The University Of Sheffield.

Interactions between stomatal density and plant disease

By

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Abstract

The work in this thesis explores the interaction between stomata and pathogen infection. Previous research in this area has focussed on the short-term response of stomatal closure in response to pathogen infection. The work presented here examines how stomatal density affects the rate at which pathogens colonise the plant, and further explores how plants regulate the formation of new stomatal openings following pathogen infection.

Using genetically engineered *Arabidopsis* and wheat plants which are altered in one or more components of the stomatal development pathway, it was shown that bacterial and fungal colonisation by stomatal pathogens is significantly affected by the stomatal density. Localised infection of *Arabidopsis* plants with *Pseudomonas syringae* pv. DC3000 was used to examine the effect of pathogen infection on the formation of stomata. Local infection was found to significantly reduce the frequency of stomata on leaves that develop following local infection of mature leaves. This is possibly an adaptive response used by plants living in environments with high risks of pathogen infection. The signalling pathway responsible for this change was examined by infiltrating a variety of *Arabidopsis* defence mutants with *Pst*DC3000, or infiltrating wild type Arabidopsis with chemical defence elicitors or signals. It was found that detection of flagellin by the FLS2 receptor, salicylic acid production and salicylic acid accumulation are required for the reduction in stomatal density.

In a separate study, an *Arabidopsis* mutant lacking CYP86A2 was found to have enhanced drought tolerance and altered tolerance to both necrotrophic and biotrophic pathogens.

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Chapter 1: Introduction

1.1 Plant pathogen infection

Numerous micro-organisms have the capacity to infect plants. Some of these are symbiotic, but many of them are pathogenic and cause disease. Depending on the plant species, plant pathogens include bacteria, fungi, viruses and oomycetes. Disease by pathogens affects all land plants. Once infected, the pathogen uses the plant's resources to feed, grow and reproduce, which is detrimental to the host plant. It is estimated that global crop losses to microbes can be as high as 24% yearly (Oerke, 2006). To infect, a pathogen must first overcome physical barriers that restrict entry into the host plant, such as rigid cell walls or a thick waxy cuticle. Over evolutionary time pathogens have evolved diverse colonisation methods to subvert these. Some pathogenic fungi and oomycetes overcome these pre-existing physical barriers by producing specialised structures called appressoria, which force their way through, or penetrate the cell wall, whilst others have biflagellate spores that target and penetrate vulnerable root hairs (Kageyama & Asano, 2009). Other pathogenic bacteria and fungi that cannot force their way into the host tissue, enter through wound sites or pre-existing natural openings, such as stomatal pores (described in section 1.2). Stomata also provide entry points for bacterial, fungal and oomycete pathogens. For instance, germ tubes of the oomycete pathogen *Plasmopara viticola* grow towards stomatal openings, where they enter through the pore and proliferate into the leaf (Espino & Nesbitt 1982), whilst terminating spores of biotrophic rust fungi are known to extend their hyphae through stomatal pores into the substomatal cavity of grasses. Rust causing pathogens infect important crop plants, such as wheat and barley, through the stomatal pores and can cause major crop losses (Staples, 2000, Mendgen *et al*., 2006).

This thesis explores the role of stomatal openings in resistance against leaf pathogens. Since the discovery that some plant species close their stomatal pores upon recognition of microbial pathogens (Melloto *et al.,* 2006), much research has focused on the molecular mechanisms by which plants regulate this stomatal immune response. Although research into the molecular mechanisms of

pathogen-induced stomatal closure is discussed in this chapter, the research presented in the following chapters concerns a much less well-studied longer-term response to localised pathogen attack, which results in reduced stomatal development in newly developing leaves, as well as the potential role of guard cells in enhancing disease resistance in plants. Both areas have received relatively little or no previous attention. However, several lines of evidence point to the induction of long-term changes in stomatal development upon localised pathogen infection. Furthermore, there is some evidence that guard cell-expressed gene products confer enhanced resistance to bacterial pathogens (Xiao *et al.,* 2004).

1.2 Stomatal function & Physiology

Stomata are microscopic pores on the epidermal surface of land plants that control the level of gas exchange between the plant and the surrounding atmosphere. Each pore overlies an air cavity inside the leaf, where gaseous exchange occurs, the pore aperture is regulated by a pair of guard cells. Alterations in stomatal apertures are regulated by the osmotic/turgor pressure of the two guard cells, which flank each stomatal opening. The turgor pressure of the guard cells determines the stomatal aperture. If the guard cells are flaccid the stomata are closed; if the guard cells are turgid, the stomata are open. The stomatal aperture responds to a number of environmental stimuli, opening in the light and under low CO_2 conditions, and closing in response to dark, high CO_2, drought and pathogen infection (Figure 1).

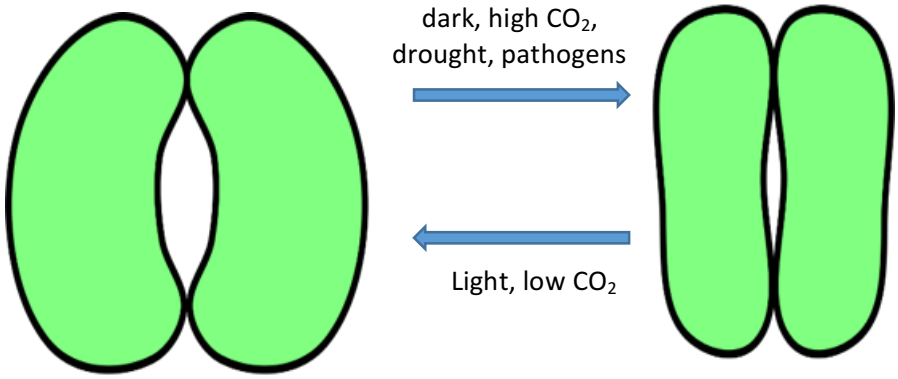


Figure 1.1. the effect of environmental stimuli on stomatal aperture. Figure shows ABA, CO_2, and dark conditions closing guard cells whilst light and low CO_2 acts to open stomatal pores.

The aperture of stomatal pores is controlled by exchange of Cl^- ions, K^+ ions, and Ca^{2+} ions across the plasma and tonoplast membrane of the guard cells. In response to drought conditions, the movement of guard cells is controlled by the abiotic stress hormone abscisic acid (ABA). Under drought conditions, ABA activates slow release (S-type) ion channels such as SLAC1, causing the efflux of Cl^- ions from the guard cells (Vahisalu *et al.,* 2008). This depolarises the plasma membrane, causing activation of K^+ ion channels and subsequence efflux of K^+ ions out of the guard cell, as well as the release of Ca^{2+} ions sequestered in the vacuole (Rolfsema *et al.,* 2004). These events result in a decreased osmotic water potential of the cell, causing reduced guard cell turgor pressure and a closing of the stomatal pore. Conversely, when Cl^- ions and K^+ move into the guard cell and Ca^{2+} ions out of the cell or into the vacuole (Rolfsema *et al.,* 2004), the cell turgor pressure increases through an increase in osmotic water potential, resulting in the opening the stomatal pore.

Much is known about how ABA is perceived and the downstream signalling pathway that results in guard cell turgor loss. ABA is detected by the PY/PYR/RCAR family of ABA-binding proteins, which form ABA-receptor complexes that induce the transcription of ABA responsive genes and activate a range of adaptive responses to (a)biotic stress (Park *et al.,* 2009). Binding of ABA to its receptors results in inactivation of the type 2 protein phosphatases ABI1 and ABI2. The inactivation of these two

phosphatases results in the activation of the OPEN STOMATA 1 (OST1) kinase, which in turn induces S-type anion channels that are responsible for the stomatal closure.

Stomatal apertures are adjusted in response to a variety of environmental stimuli, including water availability, light intensity, humidity and also in the presence of some pathogens. The mechanisms by which stomata close after pathogen attack are discussed below in section 1.3.

Several methods have been developed to study stomatal physiology. These include electrophysiology to measure guard cell ion channel activity, direct microscopic measurements of stomatal apertures using isolated epidermal strips, infra-red gas exchange analysis to measure water loss and CO_2 uptake from leaves, and infra-red thermography using leaf temperature as a proxy to estimate water loss. Each of these methods allows stomatal activity to be quantified and can be used to assess the impacts of biotic and abiotic signals on stomatal closure.

1.3 Stomatal immunity

In 2006, Melotto *et al.,* discovered a previously unknown function of stomata in defence against pathogenic bacteria. Melotto's study showed that the stomata of *Arabidopsis thaliana*, were not passive entry points, but that they respond within 1-2 hours after inoculation with *Pseudomonas syringae* (*Pst*) to restrict entry of pathogens into the apoplast. Over evolutionary time bacterial pathogens have evolved to overcome stomatal closure by producing a variety of toxins or chemical analogues which induce the reopening of guard cells. Coronatine, syringoline and the protein HopM1 secreted by *Pseudomonas syringae* have all be shown to induce stomatal re-opening and aid colonisation of the host (Melotto *et al.,* 2006, Schellenburg *et al.,* 2010, Lozano-Duran *et al.,* 2014).

Since this discovery, a number of studies have highlighted the importance of stomatal closure in defence against bacterial pathogens (Melotto *et al*., 2008, Kroupitski *et al*., 2009, Zeng *et al*., 2011). One study, using non-invasive micro electrodes, showed that stomata respond in as little as 15 minutes following application of bacterial flagellin (flg22) (Guzel-Deger *et al.,* 2015). Current studies

on stomatal immunity focus heavily on the short-term signalling responses to pathogen associated molecular patterns (PAMPs) that result in stomatal closure (Melotto *et al*., 2006, Melotto *et al*., 2008, Zeng *et al*., 2011, Montillet *et al.,* 2013, Guzel-Deger *et al.,* 2015). Since the discovery of stomatal immunity in 2006, the understanding of the signalling pathways controlling stomatal immunity has improved substantially. There has been considerable debate about the mechanisms by which stomata close in response to pathogen inoculation or PAMP application. Some studies suggest that abiotic and biotic stresses induce similar signalling components to enforce stomatal closure, whilst others have reported that the pathways controlling stomatal immunity are partially different to those controlling stomatal closure upon abiotic stress (Guzel-Deger *et al.,* 2015).

