler Time 14	18	0.3904	0.0046	0.7038	0.6762
		5	6	7	8

Genotype dmr1-2 Time 14	9	*			
Genotype dmr5 Time 7	10	0.6762	*		
Genotype dmr5 Time 11	11	0.4782	0.5521	*	
Genotype dmr5 Time 14	12	0.4365	0.5165	0.1952	*
Genotype dmr6 Time 7	13	1.1382	1.1712	1.0692	1.0512
Genotype dmr6 Time 11	14	0.8049	0.8509	0.7038	0.6762
Genotype dmr6 Time 14	15	0.5165	0.5856	0.3381	0.2761
Genotype Ler Time 7	16	0.8049	0.8509	0.7038	0.6762
Genotype Ler Time 11	17	0.7038	0.7560	0.5856	0.5521
Genotype Ler Time 14	18	0.4365	0.5165	0.1952	0.0046
		9	10	11	12

Genotype dmr6 Time 7	13	*			
Genotype dmr6 Time 11	14	1.2499	*		
Genotype dmr6 Time 14	15	1.0869	0.7304	*	
Genotype Ler Time 7	16	1.2499	0.9563	0.7304	*
Genotype Ler Time 11	17	1.1874	0.8730	0.6173	0.8730
Genotype Ler Time 14	18	1.0512	0.6762	0.2761	0.6762
		13	14	15	16

Genotype Ler Time 11	17	*	
Genotype Ler Time 14	18	0.5521	*
		17	18

Summary of analysis - infected leaves

Predictions from regression model

Time	7		11	
	Prediction	s.e.	Prediction	s.e.
Genotype				
eds1	3.375	0.4282	3.500	0.4361
dmr1-1	1.125	0.2472	1.500	0.2855
dmr1-2	0.375	0.1425	1.750	0.3083
dmr5	4.250	0.4805	6.500	0.5942
dmr6	*	*	*	*
Ler	3.500	0.6167	5.250	0.7553

Time	14	
	Prediction	s.e.
Genotype		
eds1	4.750	0.5080
dmr1-1	3.000	0.4037
dmr1-2	2.875	0.3952
dmr5	9.000	0.6992
dmr6	*	*
Ler	8.250	0.9468

Least significant differences of predictions (5% level)

Genotype eds1 Time 7	1	*
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Genotype eds1	Time 11	2	1.214	*		
Genotype eds1	Time 14	3	1.319	1.329	*	
Genotype dmr1-1	Time 7	4	0.982	0.995	1.122	*
Genotype dmr1-1	Time 11	5	1.022	1.035	1.157	0.750
Genotype dmr1-1	Time 14	6	1.169	1.180	1.289	0.940
Genotype dmr1-2	Time 7	7	0.896	0.911	1.048	0.567
Genotype dmr1-2	Time 11	8	1.048	1.061	1.180	0.785
Genotype dmr1-2	Time 14	9	1.157	1.169	1.278	0.926
Genotype dmr5	Time 7	10	1.278	1.289	1.389	1.073
Genotype dmr5	Time 11	11	1.454	1.464	1.552	1.278
Genotype dmr5	Time 14	12	1.628	1.636	1.716	1.473
Genotype dmr6	Time 7	13	*	*	*	*
Genotype dmr6	Time 11	14	*	*	*	*
Genotype dmr6	Time 14	15	*	*	*	*
Genotype Ler	Time 7	16	1.491	1.500	1.587	1.319
Genotype Ler	Time 11	17	1.724	1.732	1.808	1.578
Genotype Ler	Time 14	18	2.063	2.070	2.134	1.943
			1	2	3	4

Genotype dmr1-1	Time 11	5	*			
Genotype dmr1-1	Time 14	6	0.982	*		
Genotype dmr1-2	Time 7	7	0.634	0.850	*	
Genotype dmr1-2	Time 11	8	0.834	1.009	0.675	*
Genotype dmr1-2	Time 14	9	0.968	1.122	0.834	0.995
Genotype dmr5	Time 7	10	1.110	1.246	0.995	1.134
Genotype dmr5	Time 11	11	1.309	1.427	1.214	1.329
Genotype dmr5	Time 14	12	1.500	1.603	1.417	1.518
Genotype dmr6	Time 7	13	*	*	*	*
Genotype dmr6	Time 11	14	*	*	*	*
Genotype dmr6	Time 14	15	*	*	*	*
Genotype Ler	Time 7	16	1.349	1.464	1.257	1.369
Genotype Ler	Time 11	17	1.603	1.701	1.526	1.620
Genotype Ler	Time 14	18	1.964	2.044	1.901	1.977
			5	6	7	8