Melotto *et al.* (2006) proposed a mechanism that was shared with the ABA signalling pathway used to close stomata in response to abiotic stress. Application of flg22 or lipopolysaccharides to ABA biosynthesis mutants or the *ost1* ABA signalling mutant failed to induce stomatal closure. This provided strong evidence of a shared guard cell signalling response to abiotic and biotic stress signals. However, later studies on bacterium-triggered stomatal closure began to highlight a role for the defence signalling hormone salicylic acid (SA), which is not believed to be required for stomatal closure to abiotic stress exposure. Exogenous application of SA and ABA to mutants impaired in SA or ABA biosynthesis showed that SA could induce stomatal closure in SA-deficient plants, but could not induce stomatal closure on ABA deficient plants. However, ABA application was able to trigger stomatal closure in SA deficient mutants (Figure 2). These results suggest that the defence signalling hormone SA is acting upstream of ABA in the signalling pathway (Zheng *et al.,* 2010). Although these experiments highlight the importance of SA they do not answer whether ABA is needed in the short term to close guard cells in response to pathogens, or whether ABA is needed to prime guard cell closure before pathogen infection.

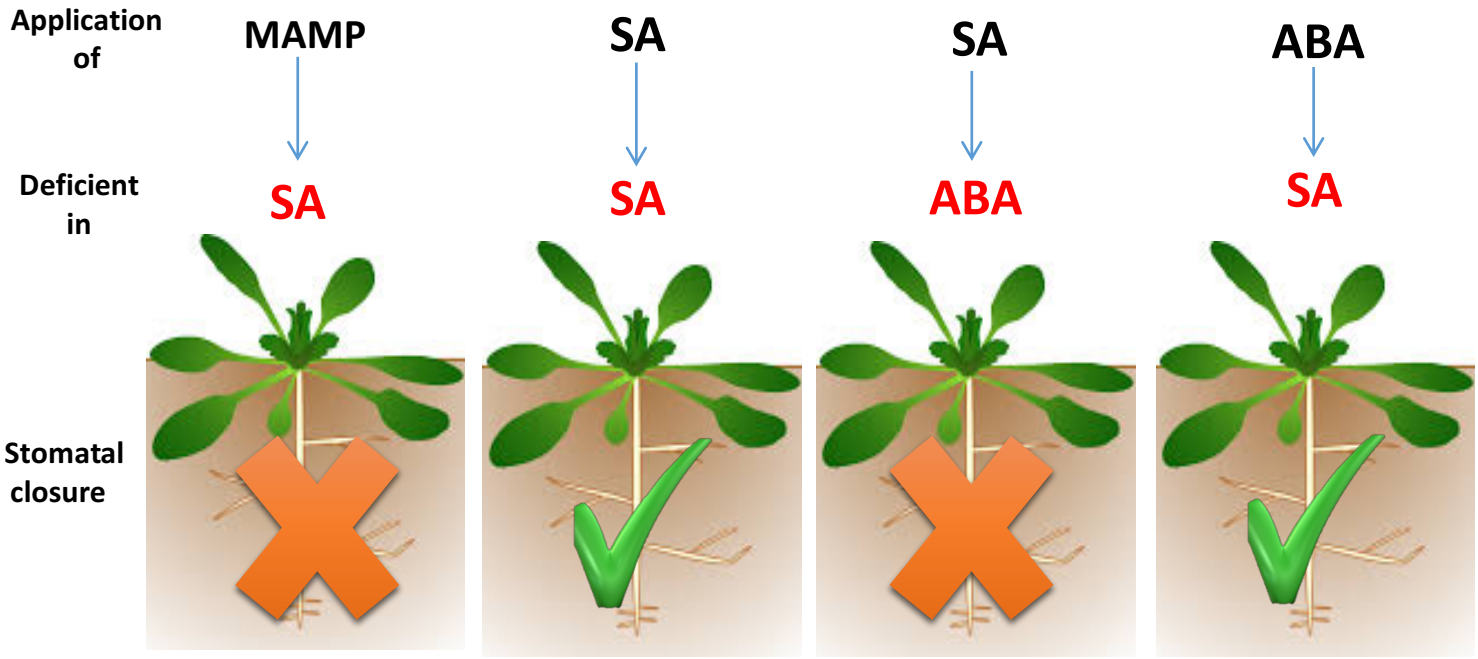


Figure 1.2. Pictorial representation of results reported by Zeng *et al.* (2010). Application of microbe associated molecular patterns, salicylic acid (SA) and abcisic acid (ABA) to SA deficient and ABA deficient mutant *Arabidopsis* plants.

Other studies have indicated an ABA-independent signalling pathway controlling stomatal immunity involving a prominent role for the 9-specific lipoxygenase encoding gene product (*LOX1*). Mutants in *LOX1* are severely compromised in their stomatal response to *Pst* and flg22 (Montillet *et al.,* 2013). This study suggested that the flagellin receptor (FLS2) triggers a signalling response through the mitogen-activated protein kinases, MPK3 and MPK6 that results in production of LOX1-dependant oxylipins, which stimulates SA biosynthesis that mediate stomata closure via SLAC1-dependent anion release. Gene expression analysis has been used to investigate the events downstream of ABA or SA application. This indicated separation between ABA and SA induced stomatal closure following treatments. The results showed significant increases in transcription of three ABA inducible genes, *RD29b*, *ABI1* and *ABI2* as early as 30 min following ABA treatment. However, there was no significant change in gene expression of these ABA-response genes following *Pst* application, suggesting that ABA perception is not required for *Pst*-induced stomatal closure. Taken together, these results

suggest stomatal closure in response to biotic and abiotic stress are controlled by partially different signalling pathways.

Further evidence for ABA-independent control of stomatal immunity came from Guzel-Deger *et al.* (2015), who used non-invasive microelectrodes to measure ion fluxes across the guard cell plasma membrane in wild-type plants and mutants that are impaired in abiotic stress-induced stomatal closure. Loss of both SLAC1 and the related anion channel SLAH3 resulted in impaired stomatal closure to either flg22 or ABA treatment. Flg22 was shown to cause stomatal closure by inhibiting uptake by K+ ion channels and stimulating S-type ion channels. Guzel-Deger *et al.* (2015) also confirmed the inability of *ost1* mutants in stomatal closure following flg22 treatment. However, the ABA-insensitive gain-of-function mutant *abi1*, which constitutively inactivates OST1 (Yoshida *et al.,* 2006), was still able to respond to flg22. Taken together, these results show that both pathways converge at the the OST1 kinase and the S-type anion channels (Figure 3).

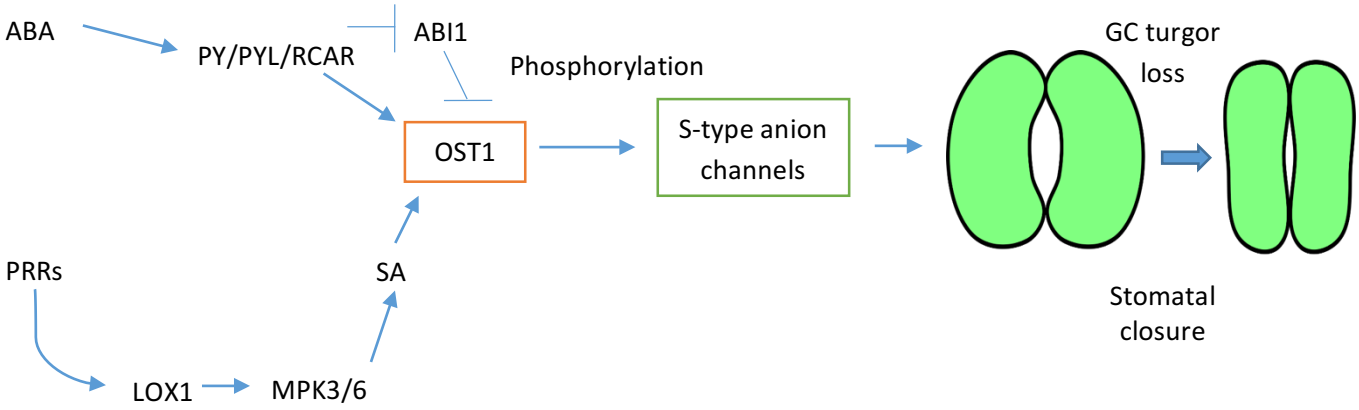


Figure 1.3. Convergence of two guard cell signalling pathways at the OST1 Kinase. The abiotic signalling pathway depends on ABA being perceived by the PY/PYL/RCAR family of ABA receptors, which inhibit ABI1 phosphatase that allows for activation of the OST1 kinase. The biotic signalling pathway depends on perception of PAMPs by pattern recognition receptors (PRRs), activating LOX1-dependent production of oxylipins. These molecules induce SA accumulation through the activation of a MAP kinase 3 and MAP kinase 6 (MPK3/6)-dependent signalling cascade, which in turn activates the OST1 kinase independently of ABI1. Downstream of OST1, both signalling pathways converge and induce phosphorylation of S-type ion channels, guard cell turgor loss and stomatal closure.

1.4 Stomatal development and patterning

1.4.1 Development

The cellular divisions and differentiations leading to the production of stomatal complexes have been well studied, particularly in *Arabidopsis thaliana*. Within the early developing leaf epidermis or protoderm stomatal precursor cells called meristemoid mother cells (MMCs) are selected by an unknown mechanism to enter the stomatal lineage through an asymmetric cell division to form a stomatal lineage ground cell (SLGC) and a smaller sister cell known as a meristemoid (Shpak *et al*., 2005). Meristemoids may then undergo usually two or three subsequent asymmetric amplifying divisions each producing another SLGC, and regenerating the meristemoid located within the centre of resulting SLGCs, which ultimately differentiates into a guard mother cell (GMC). The GMC then undergoes a symmetric division, forming the pair of guard cells that flank the stomatal pore. The SLGCs usually differentiate into epidermal pavement cells but may retain meristematic activity and divide asymmetrically to produce another secondary meristemoid which will ultimately form an additional guard cell pair. The cell walls between the guard cells separate to form the pore and an air space (or stomatal cavity) between mesophyll cells below. During stomatal development, cell differentiation and division are controlled by the related group of basic helix-loop-helix (bHLH) transcription factors SPEECHLESS, MUTE and FAMA, which each interact with the bHLH regulatory transcription factors SCRM and SCRM2 at each stage of cell division. SPEECHLESS controls the asymmetric divisions and entry of cells into the stomatal lineage, MUTE regulates the transition between a meristemoid and GMC, and FAMA controls the division of GMCs into two guard cells. The Mechanics regulating pore and stomatal cavity formation remain unknown.