Genotype dmr1-2	Time 14	9	*			
Genotype dmr5	Time 7	10	1.235	*		
Genotype dmr5	Time 11	11	1.417	1.518	*	
Genotype dmr5	Time 14	12	1.595	1.685	1.822	*
Genotype dmr6	Time 7	13	*	*	*	*
Genotype dmr6	Time 11	14	*	*	*	*
Genotype dmr6	Time 14	15	*	*	*	*
Genotype Ler	Time 7	16	1.454	1.552	1.701	1.851
Genotype Ler	Time 11	17	1.693	1.778	1.908	2.044
Genotype Ler	Time 14	18	2.037	2.108	2.220	2.337
			9	10	11	12

Genotype dmr6	Time 7	13	*			
Genotype dmr6	Time 11	14	*	*		
Genotype dmr6	Time 14	15	*	*	*	
Genotype Ler	Time 7	16	*	*	*	*
Genotype Ler	Time 11	17	*	*	*	1.936
Genotype Ler	Time 14	18	*	*	*	2.244
			13	14	15	16

Genotype Ler	Time 11	17	*	
Genotype Ler	Time 14	18	2.405	*
			17	18

Figure 6.3 – Fg infection of *dmr1* mutant alleles

Regression analysis

=====

Distribution: Poisson
Link function: Log
Fitted terms: Constant + Genotype + Time + Genotype.Time

Summary of analysis - Green siliques

Predictions from regression model

Response variate: G

	TIME	7		11	
		Prediction	s.e.	Prediction	s.e.
Genotype					
eds1		0.6250	0.1637	0.3750	0.1268
dmr1-1		2.5714	0.3549	2.2500	0.3105
dmr1-2		0.5000	0.1464	0.0000	0.0004

	TIME	14	
		Prediction	s.e.
Genotype			
eds1		0.0000	0.0004
dmr1-1		0.0000	0.0004
dmr1-2		0.0000	0.0004

Least significant differences of predictions (5% level)

Genotype eds1	TIME 7	1	*			
Genotype eds1	TIME 11	2	0.4138	*		
Genotype eds1	TIME 14	3	0.3272	0.2534	*	
Genotype dmr1-1	TIME 7	4	0.7813	0.7534	0.7094	*
Genotype dmr1-1	TIME 11	5	0.7017	0.6705	0.6208	0.9427
Genotype dmr1-1	TIME 14	6	0.3272	0.2534	0.0010	0.7094
Genotype dmr1-2	TIME 7	7	0.4389	0.3871	0.2926	0.7674
Genotype dmr1-2	TIME 11	8	0.3272	0.2534	0.0010	0.7094
Genotype dmr1-2	TIME 14	9	0.3272	0.2534	0.0010	0.7094
			1	2	3	4

Genotype dmr1-1	TIME 11	5	*			
Genotype dmr1-1	TIME 14	6	0.6208	*		
Genotype dmr1-2	TIME 7	7	0.6863	0.2926	*	
Genotype dmr1-2	TIME 11	8	0.6208	0.0010	0.2926	*
Genotype dmr1-2	TIME 14	9	0.6208	0.0010	0.2926	0.0010
			5	6	7	8

Genotype dmr1-2	TIME 14	9	*			
			9			

Summary of analysis - infected leaves

Predictions from regression model

Response variate: IL

	Prediction	s.e.
--	------------	------

Genotype			
eds1	5.016	0.2647	
dmr1-1	2.516	0.1874	
dmr1-2	2.765	0.1997	

Least significant differences of predictions (5% level)

Genotype eds1	1		*		
Genotype dmr1-1	2	0.6483		*	
Genotype dmr1-2	3	0.6628	0.5475		*
		1	2		3

Summary of analysis - Siliques

Predictions from regression model

Response variate: S

TIME	7		11	
	Prediction	s.e.	Prediction	s.e.
Genotype				
eds1	4.875	0.3741	6.000	0.4150
dmr1-1	4.875	0.3741	5.625	0.4018
dmr1-2	1.571	0.2270	2.500	0.2679