1.4.2 Patterning

The ERECTA family of transmembrane leucine rich repeat receptor like kinases (LRR RLKs), the somatic embryogenesis receptor kinases (SERKs), along with the LRR receptor like protein TOO MANY MOUTHS (TMM), and their extracellular ligands the epidermal patterning factors, regulate stomatal spacing and patterning. In early leaf development ERECTA expression is strong in the developing protoderm, whilst ERECTA LIKE 1 (ERL1) and ERL2 are expressed in stomatal precursors later during development (Shpak *et al.*, 2005), reflecting their distinct roles during stomatal development. The ERECTA receptor (expressed early) negatively regulates entry of cells into the stomatal lineage, whist ERL1 (expressed later) regulates asymmetric division of meristemoids surrounding the developing stomata and prevents stomatal or meristemoid differentiation in directly adjacent cells. This ensures that two pairs of guard cells are not positioned next to one another in the mature epidermis. Recent evidence presented by Meng *et al*. (2015) highlight an important role for SERKs in regulating stomatal patterning in *Arabidopsis*, adding further intricacy to the stomatal development regulatory signalling network. Examination of 14 SERK receptor mutant lines revealed that SERK1, SERK2, SERK3 and SERK4 receptors were all essential for normal stomatal patterning, although more severe stomatal clustering was seen in SERK3 and SERK2 mutants (Meng *et al.,* 2015).

Recently, several ligands have been identified as being responsible for controlling stomatal density through interaction with the ER/ERL receptors (Lee et al., 2012, Lee *et al.,* 2014).. These belong to the epidermal patterning factor-like (EPF/EPFL) family of cysteine-rich secreted peptides. Currently four EPF/EPFL peptides are known to regulate stomatal development: EPF1, EPF2 EPFL6/CHALLAH and EPFL9/STOMAGEN. EPF2, EPF1 (both expressed in meristemoids SLGCs) and EPFL6 (expressed in the hypocotyl) negatively regulate stomatal density whilst EPFL9 (expressed in the mesophyll cells) positively regulates stomatal density. Recent evidence suggests that these putative ligands compete for the ERECTA, ERL1 and ERL2 receptor binding sites to regulate stomatal patterning (Lee *et al.,* 2014).

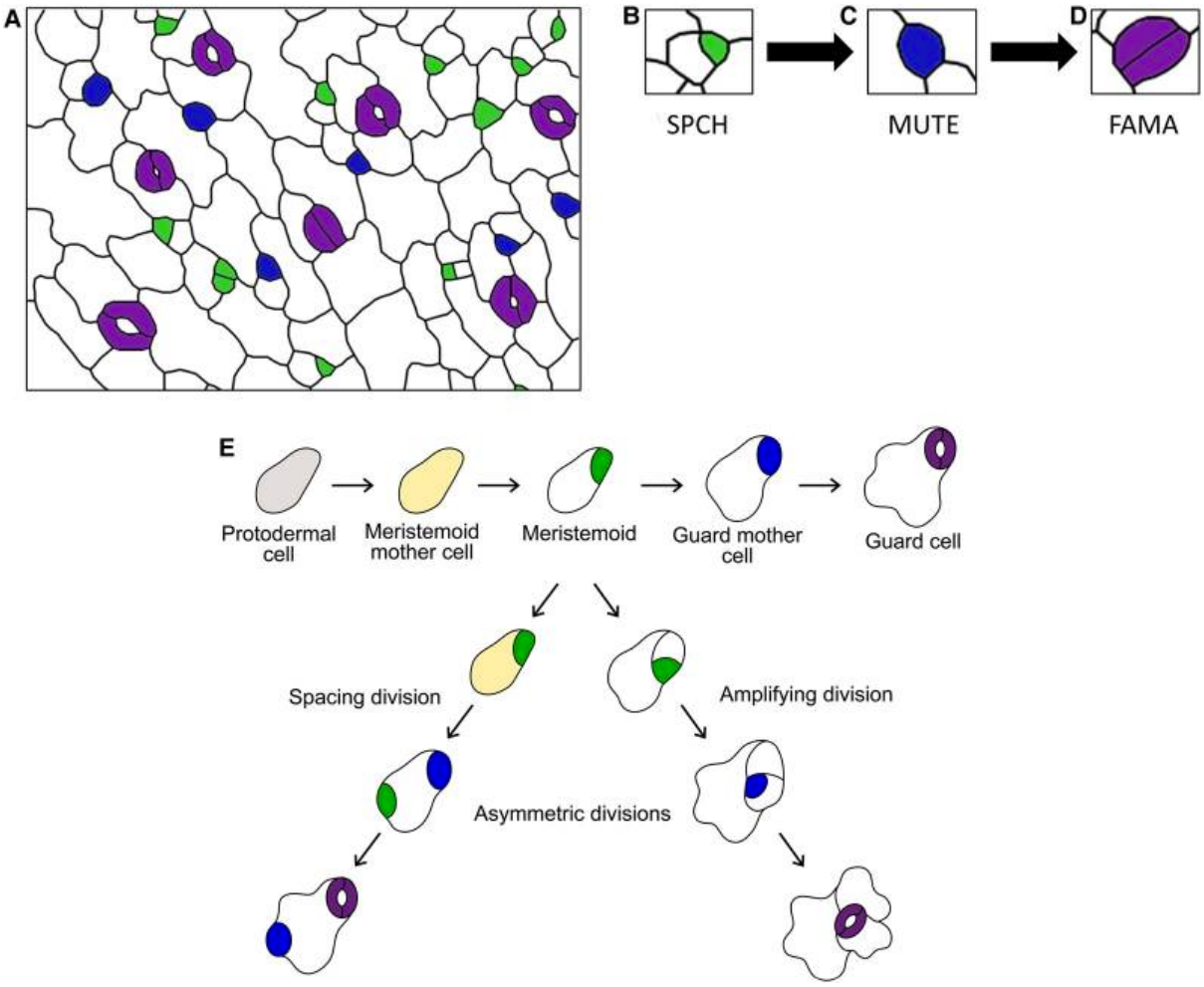


Figure 1.4. diagram of the stomatal development pathway in *Arabidopsis thaliana* (Zoulias *et al.,* 2018). "(**A**) Vectorised confocal image of a young developing *Arabidopsis* abaxial leaf epidermis. This representative epidermis contains cells expressing each of the three bHLH transcription factors that control stomatal development. MMCs and meristemoids which contain SPCH are colored in green (**B**), while GMCs are in blue and contain MUTE (**C**). Newly formed and maturing guard cells are indicated by purple and express FAMA (**D**). Together with the pavement (white) and stomatal lineage ground cells (white), this forms the progression of protodermal cells through the stomatal lineage. (**E**) Cartoon to illustrate the controlled cell divisions and cell fate transitions that regulate stomatal development in the *Arabidopsis* early leaf epidermis."

1.4.3 Genetic engineering of EPF peptides alters stomatal density of plants

Genetic manipulation of genes encoding EPF signalling peptides has proved to be a useful tool in engineering plants with different stomatal traits. Through this approach, different Arabidopsis genotypes have been created that vary in stomatal density, ranging from 20% to 300% of the stomata density of Col-0 wild-type plants (Doheny-Adams *et al*., 2012). Due to the highly-conserved coding sequence between EPF genes of Arabidopsis and EPF genes of monocots, it has also been possible to engineer barley plants with significant reductions in stomatal density (Hughes *et al.,* 2017). These mutants have allowed a broad range of physiological studies into guard cell conductance, gas exchange, drought and other photosynthetic parameters. In the current project, Arabidopsis genotypes and transgenic wheat varieties with altered stomatal densities were used to investigate whether there is link between pathogen infection, stomatal density and disease resistance.

1.5 Genetic control of stomatal density in response to environmental stimuli

Stomatal gas exchange is influenced by both the aperture of the stomata and the number of stomatal pores, each of which can be altered by the plant to suit the external environmental conditions. The subsequent sections explore both the biotic and abiotic stimuli that can influence the frequency that stomata develop.

1.5.1 Stomatal density response to abiotic stress

The density of stomata in the epidermis of mature leaves influences the maximum and minimum rates of carbon uptake and water loss achievable by the plant. As well as altering stomatal aperture, plants can also alter stomatal density (*SD*) in response to environmental variations including CO_2, light intensity and humidity. The environmental conditions experienced by the mature leaves, alter the stomatal density of developing leaves via a long distance systemic signalling pathway. The plant can regulate the *SD* of mature leaves by two main process. Firstly, *SD* can be regulated by the level of EPF peptide production or the activity of their associated transcription factors and ligands (as discussed

earlier). Via this method, plants can control differentiation of each cell, essentially deciding whether the cells become guard cells or epidermal cells. In this way, the plant alters the ratio of guard cells to epidermal cells. This developmental response can be quantified by the stomatal index (SI), which calculates the percentage of stomatal cells relative to the total epidermal cell number and stomata. Plants can also control their *SD* through regulation of the number of cell cycles, cell divisions and cell expansion rates. This leads to leaves with altered numbers if epidermal cells, including stomatal cells, but with the same ratio of guard cells to epidermal cells, hence unaltered SIs.

Atmospheric CO_2 concentration, light intensity and low relative humidity are known to influence the frequency at which stomata occur on developing leaves (Schoch *et al.,* 1980, Woodward, 1987, Thomas *et al*., 2004). The change in stomatal density and patterning observed is a result of the environmental conditions experienced by mature leaves, rather than a response that is perceived by the whole plant, suggesting that long-distance signalling molecules are able to travel from mature leaves to developing leaves to modulate stomatal development. Exposure of mature Arabidopsis leaves to high concentrations of CO_2 results in newly formed leaves under ambient CO_2 levels with reduced SI (Lake *et al.,* 2001). Similarly, Schoch *et al*. (1980) showed in cowpea (*Vigna sinensis*) that the light intensity experienced by the mature leaves determines the SI and *SD* of newly formed leaves. Despite recent advances in our understanding of developmental processes responsible for the formation of stomata, less is known about the nature of the long distance regulatory signals emanating from mature leaves. Experiments with Arabidopsis mutants have suggested a role for ABA, ethylene, jasmonic acid and reactive oxygen species in the systemic signalling pathway(s) controlling *SD* changes in response to exposure to altered CO_2 and light (Lake *et al.,* 2002).

1.5.2 Stomatal density response to biotic stress

There is a growing body of evidence showing *SD* and stomatal development can also be influenced by biotic stresses. For instance, it is known that viral infection of plants results in reduced *SD* and *SI* in new leaves developing after infection (Lake & Wade 2009, Meng *et al*., 2015, Murray *et al*., 2015). However, the mechanisms controlling this response have not been explored. Furthermore, infection of Arabidopsis with powdery mildew (*Erysiphe cichoracearum*) has been shown to increase the *SD* by 15% and 38% on the abaxial leaf epidermis under ambient CO_2 and elevated CO_2 conditions, respectively (Lake & Wade 2009). Interestingly, this study also reported increased trichome density following infection. Hence, infection by viruses and fungi can affect epidermal leaf morphology, in terms of *SD*, *SI* and trichome density. These studies also raise the possibility that infection by other pathogens may affect epidermal cell patterning. More recently, it was found that genetic expression of the bacterial effectors *AvrPto* and *AvrPtoB* in Arabidopsis alters stomatal development, resulting in plants with increased *SD* and *SI*. Interestingly, the transgenic expression of these bacterial effectors also resulted in a violation of the one cell spacing rule and allowed for stomatal clustering (Figure 5) (Meng *et al.,* 2015). Interestingly the SERK receptors are known to have important functions in controlling both stomatal development (see section 1.4.2) and immune responses. In the absence of SERK3 early flg22 induced defence responses are severely reduced (Heese *et al.,* 2007). While this suggests that pathogen effectors can enhance stomatal development, it remains unclear whether this also occurs during natural infection by bacteria.

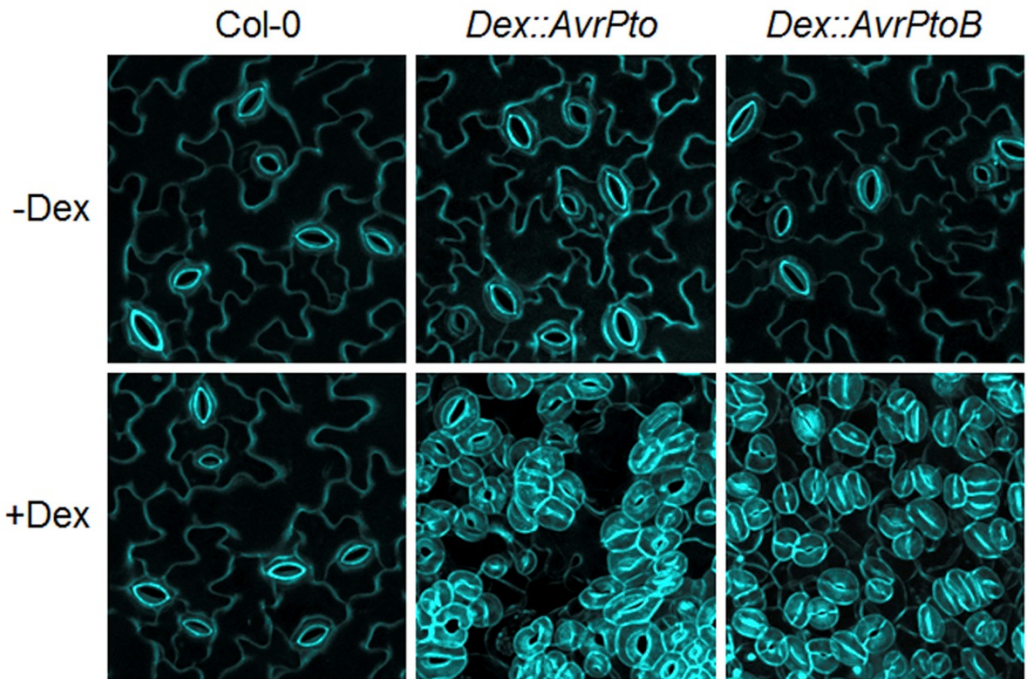


Figure 1.5. Differences in stomatal patterning following Dex inducible expression ectopic overexpression of the bacterial virulence genes *AvrPto* and *AvrPtoB* in *Arabidopsis thaliana* (Meng *et al.,* 2015).

1.6 Long distance pathogen defence signalling

Localised pathogen attack can induce a 'primed' immune state in distal plant parts, which mediates a stronger and/or faster defence responses on subsequent challenge by the same or other pathogens (Ryals *et al*., 1996, Conrath *et al.,* 2006) (Figure 5). This systemic immune response is known as 'systemic acquired resistance' (SAR) or 'Induced systemic resistance" (ISR). SAR or ISR rely on one or more systemic signals that move from the local tissue throughout the whole plant and prime the distal tissues against future pathogen attack. SAR and ISR are induced by different stimuli but can both result in systemic resistance to a variety of pathogen species.

SAR is induced as part of a hypersensitive response to pathogenic microbes and requires activation of the SA acid plant defence signalling pathway. After infection, SA accumulates in both inoculated leaves and systemic leaves distal from the original site of infection (Malamy *et al.,* 1990). Early research suggested that SA might be a mobile signal controlling acquired resistance (SAR). However,

later studies with radio-labelled SA and transgenic *NahG* plants that rapidly degrade SA into inactive catechol, revealed that accumulation of SA in systemic tissues was more likely the result of *de novo* induction of SA biosynthesis in systemic tissues, rather than transport of the hormone itself (Vernooj *et al.,* 1994, Molders *et al.,* 1996).

In recent years, mass spectrometry-based analyses of metabolites in petiole exudates from pathogen-inoculated leaves have identified a range of possible long-distance signals controlling SAR. Jung *et al.,* (2009) identified high concentrations of azelaic acid (AzA) in petiole exudates of infected Arabidopsis. Deuterium labelling of azelaic acid confirmed that this bi-carboxylic acid acts as a mobile signal from the inoculated leave to the systemic tissues. In addition, Navarova *et al.* (2012) identified the amino acid pipecolic acid, which accumulates in both the systemic leaves and in the petiole exudates of inoculated leaves. Although the movement of pipecolic acid was not tracked throughout the plant, the authors provided evidence that this amino acid can act as a mobile defence signal. For example, mutants deficient in the AGD2-LIKE DEFENCE RESPONSE PROTEIN (ALD1) gene are deficient in SAR, and exogenous application of pipecolic acid restores SAR in the ald1 mutant. Similarly, plants pre-treated with AzA showed higher levels of SA induction upon infection. It therefore seems likely that both AzA and pipecolic acid have a role in the systemic priming response of SAR-expressing plants (Jung *et al.,* 2009). While it is widely accepted that AzA is a mobile signal, SA and pipecolic acid are currently mostly regarded as a systemic amplifiers of SAR signals, rather than the SAR signal itself (Stahl *et al.,* 2016). In addition to these metabolites, two lipid transfer proteins (LTPs) known as Azaleic Acid Induced 1 (AZI1) and Defective in Induced Resistance (DIR1), have been shown to be essential for SAR (Jung *et al.,* 2009; Maldonado *et al.,* 2002). In addition, there is evidence for other metabolites acting as long-distance SAR signals, including methylsalicylate (MeSA; Park *et al.,* 2007), glycerol-3-phosphate (Chanda *et al.,* 2011) and the diterpenoid dehydroabietinal (Lorene-Kukula *et al.,* 2012). It therefore seems plausible that SAR relies on a multitude of long-distance signals, the importance of which varies according to the growth conditions (Liu *et al.,* 2011).

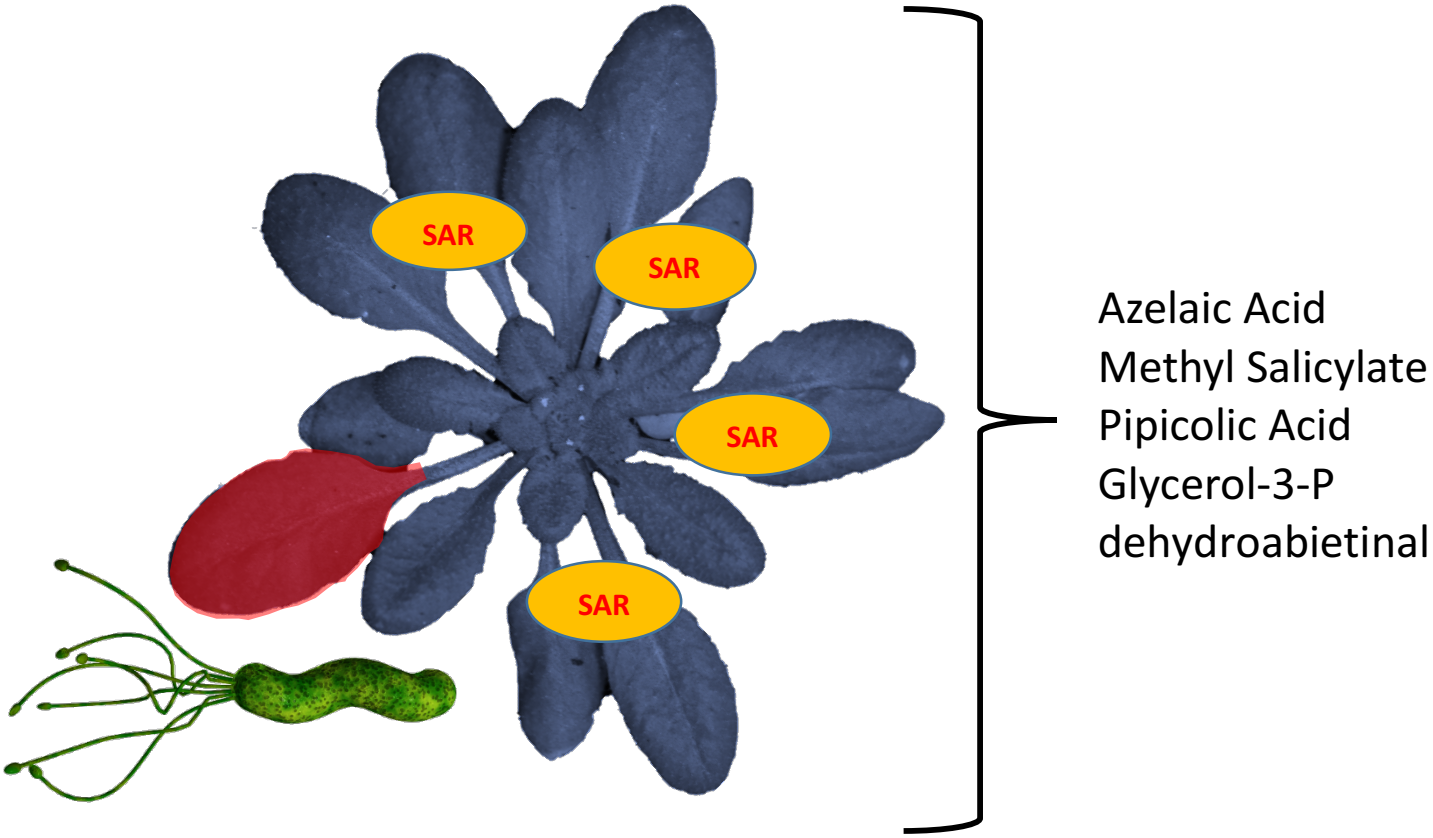


Figure 1.6. Local infection induces a range of systemic signals that can prime distal tissues for SA-dependent defences. Azelaic acid, pipecolic acid, glycerol-3 phosphate, dehydroabietinal and methyl salicylate have all been implicated as mobile signals from locally inoculated leaves to systemic tissues to induce systemic acquired resistance (SAR).