TIME	14	
	Prediction	s.e.
Genotype		
eds1	7.000	0.4483
dmr1-1	6.625	0.4361
dmr1-2	5.500	0.3973

Least significant differences of predictions (5% level)

Genotype eds1	TIME 7	1		*			
Genotype eds1	TIME 11	2	1.117		*		
Genotype eds1	TIME 14	3	1.167	1.221		*	
Genotype dmr1-1	TIME 7	4	1.058	1.117	1.167		*
Genotype dmr1-1	TIME 11	5	1.097	1.155	1.203	1.097	
Genotype dmr1-1	TIME 14	6	1.148	1.203	1.250	1.148	
Genotype dmr1-2	TIME 7	7	0.875	0.946	1.004	0.875	
Genotype dmr1-2	TIME 11	8	0.920	0.987	1.044	0.920	
Genotype dmr1-2	TIME 14	9	1.091	1.148	1.197	1.091	
			1	2	3		4

Genotype dmr1-1	TIME 11	5		*			
Genotype dmr1-1	TIME 14	6	1.185		*		
Genotype dmr1-2	TIME 7	7	0.923	0.983		*	
Genotype dmr1-2	TIME 11	8	0.965	1.023	0.702		*
Genotype dmr1-2	TIME 14	9	1.130	1.179	0.915	0.958	
			5	6	7		8

Genotype dmr1-2	TIME 14	9		*			
				9			

Figure 6.4 – Fc infection of *dmr1* mutant alleles

Regression analysis

=====

Distribution: Poisson

Link function: Log

Fitted terms: Constant + Exp + Geno + Time + Geno.Time

Summary of analysis - siliques

Predictions from regression model

Response variate: S

	Prediction	s.e.
Geno		
eds1-2	5.453	0.2703
dmr1-1	4.970	0.3870
dmr1-2	3.313	0.3113
dmr1-3	4.313	0.2401
dmr1-4	4.264	0.2387
dmr1-6	4.710	0.2510

Least significant differences of predictions (5% level)

Geno eds1-2	1							*
Geno dmr1-1	2	0.9519						*
Geno dmr1-2	3	0.8299	0.9537					*
Geno dmr1-3	4	0.7149	0.9164	0.7900				*
Geno dmr1-4	5	0.7131	0.9148	0.7882	0.6697			*
Geno dmr1-6	6	0.7292	0.9289	0.8041	0.6870	0.6851		*
		1	2	3	4	5		

Geno dmr1-6	6							*
								6

Summary of analysis - infected leaves

Predictions from regression model

Response variate: IL

	Time	7		13	
		Prediction	s.e.	Prediction	s.e.
Geno					
eds1-2		3.580	0.3788	5.612	0.4750
dmr1-1		0.693	0.2578	3.465	0.5879
dmr1-2		0.924	0.2984	3.003	0.5457
dmr1-3		2.225	0.2984	4.741	0.4363
dmr1-4		0.581	0.1522	3.870	0.3940
dmr1-6		2.612	0.3234	5.418	0.4667

Least significant differences of predictions (5% level)

Geno eds1-2 Time	7	1						*
Geno eds1-2 Time	13	2	1.204					*
Geno dmr1-1 Time	7	3	0.914	1.078				*
Geno dmr1-1 Time	13	4	1.400	1.516	1.266			*
Geno dmr1-2 Time	7	5	0.962	1.120	0.780	1.298		
Geno dmr1-2 Time	13	6	1.329	1.451	1.190	1.560		
Geno dmr1-3 Time	7	7	0.957	1.112	0.786	1.317		
Geno dmr1-3 Time	13	8	1.145	1.277	1.011	1.467		