ISR is triggered by the infection of the plant by plant-growth promoting rhizobacteria (PGPR) that cause no visible damage to the plant (Loon *et al.,* 1998). In contrast to SAR, ISR does not involve the accumulation of SA or defence proteins associated with the SA defence signalling pathway and instead relies on signalling pathways regulated by jasmonic acid and ethylene (Pieterse *et al.,* 1998). PGPR produce a variety of chemicals that are thought to trigger systemic phyto-hormonal signalling that can induce ISR, however the exact systemic signals remain unclear (Huran-or-rashid & Chung

2017). Candidates for future research include secondary metabolites such as flavonoids both known to be found in root exudates during root colonisation of PGPR and known to act as feeding inhibitors for insects and microbes (Zamioudis & Pieterse, 2012), and fatty acid products of lypoygenase enzymes known to be transcriptionally induced by colonisation of certain species of PGPR (Wu *et al.,* 2010).

1.7 Project aims

The aim of the work presented in this thesis was to improve our understanding of the relationship between pathogen infection, stomatal density, and disease resistance. A primary aim was to understand whether stomatal density determines the level of resistance against fungal and bacterial pathogens. Having determined that this is the case, it was then important to understand whether and how pathogen infection influences stomatal development in systemic newly developing leaves. Additionally, the potential for guard cell-specific defence mechanisms contributing to plant innate immunity was examined.

1.7.1 Chapter 3 - the effect of *SD* on disease severity was investigated. Using Arabidopsis and wheat genotypes with altered *SD*s (Doheny-Adams *et al.,* 2012), this study addresses the role of *SD* on pre- and post-invasive disease resistance. Non-stomatal pathogens and syringe infiltrations that bypass stomatal entry were used to test whether *SD* manipulation through constitutive overexpression of EPF peptides compromised post-invasive defences.

The aims of chapter 3 were as follows:

1. To understand how altering stomatal density through manipulation of the stomatal development pathway influences pre-invasive immunity.
2. To understand how altering stomatal density through manipulation of the stomatal development pathway influences post-invasive immunity.
3. To understand how altering stomatal density through manipulating stomatal development pathways influences basal resistance against non-stomatal pathogens.

1.7.2 Chapter 4 - investigated the effects of *Pst* infection on the stomatal development and stomatal patterning of Arabidopsis. This study analysed the stomatal development response in a range of local and systemic defence signalling mutants following *Pst* infection. Furthermore, mutants altered in DNA methylation and progenies from severely diseased wild-type plants that are transgenerationally primed for SA-dependent defences (Luna *et al.,* 2012; Lopez *et al.,* 2016) were used to determine

whether the *Pst*-induced *SD* reduction in newly developed leaves involves epigenetic regulatory mechanisms.

The aims of chapter 4 were as follows:

1. To investigate the effect of localised inoculation of mature leaves with the pathogenic leaf pathogen *Pst*DC3000 on stomatal development in newly formed leaves.
2. To investigate the local signalling pathway responsible for the systemic *SD* response to *Pst*DC3000 inoculation.
3. To investigate the systemic signalling pathways controlling the *SD* response to *Pst*DC3000 inoculation.
4. To explore the genetic and/or epigenetic mechanisms controlling the systemic *SD* response.

1.7.3 Chapter 5

- The role of the *ATT1* gene in *Arabidopsis* was investigated. *ATT1* encodes the cytochrome P450 enzyme CYP86A2, an enzyme belonging to a larger group of enzymes that modify or degrade long chain fatty acids. In addition to being expressed in the mature ovary, CYP86A2 is specifically expressed in the guard cells and has previously been reported to regulate defence against *Pst*DC3000. Experiments were carried out to understand the mechanisms by which *ATT1* causes disease resistance in Arabidopsis.

The aims of chapter 5 were as follows:

1. To understand how CYP86A2 contributes to disease resistance.
2. To investigate whether *att1* mutants lacking CYP86A2 exhibit morphological differences that could lead to disease resistance.
3. To explore how knockdown lines of Arabidopsis in *ATT1* respond to inoculation by stomatal and non-stomatal pathogens.
4. To explore why null mutants of *ATT1* exhibited enhanced drought tolerance compared to Col-0 wild-type plants.

Chapter 2: Materials & Methods

This chapter details the materials and methods used in my PhD project.

2.1 Arabidopsis growth conditions & propagation

Arabidopsis thaliana plants (background ecotype Col-0 or Ws) and mutant plants derived from these genetic backgrounds were grown under short day conditions in a Conviron walk in controlled environment chamber (9 hours light, 160µmol m^{-2} s^{-1}at 22°C day, 16°C night). Seeds were stratified for 2 days at 4°C and were grown in a 3:1 mixture of John Innes M3 peat compost and perlite in $23cm^3$ pots. Plants were watered approximately twice a week from below. Fourty-four millimetre Jiffy-7 Peat pellets were used to grow plants in that were dip inoculated with *Pst*DC3000.

2.2 *Arabidopsis thaliana* mutants

The following table details all the *Arabidopsis thaliana* mutants used in this study, the characteristics of their genetic mutations and details of the original citation.

Gene	Mutant name	Notes
Flagellin-sensitive 2	*fls2c*	Lacks the flagellin receptor responsible for detecting bacteria flagellin (Zipfel *et al.,* 2004)
Nonexpressor of PR genes 1	*npr1-1*	Lacking a coregulatory protein that has a binding pocket for SA. (Cao *et al.,* 1994)
Salicylic acid induction deficient 2	*sid2-1*	Lacks a protein (isocharismate synthase 1) essential for the production of SA. Mutants do not accumulate SA. (Nawrath & Metraux, 1999)
SA-degrading salicylate hydroxylase	NahG	Carry the NahG transgene, a salicylate hydroxylase that converts SA to catechol (Van Wees & Glazebrook, 2003)
Defective in induced resistance	*dir1-1*	Lacks a apoplastic lipid transfer protein. Mutants are compromised in SAR (Maldonado *et al.,* 2002)
Azelaic acid insensitive	*azi1-1*	Lacks a lipid transfer protein important for moving lipids out of cells. Thought to move a mobile signal away from localised infection. Responsible for inducing SAR. (Jung *et al.,* 2009)
AGD2-Like defence response protein 1	*ald1-1*	Lacks an amino transferase enzyme which is critical for pipecolic acid biosynthesis. (Navarrova *et al.,* 2012)
DNA-directed RNA polymerase V subunit 1	*npre1-11*	Causes loss of directed DNA methylation through disruption of the Pol V. (Kanno *et al.,* 2004)

Repressor of silencing 1	*ros1-4*	The *ros1* gene encodes an endonuclease III domain nuclear protein. Disruption of the gene causes of loss of *ros1* directed DNA demethylation. (Alonso *et al.,* 2004)
9-cis epoxycarotenoid dioxygenase 3 /9-cis epoxycarotenoid dioxygenase 5	*nced3/nced5*	Deficient in the two isoforms of NCED3 and NCED5 most highly expressed in guard cells. NCED3 is the role limiting step in ABA biosynthesis and mutants have very low levels of ABA (Frey *et al.,* 2012).
Aberrant induction of type three genes	*att1-1 & att1-2*	Encodes a cytochrome p450 gene important in cutin synthesis and pathogen response
Epidermal patterning factor 2 over expresser	*EPF2 OE*	Overexpression of a negative regulator of *SD*, mutants have significantly fewer stomata. (Hunt & Gray, 2009)
Epidermal patterning factor-like 7	*EPFL7 0E*	Overexpression of a negative regulator of *SD*, mutants have significantly fewer stomata (Dutton *et al.,* In submission)
Epidermal patterning factor-like 9	*EPFL9 0E*	Overexpression of a positive regulator of *SD*, mutants have significantly more stomata. (Hunt, Bailey, Gray, 2010)
Breaking of asymmetry in the stomatal lineage and to many mouths double mutant	*bas*tmm	Double knockout mutant lacking BASL responsible for asymmetric divisions and a transmembrane receptor-like protein that blocks stomatal development (Doheny-Adams *et al.,* 2011). Mutants have extremely high *SD* and show heavy stomatal clustering. (Hunt & Gray, 2010)

Table 2.1; table of mutants used during the study, including original citation and details of the mutation.

2.3 Wheat mutants

Wheat transformants were created in collaboration with NIAB Cambridge under the BBSRC funded community resource for wheat transformation. The wheat variety for the transformation process was Fielder (a US spring hexaploid variety). Gene discovery, sequence alignment and phylogeny was conducted by Dr Lee Hunt at the University of Sheffield. A construct was created by which introducing the wheat epidermal patterning factor like gene TRIAE_CS42_U_TGACv1_641036_AA2082860.1 under the control of the rice actin promotor. Plants were regenerated and transgene expression and copy number analysed prior to the start of this study.

2.4 Pathogens and pathogen infection methods

The following section outlines the strains of pathogens used in this study and the infection protocols used for each species of pathogen.

2.4.1 *Pseudomonas syringae* Strains

Three strains of bacterial pathogen were used

1. *Pseudomonas syringae* DC3000 – Originally isolated from tomato plants, causes leaf speck on tomatoes and also infects Arabidopsis plants. Referred to in this study as *Pst*DC3000 (Zipfel *et al.,* 2004).
2. *Pseudomonas syringae* Avrrpt2 – An avirulant strain of *Pst* that has been modified so *Arabidopsis* plants can identify infection and mount effective defence responses (Whalen *et al.,* 1991).
3. Green fluorescent protein tagged *Pseudomonas syringe* DC3000 – GFP tagged *Pst* used to visualise bacteria on the leaf epidermis and in the stomatal cavity (Yu *et al.,* 2013).

2.4.2 Bacterial Cultures

Pseudomonas syringae pv. DC3000 were grown overnight in 250 ml conical flasks containing 50 ml of Kings B medium (containing 10g/L of proteose peptone, 1.5g/L of anhydrous K_2HPO_4 and 15g/L of glycerol). Media was adjusted to pH 7.0 and autoclaved before being supplemented with the appropriate antibiotics and 250 µl of 1M $MgSO_4$ as an osmolite. Cell cultures were pelleted in a centrifuge at 2000g for 11 minutes and suspended in 10mM $MgSO_4$. The optical density (OD) of the culture was measured at 600nm and subsequently diluted to the desired cell density.

2.4.3 Dip inoculation of *Arabidopsis* with *Pst*

For dip inoculation of Arabidopsis, final cell density was adjusted to OD_{600} 0.2 with 10mM $MgSO_4$ and silwet detergent L77 added to a concentration of 0.002%. The plant (grown in a peat pellet) was inverted and the entire rosette of the plant was submerged in the bacterial solution and gently swirled for 5 seconds. Plants were immediately placed into a tray and covered with a propagator lid to maintain high humidity. Propagator lids were left on for 24 hours and left to grow in the plant growth chamber as described in section 2.1.1. Control plants were treated in the same way and dipped in $MgSO_4$ and silwet solution with the bacteria excluded.

2.4.4 Syringe inoculation of *Arabidopsis* with *Pst*

For syringe infiltration three leaves of each *Arabidopsis* plant were infiltrated with up to 0.2 ml bacteria at an OD_{600} of 0.01 suspended in 10mM $MgSO_4$, whilst still attached to the plant 5 weeks after germination. To do this bacterium were forced into the leaf using a 1ml syringe. Leaves were infiltrated until the whole leaf had turned a dark green colour indicating the solution had filled the entire leaf area apoplast.

2.4.5 Incubation of GFP-tagged bacteria with leaves for microscopy

For the visualisation of bacteria under florescent microscopy a culture of GFP tagged *Pst* at 0.2 OD_{600} was incubated in solution with excised whole *Arabidopsis* leaves in a closed Petri dish inside the growth chamber. Leaves were left for 12 hours to infect before inspection under UV microscopy using a Leica DM6000 fluorescent light microscope.

2.4.6 Extraction of bacteria from leaves

Twenty-eight millimetre squared leaf discs were taken using a leaf borer and washed gently for 10 seconds in 70% ethanol. Disks were further rinsed in sterile 10mM $MgSO_4$ for 30 seconds. Leaf disks were placed in Eppendorf tubes containing 500µl of sterile 10mM $MgSO_4$ and ground by hand with a micropestle until all leaf tissue was macerated. Samples were then vortexed and serially diluted. Serial dilutions were then plated onto Kings B agar medium supplemented with the appropriate antibiotics and left to grow overnight. Colonies formed on the agar were counted and used as a proxy for the number of bacteria in the plant leaf disk at the time of extraction.

2.4.7 *Hyaloperonospora arabidopsidis* infection and quantification

The oomycete pathogen *Hyaloperonospora arabidopsidis* strain WAC09 was used to assess the basal level of resistance to biotrophic pathogens. The pathogen was propagated for 1 generation prior to infection on highly susceptible *sid2-1 Arabidopsis* plants. Spores were rinsed from infected leaves with distilled water and the suspension strained through a muslin cloth to remove any soil or plant tissue. Spores were counted under a microscope using a haemocytometer and diluted to 10^6 spores/ml with sterile water.

Fourteen-day-old *Arabidopsis* seedlings were sprayed with the oomycete spore suspension and grown in plant propagators with the pathogen for 5 days in the plant growth chamber. Following this plants were taken for infection scoring.

Infection by the pathogen and the development of the oomycete was analysed in whole leaves stained with lactophenol-trypan blue (200mg/L Trypan Blue, 100mg/L phenol, 100ml/L of 100% glycerol, 100 ml/L lactic acid, 600m/Ll absolute ethanol, 200ml/L distilled water). Leaves were boiled for 1 minute in Trypan Blue solution and placed in chloral hydrate to destain leaves (as described by Koch & Slusarenko, 1990). Fifty leaves of each genotype were analysed under a compound light microscope. For each leaf the level of colonisation by the pathogen was assigned into one of four classes (as described by Luna *et al.,* 2012);

- I – No pathogen growth visible.
- II – Hyphal colonisation without conidiophores.
- III – Hyphal colonisation with conidiophores and sporadic oospores (i.e. less than 5 oospores).
- Iv - Extensive colonisation, conidiophores and frequent oospores.

2.4.8 *Plectosphaerella cucumerina*

Spores of *Plectosphaerella cucumerina* strain BMM (Ton & Mauch-Mani, 2004) were kindly provided by David Pardo from the Ton Lab. Five-week old *Arabidopsis* plants were inoculated with spores at a density of 10^6 spores per ml. A 20µl droplet was inoculated onto the surface of three mature leaves, the droplet was left to dry and the plants were immediately sealed inside propagators with parafilm to maintain a humid environment. Lesion diameter was measured at 6 and 12 days post infection with Vernier calipers.

2.4.9 Brown Rust (*Puccinia triticina*)

Brown rust spores (wheat isolate BRW15/521) were obtained from NIAB (Dr Jane Thomas) and used for infection of wheat stomatal density mutants (TaEPF overexpressing). Brown rust spores were weighed and diluted with talcum powder in a 1:100 ratio of rust spores to powder and mixed well. Sections of leaf 2 (the second leaf to develop after the cotyledon) were removed from wheat plants

and placed in a Petri dish lined with damp tissue paper. Leaves were brushed with the spores using a small paint brush until the leaf was visually covered in powder. Leaves were incubated overnight in the dark at 14°C. Leaf sections were stained using Calcofluor-white stain (Sigma-Aldrich) and a drop of 10% KOH. Infected leaves were views under 20x fluorescence microscopy using a Leica DM6000 to visualise fungal hyphal growth.

2.5 Stomatal density (*SD*) and index (SI) determination

2.5.1 Epidermal cell counting

For leaf epidermal cell counting, ImpressPLUS dental resin (Perfection Plus Ltd, Hants) was applied to the abaxial surface of fully expanded leaves and allowed to set before the leaf was removed from the resin. Nail varnish peels were taken from the set resin impression. Nail varnish peels were mounted onto glass slides and cell measurements and cells counts were taken from three 0.25mm^2 areas of the leaves on 8 separate plants belonging to each genotype. One leaf per plant was analysed with 3 counts being taken from the widest part of the leaf to the side of the mid vein. Mean stomatal density was calculated for each plant, and for each genotype/ treatment. *SI* was calculated using the formula presented below.

Stomatal index = (stomatal number / (epidermal cell number + stomatal number)) x 100

2.5.2 Epidermal cell counts following pathogen infection

To investigate whether infection by *Pseudomonas syringae* has an effect on stomatal density three mature leaves of 5 week old Arabidopsis plants were syringe infiltrated with *Pst*. At the time of infection the youngest developing leaves were marked as a reference so the leaves that developed after infection could be identified and analysed approximately 2-3 weeks later. Fully expanded leaves that developed after infection were taken for epidermal cell counts as described above. Figure 2.1 illustrates the process.

Figure 2.1 Representation of infection of 5 week old Arabidopsis plants with *Pseudomonas syringae*. Leaves coloured red represent three syringe infiltrated mature leaves, white dots represent the youngest leaves marked at the time of infection. The purple rosette centre indicates the leaves developing at the time of infection and these were the leaves that stomatal density counts were conducted on after they had fully expanded.

2.6 Treatment of plants with chemical elicitors or defence hormones

flg22 (Bioscience) peptide and LPS purified from *Pseudomonas aeruginosa* serotype 10 (Sigma Aldrich) were diluted in 10mM $MgSO_4$ to final concentrations of 200nM (flg22) and 100ug ml^{-1} (LPS) and syringe infiltrated into three leaves of each plant. SA and L-Pipecolic (Sigma-Aldrich) acid were syringe infiltrated into leaves at a concentration of 1mM. For azelaic acid (AzA) treatments 1mM AzA dissolved in 5mM MES was syringe infiltrated into three leaves of each plant from a Col-0 background, appropriated mock treatments were applied to controls. Control plants were infiltrated with 10mM $MgSO_4$ or 5mM MES.

2.7 Stomatal aperture measurements following flagellin treatment

For stomatal aperture measurements 5 well-watered plants of each genotype were treated with 200nm/ml of flagellin dissolved in sterile H_2O, and mock plants were treated with the appropriate H_2O mock treatment. The plants were left for 1 hour, before epidermal peels were taken from flg22 treated leaves and imaged on a light microscope (Olympus BX51). Aperture measurements were taken from x40 field of view from 1 leaf of 8 plants per genotype using ImageJ software and mean stomatal apertures were calculated from measurements of the pore width at its widest point.

2.8 Statistical analysis

All statistical tests and analysis were performed using GraphPad Prisms (version 7) from Graph Pad Software. Before conducting statistical tests, data was analysed for normal distribution and corrected for if necessary. Specific details of statistical tests are detailed throughout in figure legends. Statistically significant differences are reported throughout using a minimum alpha level of $P<0.05$, whist error bars on graphs represent 1 standard error (SE) from the mean.

2.9 DNA Extraction from Arabidopsis

Leaf tissue samples were ground in Eppendorf tubes using a micro-pestle in 300 µl of DNA extraction buffer (200mM Tris, 250mM NaCl, 25mM EDTA and 0.5% SDS). Ground samples were vortexed and then centrifuged for at 13,000 rpm for 2 minutes and the supernatant decanted. The supernatant was mixed at a 1:1 ratio of supernatant to isopropanol and centrifuged at 13,000 rmp for 5 minutes. The supernatant was removed and the DNA was re-suspended in 100 µl of water.