Geno	dmr1-4	Time	7	9	0.811	0.991	0.596	1.209
Geno	dmr1-4	Time	13	10	1.083	1.223	0.939	1.417
Geno	dmr1-6	Time	7	11	0.988	1.139	0.824	1.341
Geno	dmr1-6	Time	13	12	1.191	1.318	1.064	1.506
					1	2	3	4
Geno	dmr1-2	Time	7	5	*			
Geno	dmr1-2	Time	13	6	1.224	*		
Geno	dmr1-3	Time	7	7	0.842	1.242	*	
Geno	dmr1-3	Time	13	8	1.055	1.400	1.048	*
Geno	dmr1-4	Time	7	9	0.667	1.128	0.665	0.918
Geno	dmr1-4	Time	13	10	0.986	1.347	0.980	1.165
Geno	dmr1-6	Time	7	11	0.878	1.267	0.873	1.077
Geno	dmr1-6	Time	13	12	1.106	1.440	1.098	1.265
					5	6	7	8
Geno	dmr1-4	Time	7	9	*			
Geno	dmr1-4	Time	13	10	0.839	*		
Geno	dmr1-6	Time	7	11	0.710	1.011	*	
Geno	dmr1-6	Time	13	12	0.975	1.210	1.126	*
					9	10	11	12

Figure 6.8 – Amino acid treatment (DHS, LHS, THR)

Regression analysis

=====

Distribution: Poisson

Link function: Log

Fitted terms: Constant + Treat + block + Treat.block

Predictions from regression model

Response variate: Buds

	Prediction	s.e.
Treat		
Water	3.484	0.3523
DHS	4.266	0.3915
LHS	0.234	0.0886
THR	4.219	0.4111

Least significant differences of predictions (5% level)

Treat	Water	1	*			
Treat	DHS	2	1.1166	*		
Treat	LHS	3	0.7701	0.8509	*	
Treat	THR	4	1.1478	1.2035	0.8916	*
			1	2	3	4

Predictions from regression model

Response variate: Flowers

	Prediction	s.e.
Treat		
Water	4.016	0.2917
DHS	4.375	0.3040
LHS	2.500	0.2293
THR	5.000	0.3385

Least significant differences of predictions (5% level)

Treat Water	1	*			
Treat DHS	2	0.8930	*		
Treat LHS	3	0.7865	0.8072	*	
Treat THR	4	0.9472	0.9644	0.8667	*
		1	2	3	4

Predictions from regression model

Response variate: Siliques

	Prediction	s.e.
Treat		
Water	4.016	0.1295
DHS	5.234	0.1473
LHS	5.141	0.1464
THR	5.000	0.1502

Least significant differences of predictions (5% level)

Treat Water	1	*			
Treat DHS	2	0.4158	*		
Treat LHS	3	0.4144	0.4402	*	
Treat THR	4	0.4204	0.4459	0.4446	*
		1	2	3	4

Predictions from regression model

Response variate: Infected Leaves

	Prediction	s.e.
Treat		
Water	2.359	0.2686
DHS	2.500	0.2774
LHS	2.500	0.2773
THR	4.344	0.3716

Least significant differences of predictions (5% level)

Treat Water	1	*			
Treat DHS	2	0.8186	*		
Treat LHS	3	0.8185	0.8315	*	
Treat THR	4	0.9720	0.9830	0.9829	*
		1	2	3	4

Figure 6.10 – HS silique treatment

Analysis of variance
=====

Tables of means
=====

Variate: dist

Grand mean 6.65

Treatment	DHS	LHS	Water
	7.04	3.22	9.68

Standard errors of means

Table	Treatment
rep.	12
d.f.	31
e.s.e.	0.666

Least significant differences of means (1% level)

Table	Treatment
rep.	12
d.f.	31
l.s.d.	2.585

Figure 6.12 LHS on *eds1-2* and *dmr1-2*

Regression analysis
=====

Distribution: Poisson
Link function: Log
Fitted terms: Constant + Genotype + treatment + Genotype.treatment

Summary of analysis – BUDS

Predictions from regression model

treatment	DHS		LHS	
	Prediction	s.e.	Prediction	s.e.
Genotype				
dmr1-2	3.750	0.6651	1.250	0.3839
eds1-2	3.875	0.6760	1.875	0.4703

treatment	Water	
	Prediction	s.e.
Genotype		
dmr1-2	3.667	0.7594
eds1-2	3.833	0.7764

Least significant differences of predictions (5% level)

Genotype dmr1-2 treatment DHS 1 *

Genotype dmr1-2 treatment LHS	2	1.555	*	
Genotype dmr1-2 treatment Water	3	2.043	1.723	*
Genotype eds1-2 treatment DHS	4	1.920	1.574	2.058
Genotype eds1-2 treatment LHS	5	1.649	1.229	1.808
Genotype eds1-2 treatment Water	6	2.070	1.753	2.199
		1	2	3
Genotype eds1-2 treatment DHS	4	*		
Genotype eds1-2 treatment LHS	5	1.667	*	
Genotype eds1-2 treatment Water	6	2.084	1.838	*
		4	5	6