2.10 RNA Extraction from Arabidopsis

Leaf tissue samples were frozen in liquid nitrogen and homogenised using a micro- pestle in Eppendorf tubes. A Sigma spectrum™ plant total RNA kit was used to extract RNA from lyophilised samples following the manufacturer's protocol. RNA concentration was measured using a NanoDrop™ 8000 Spectrophotometer.

2.11 cDNA synthesis

Extracted RNA was diluted to a concentration of 100ng/µl, then 0.8µl of RNase Free DNase 1 (NEB) and 1.2µl of 10x DNase 1 buffer was added to 10µl of RNA and incubated at 37°C for 20-30 minutes. After incubation 2µl of DNA-free™ DNase activation reagent was added and incubated at 20°C for 2 minutes, centrifuged and the supernatant taken for cDNA synthesis.

The Maxima H Minus Reverse transcriptase (Thermos Scientific) kit was used to synthesise cDNA following the manufacturer's instructions using random hexamer primers.

2.12 Quantitative RT-PCR and data analysis

qPCR experiments were performed using a Corbett Rotor Gene 6000. Reactions were comprised of 5µl 2x Rotor-Gene SYBR green PCR master mix, 1µl of each oligonucleotide primer (final concentration 0.25 µl) and 2µl of cDNA (containing 8ng of cDNA in the final reaction) and the volume adjusted to 10 µl by the addition of H_2O. Two step qPCRs were run with the following temperature cycles, 10 minutes at 95°C, 35 cycles (95°C for 10 seconds, 60°C for 55 seconds and acquiring at the end of the annealing time at wavelength of 510 nm). Fold changes in gene expression were calculated using the delta-delta Ct method described by Livak & Schmittgen (2001) with corrections made for the PCR amplification efficiency of each sample. Amplification values for gene(s) of interest were normalised against amplification values for genes encoding at least two of the house keeping genes *GAPDH*, *UBC* or *SAND* (sequences and AGI numbers given in table 2.2) which have been shown to be expressed at relatively consistant levels (Chechowski *et al.,* 2005).

AGI	Annotation	Forward	Reverse	Citation
AT5G25760	UBC	CTGCGACTCAGGGAATCTTCTAA	TTGTGCCATTGAATTGAACCC	Czechowski *et al.,* 2005
AT2G28390	SAND	AACTCTATGCAGCATTTGATCCACT	TGATTGCATATCTTTATCGCCATC	Czechowski *et al.,* 2005
AT1G16300	GAPDH	GCCATCCCTCAATGGAAAATT	GAGACATCAACGGTTGGAACAC	Czechowski *et al.,* 2005
AT2G14610	PR1	GTCTCCGCCGTGAACATGT	CGTGTTCGCAGCGTAGTTGT	(Sanchez *et al.,* 2016)
AT4G00360	ATT1	GTGGCGGCACTTATCAGAC	GGTCACGAGACCTTGCTTTT	Menard *et al.,* 2014
AT1G34245	EPF2	TCAAACGCACCACAAGAAGG	AGCTTGATCCTGTTGGGTAC	Casson & Hetherington 2014
AT4G12970	EPFL9	GTTCAAGCCTCAAGACCTCG	CCTTCGACTGGAACTTGCTC	Casson & Hetherington 2014
AT5G53210	SPCH	AACGGTGTCGCATAAGATCC	CAAGAGCCAAATCTTCAAGAGC	Casson & Hetherington 2014
AT3G06120	MUTE	AACGTCGAAAGACCCTAAACCG	TTAGCATGAGGGGAGTTACAGC	Casson & Hetherington 2014
AT3G24140	FAMA	GCTGCTAGGGTTTGACGCCATGA	GGAGTAGAGGACGGTTTGTTCC	Casson & Hetherington 2014

Table 2.2. Table of all qPCR primers used during this study including original citations of primers first use.

2.13 Agarose gel electrophoresis

DNA fragments were size separated and visualised using gel electrophoresis. One-two % agarose gels (depending on the size of the DNA fragments) were prepared using Tris acetate EDTA buffer (20mM glacial acid, 40mM Tris and 1mM EDTA). Gels contained approximately 0.5µg/ml of ethidium bromide which was used to visualise DNA samples under UV. DNA was loaded into gels mixed with DNA loading buffer and electrophoretically separated at 100V for the appropriate amount of time alongside a ladder of fragments of a known size.

2.14 Polymerase chain reaction (PCR)

For genotyping and assessing the presence or absence of particular genes BioMix^{TM} red (Bioline) was used. This non-proof reading *Taq* polymerase enzyme was used in 10 µl reactions containing 5µl of biomix red, 2µl of each primer mix (0.1µM concentration) and 1µl of template DNA. cDNA or genomic DNA amplifications were typically carried out over 35-40 thermocycles cycles.

2.15 Gas exchange measurements

A LI-6800 portable photosynthesis system (Licor, Lincoln, NE) was used to carry out InfraRed Gas Analysis (IRGA) on mature, fully expanded Arabidopsis leaves that were attached to live plants. During experiments relative humidity inside the IRGA florescent chamber head was maintained at 70% using self-indicating desiccant. The block temperature was set at 20^{o}C, reference CO_2 maintained at 400ppm and light intensity at 300µmol.m^{-2}.s^{1}. Analysis was carried out inside the plant growth chamber. Following attachment of the Licor chamber to each leaf the apparatus was left until gas exchange values had stabilised (approximately 20 minutes) before readings were taken.

2.16 Infrared thermal imaging

An FLIR T650sc infrared thermal imaging camera was to used to assess leaf surface temperatures and capture infrared images of Arabidopsis plants grown inside the growth chamber. Twentyimages of plants were taken over a period of 30 minutes, the first 5 images were omitted from the analysis to allow for plants to adapt to any movement and disturbance in the growth chamber whist setting up the equipment. Images were analysed using ThemaCAM Researcher Profession software provided by FLIR systems.

2.17 Measuring cuticular conductance

Plants for cuticular conductance measurements were grown in 4:2:3 v/v mixture of peat, vermiculite and water as described (Kollist *et al.*, 2007) in growth chambers (MCA1600, Snijders Scientific, Drogenbos, Belgium) at 12 h/12 h day/night cycle, 23°C/20°C temperature, 100 µmol m^{-2} s^{-1} light, and 70% relative humidity (RH). Twenty-five to thirty days old plants were used for experiments. A custom-built device (Kollist *et al.*, 2007) was used for measuring gas-exchange in intact plants. Cuticular conductance was determined as in (Jakobson *et al.*, 2016) with the following modifications. Plants were acclimated in measurement cuvettes at 65-70% RH, in darkness and 1300 ppm CO_2 to induce stomatal closure. When leaf conductance had stabilised, rosettes were excised from the roots and the following drop in leaf conductance was recorded. Cuticular conductance was calculated with the assumption that the stomatal conductance of all mutants reached zero at the same time as the wild-type, by 64-88 min after rosette excision. The remaining leaf conductance at this time period was termed cuticular conductance.

2.18 Leaf area measurements

Total leaf area was calculated by removing whole leaves. Leaves were then flattened and laid on white paper. Digital images were taken from a fixed distance and uploaded to ImageJ where total leaf area was calculated using the number of pixels as a proxy for leaf area.

2.19 Measurements of light adapted quantum yield of photosystem II

Light adapted quantum yield of photosystem II 380 (ΦPSII) was measured throughout the terminal drought experiment with ATT1 plants. Each day following from when watering ceased one mature fully expanded *Arabidopsis* was chosen for the measurement at day 1 and the same leaf was then monitored throughout the course of the drought experiment. Photosystem II measurements were taken using a FluorPen FP100 (Photon Systems Instruments) with a saturating pulse of 3000 µmol m^{-2} s^{-1} light.

2.20 Quantification of signalling hormones using UPLC-qTOF-Mass spectrometry

Jasmonic acid, salicylic acid and abscisic acid quantities were analysed by UPLC-qTOF-Mass spectrometry. For initial sample preparation, 100-200mg fresh weight of Arabidopsis leaves were collected and weighed into 2ml Eppendorf tubes with 3 stainless steel beads and immediately frozen in liquid nitrogen. Tissue was Homogenised using a mixer mill for 2 minutes at 30 Hz. One millilitre of ice-cold extraction buffer (methanol (95%): water (4.95%): formic acid (0.5%)) was added, and again placed into the mixer mill for 2 minutes at 30 Hz. The sample was then centrifuged at 4^{o}C for 10 minutes at 14,000 g and 800µl of the supernatant was transferred into a fresh Eppendorf. The resulting pellet was then re-extracted in 500µl as described above, after which 400µl of the supernatant was pooled with the previous 800µl and split into two fresh Eppendorf's containing 600µl of supernatant each. The two samples were subsequently placed into a speed-vac until completely dry (approximately 2 hours). Samples were then stored at -80^{o}C until MS analysis.

Before analysis samples were re-suspended in 100µl of extraction buffer (methanol (95%): water (4.95%): formic acid (0.5%)) in preparation for UPLC-qTOF-MS analysis. Samples were sonicated in cold water for 10 minutes, vortexed and centrifuged at 4^{o}C for 15 minutes at 14,000g to remove any particles in the solution. Supernatant was then transferred into a glass bottle with an insert.

Samples were ran using a MS Waters Synapt G25Si LC-MS and a Water Acquity i-class UPLC. The machine settings used were as follows; polarity - ES, Capillary - 3.0000Kv, source temperature - 120^{o}C, sampling cone - 60.0000, source offset - 80.0000, source gas flow - 0.00 mL/min, Desolvation temperature - 350^{o}C, Cone gas flow - 60 L/Hr, Desolvation gas flow - 800L/Hr, Nebuliser gas flow - 6.5 bar. Phytohormone standards containing 0.1ug/mL, 1ug/mL and 1ug/mL of SA, JA and ABA were used to identify the exact fragmentation patterns of the hormones of interest.