Summary of analysis - SILIQUES

Predictions from regression model

Response variate: S

treatment	DHS		LHS	
	Prediction	s.e.	Prediction	s.e.
Genotype				
dmr1-2	3.000	0.3682	1.750	0.2805
eds1-2	5.125	0.4816	4.125	0.4319

treatment	Water	
	Prediction	s.e.
Genotype		
dmr1-2	2.333	0.3747
eds1-2	5.000	0.5492

Least significant differences of predictions (5% level)

Genotype dmr1-2 treatment DHS	1	*		
Genotype dmr1-2 treatment LHS	2	0.937	*	
Genotype dmr1-2 treatment Water	3	1.063	0.947	*
Genotype eds1-2 treatment DHS	4	1.227	1.128	1.235
Genotype eds1-2 treatment LHS	5	1.149	1.043	1.157
Genotype eds1-2 treatment Water	6	1.339	1.248	1.346
		1	2	3
Genotype eds1-2 treatment DHS	4	*		
Genotype eds1-2 treatment LHS	5	1.310	*	
Genotype eds1-2 treatment Water	6	1.479	1.414	*
		4	5	6

Figure 6.13 morphology and senescence

Analysis of Variance - Rosette Diameter

=====

Variate: Rosette Diameter

Grand mean 75.1

Genotype	dmr1-1	dmr1-2	eds1
	77.9	61.0	86.4

Standard errors of differences of means

Table	Genotype
rep.	9
d.f.	24
s.e.d.	3.85

Least significant differences of means (5% level)

Table	Genotype
rep.	9
d.f.	24
l.s.d.	7.94

Least significant differences of means (1% level)

Table	Genotype
rep.	9
d.f.	24
l.s.d.	10.76

Regression analysis - Senescent leaves

=====

Distribution: Poisson

Link function: Log

Fitted terms: Constant + Genotype + Day + Genotype.Day

Predictions from regression model

Prediction	s.e.
Genotype	
dmr1-1	2.542 0.2507
dmr1-2	1.833 0.2129
eds1	4.500 0.3335

Least significant differences of predictions (5% level)

Genotype dmr1-1	1	*		
Genotype dmr1-2	2	0.6571	*	
Genotype eds1	3	0.8337	0.7907	*
		1	2	3

Figure 6.14 Apogee *F. graminearum* HS

Analysis of variance
=====

Variate: spikelets

Tables of means
=====

Grand mean 8.5714

TREATMENTB	DHS	H2O	LHS
mean	9.0000	9.4000	7.4000
rep.	4	5	5

Standard errors of differences of means

TREATMENTB	DHS	1	*		
TREATMENTB	H2O	2	0.7122	*	
TREATMENTB	LHS	3	0.7122	0.6715	*
			1	2	3

Minimum standard error of difference	0.6715
Average standard error of difference	0.6987
Maximum standard error of difference	0.7122

Least significant differences (at 5%)

1	*		
2	1.5676	*	
3	1.5676	1.4780	*
	1	2	3

Minimum least significant difference	1.478
Average least significant difference	1.538
Maximum least significant difference	1.568

Figure 7.4 – *f6'H1* and Col-0 silique infection *F. culmorum* and *F. graminearum*

Analysis of variance
=====

Tables of means
=====

Variate: infection

Grand mean 9.37

Arabidopsis	Col0	F6H1
	9.35	9.39

Fusarium	Fc	Fg
	9.86	8.88

Arabidopsis	Fusarium	Fc	Fg
-------------	----------	----	----

Col0	9.91	8.78
F6H1	9.80	8.98

Standard errors of means

Table	Arabidopsis	Fusarium	Arabidopsis Fusarium
rep.	14	14	7
d.f.	24	24	24
e.s.e.	0.227	0.227	0.320

Least significant differences of means (5% level)

Table	Arabidopsis	Fusarium	Arabidopsis Fusarium
rep.	14	14	7
d.f.	24	24	24
l.s.d.	0.661	0.661	0.935

Figure 7.5 – The effect of *erecta*

Regression analysis
=====

Response variate: Flowers
Distribution: Poisson
Link function: Log
Fitted terms: Constant + Genotype + Day + Tray + Genotype.Day +
Genotype.Tray + Day.Tray + Genotype.Day.Tray

Predictions from regression model

Response variate: Flowers

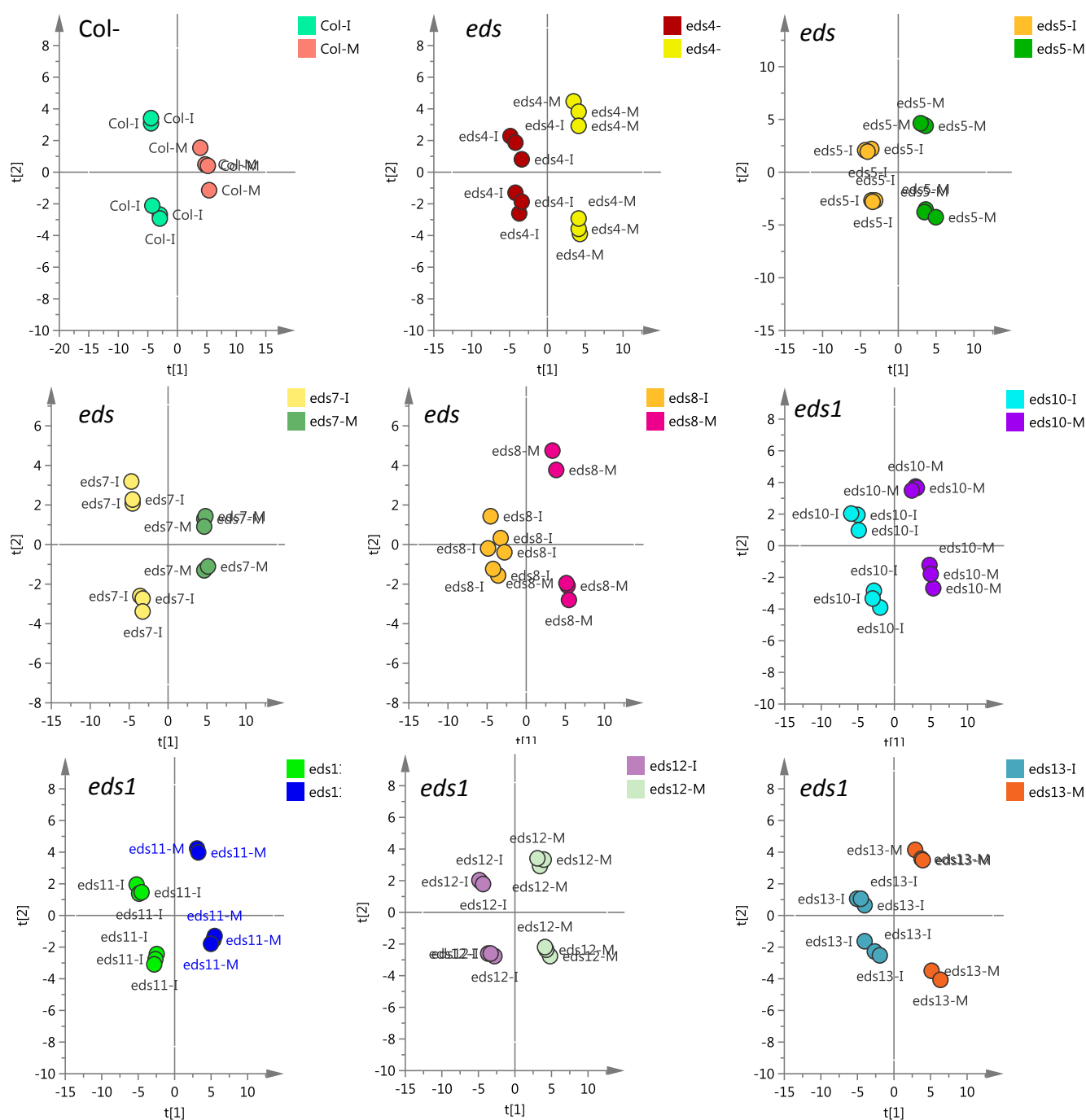
	Prediction	s.e.
Genotype		
Col0	0.000	0.0001
Coler	4.437	0.1407
dmr1-2	5.812	0.1611
eds1-1	5.375	0.1549

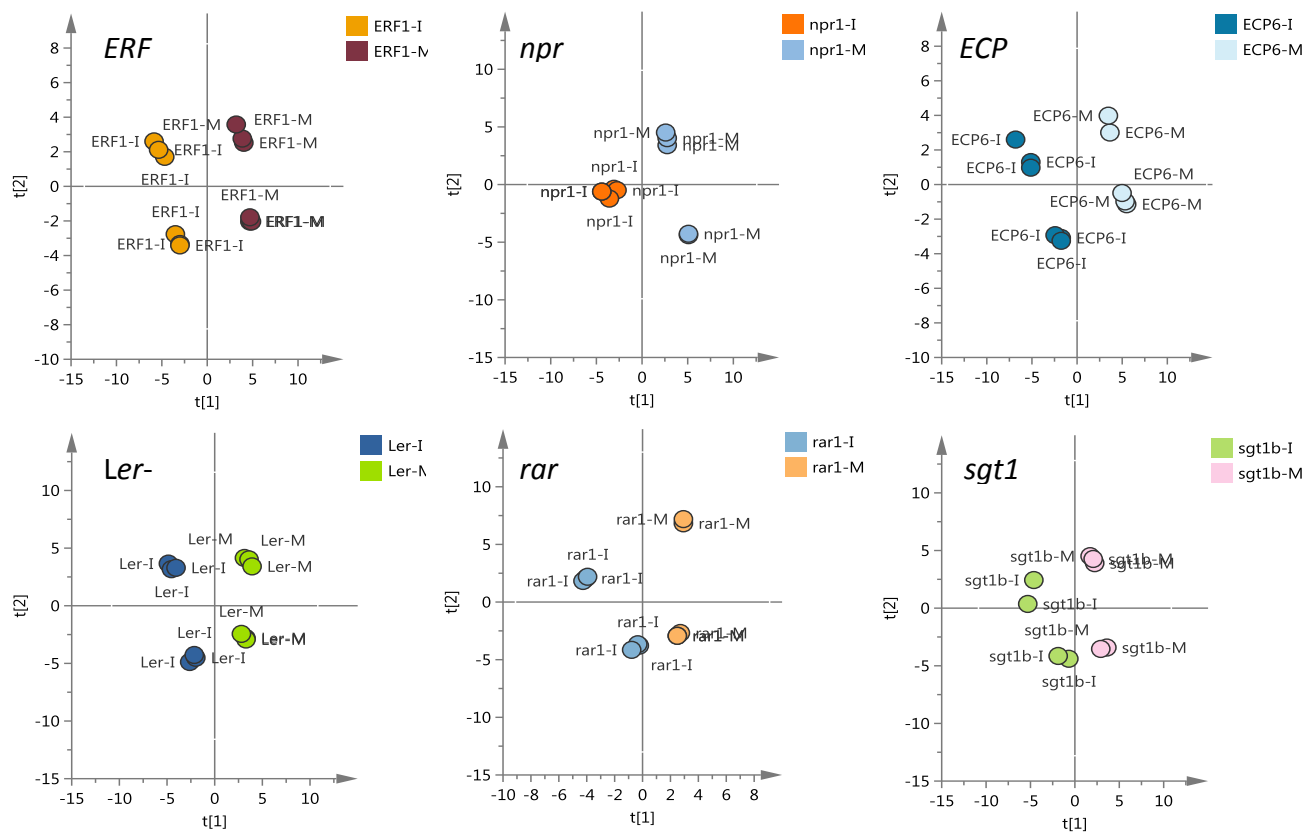
Least significant differences of predictions (5% level)

Genotype Col0	1		*		
Genotype Coler	2	0.2829		*	
Genotype dmr1-2	3	0.3238	0.4300		*
Genotype eds1-1	4	0.3114	0.4207	0.4493	*
		1	2	3	4

Appendix 6: PCA plots of individual genotypes

PCA plots of metabolic profiles of mock vs *F. culmorum* infected pedicel-stem junctions of 15 Arabidopsis genotypes. PCA constructed from ¹H-NMR data using extracted regions of known characteristic regions. Models constructed using unit variance scaling. Generated by Jane Ward, MeT-RO.





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