Chapter 3 - Stomatal density and Its effects on plant immunity

3.1 Introduction

As discussed in Chapter 1, stomata represent important entry sites for many plant pathogens including those of agricultural importance. Upon detection of pathogens on the leaf surface, at least some plant species are known to rapidly close their stomatal pores to restrict entry into the host (Melloto *et al.,* 2006). To overcome this, some pathogens have evolved ways to circumvent host defences by either triggering the plant to reopen its stomata, or through the use of specialised structures that can force through the closed guard cells.

Studies of the developmental signalling pathway that regulates stomatal development has allowed the creation of plants with altered *SD* and stomatal patterning. For example, through the manipulation of the EPF signalling family of peptides, *Arabidopsis*, poplar, barley and wheat stomatal mutants have been created with a range of stomatal densities (Doheny-adams *et al.,* 2012, Wang *et al.,* 2016, Hughes *et al.,* 2017). In this chapter experiments were carried out using Arabidopsis and wheat EPF mutants to test whether altered stomatal density could affect resistance to early colonisation by stomatal pathogens.

3.1.1 Aims

The effect of *SD* on pathogen entry and colonisation has never been directly studied. This chapter investigates the relationship between stomatal density and pre-invasive immunity to pathogens.

The aims of the research presented in this chapter were;

- Firstly, to understand how stomatal density contributes to the number of bacterial or fungal pathogens entering the plant in the early stages of infection. To do this genetically modified *Arabidopsis* and wheat plants were treated with *Pseudomonas syringae* or brown rust and early infection quantified.

- Secondly, to investigate the possibility that genetically modifying *Arabidopsis SD* by constitutively over expressing the EPFs could influence post-invasive immunity. This was done by (i) quantifying infection in stomatal density mutants to pathogens that had not entered through the stomata and (ii) the use of mass spectrometry to identify the quantities of key regulatory hormones inside the leaf.

3.1.2 Introduction to the pathogens used in this Chapter

The two stomatal pathogens used in this chapter were the bacterial pathogen of *Arabidopsis Pseudomonas syringae* strain DC3000 (*Pst*DC3000), which is an established laboratory model used to study plant pathogen interactions, and the fungal pathogen of wheat, brown rust (*Puccinia triticina*). Both pathogens are known to entre plants primarily through the stomatal pores but probably use different mechanisms to find stomatal pores and infect plants.

Pst is a motile Gram-negative bacterium, which infects host plants through stomatal pores or through open wounds. It is believed that foliar pathogens such as *Pst* may be attracted to open stomata through unknown chemotactic signals emitted from the guard cells. Experiments with lettuce leaves incubated with *Salmonella enterica* in the light and in the dark reveal an aggregation of bacteria around the stomata only when plants were incubated under light. Scattered distributions of bacteria are seen when incubated under darkness (Kroupitski *et al.,* 2009), indicating that the bacteria may need photosynthetic or gas exchange signals to locate the guard cells and pore. Once they are located close to a stomatal pore *Pst* and other bacteria use a form of cell-cell communication known as quorum sensing to form a co-ordinated colony strategy to infect the plant in larger numbers. This increases the likelihood of a successful infection and helps to protect a sufficient number bacteria from induced antimicrobial activity of plants. *Pst*DC3000 produces 3-oxo-hexanoyl-homoserine lactone, part of a larger class of molecules used by bacteria as transcriptional regulators. Once a threshold concentration of the molecule has been reached, indicating a large enough colony of

bacteria, the transcription of virulence genes is upregulated, helping the bacteria enter the plant and overcome induced host defences (Quiñones *et al.,* 2005), thus aiding successful infection.

Several fungal pathogen species cause rust diseases. Rusts are prolific agricultural pathogens. Once germinated on the leaf surface 90% of spores find and form appressoria over the guard cells. It is thought that the growing hyphae of rust pathogens of cereals may have an ability to detect topographical changes in the leaf epidermis and using these features to successfully navigate towards guard cells (Hoch *et al.,* 1987). When the hyphae reach a guard cell pair they form an appressorium which is an infection structure used to press and penetrate closed stomatal pores. The epidermal cells of cereals are arranged in parallel rows known as files, with stomata typically forming only in some files interspersed by epidermal pavement cells. Germinating spores produce hyphae that grow perpendicularly to the files of epidermal cells and stomatal cells of grasses. Hoch *et al.* (1987) through germinating spores of different rust species on artificial ridged surfaces, demonstrated that appressorium formation was seen after the hyphae crossed ledges of 0.5um or greater. This ledge height correlates with the height of the cuticular ledge, a guard cell structure that surrounds the pores of stomata of their host plants. Hence it is believed that hyphae of rust species grow across the epidermal cells until they detect the topographical features of a stomatal pore. They cease growing and form appressorium to penetrate the pore.

The non-stomatal pathogen used in this chapter was the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*) which is and obligate biotrophic pathogen of *Arabidopsis*. Hpa infection of Arabidopsis is an extensively studied system which has been used effectively to assess the susceptibility of many genetic mutants to biotrophic infection (Asai *et al.,* 2015). The infection process begins when the spore germinates on the surface of the host, from here the hyphae grow and penetrating usually between two adjoining mesophyll cells. The mechanism used by the pathogen to detect the presence of two adjoining cells remains unknowm. Once inside the plant the hyphae remain outside the host cells in the intracellular space, however, the pathogen produces specialised feeding mycelium which

penetrate the host cells forming a web-like structure inside the host cell used to feed the pathogen (Soylu *et al.,* 2003)

Arabidopsis Wheat

a) b)

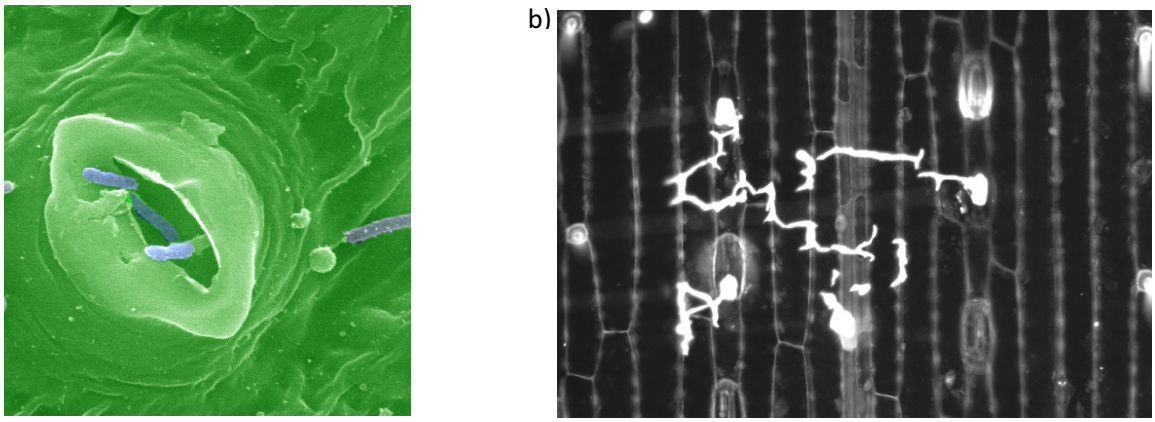


Figure 3.1. Stomatal pathogens entering through the guard cells. a) SEM of *Pseudomonas syringae* entering *Arabidopsis* stomatal pores (image captured by Dr Lee Hunt, University of Sheffield). b) Germ tubes of wheat rust fungal pathogen forming appressoria over wheat stomatal pores.

3.1.3 *Arabidopsis thaliana* mutants used in this chapter

Arabidopsis thaliana stomatal density mutants used were Epidermal patterning factor 2 over expresser (EPF2 OE), Epidermal patterning factor-like 7 overexpressor (EPFL7 0E), Epidermal patterning factor-like 9 overexpressor (EPFL9 0E) and Breaking of asymmetry in the stomatal lineage and Too many mouths double knock out mutant (basltmm). The mutants are previously detailed in section 2.2 in Chapter 2.

3.2 Results

3.2.1 Understanding how stomatal density affects pathogen colonisation

To understand the link between stomatal density and bacterial infection *Arabidopsis SD* mutants that were genetically altered in their levels of EPF signalling peptides or other components of stomatal development were challenged with both stomatal and non-stomatal pathogens. In the experiments described here *SD* mutants had *SD*s ranging from 30-200% of the density of stomata of Col-0 control plants. The stomatal density of the mutants ranging from 80mm^{-2} to 470mm^{-2} in comparison Col-0 with 200mm^{-2} (Figure 2a). The plants overexpressing EPF2, EPF7 and EPFL9 had stomata that were well spaced (ie with the intervienting parent cell) and the basltmm mutant showed stomatal clustering.

To investigate whether *SD* affects the level of pre-invasive immunity, all five mutants were challenged with the bacterial pathogen *Pseudomonas syringae* (*Pst*) strain DC3000, at a known titre sprayed onto the leaf. The bacteria that had entered the leaves of plants were extracted and serially diluted onto agar plates at 4 and 24 hours after inoculation.

Results showed that at 4 hours after inoculation (hpi), the stomatal density (*SD*) of leaves had significantly affected the number of bacteria entering the leaf. Extractions showed that the *EPF2 OE* and *EPFL7 OE* lines with fewer stomata allowed significantly lower bacterial titres inside the leaf than Col-0 plants, whilst *EPFL9 OE* and *baltmm* plants with more stomata allowed significantly higher bacterial titres in their leaves compared to Col-0 plants (Figure 2b). At 24 hpi similar trends were observed in 3 out of the 4 *SD* mutants. Again, compared to the Col-0 wild-type line, the *EPFL7 OE* line with fewer stomata allowed reduced colonisation rates by *Pst*DC3000, whilst *EPFL9 OE* and *basltmm* plants with more stomata sustained higher rates of *Pst*DC3000 colonisation (Figure 2c). This experiment was repeated on a number of occasions with similar results. Hence, leaf colonisation of spray-inoculated *Pst*DC3000 correlates positively with *SD*.

To understand whether the altered rates of *Pst*DC3000 colonisation in the stomatal development genotypes could be attributed to entry through stomatal pores, GFP-tagged *Pst*DC3000 was used to visualise the location of bacterial attachment to the leaf surface. At 12 hpi, fewer bacteria were attached to the leaf surface of plants with reduced *SD*. Contrastingly, greater numbers of GFP-expressing bacteria could be observed on leaves with increased *SD*. Furthermore, bacteria attached to leaves were primarily associated with the stomatal complexes where they can gain entry (Figure 2d).

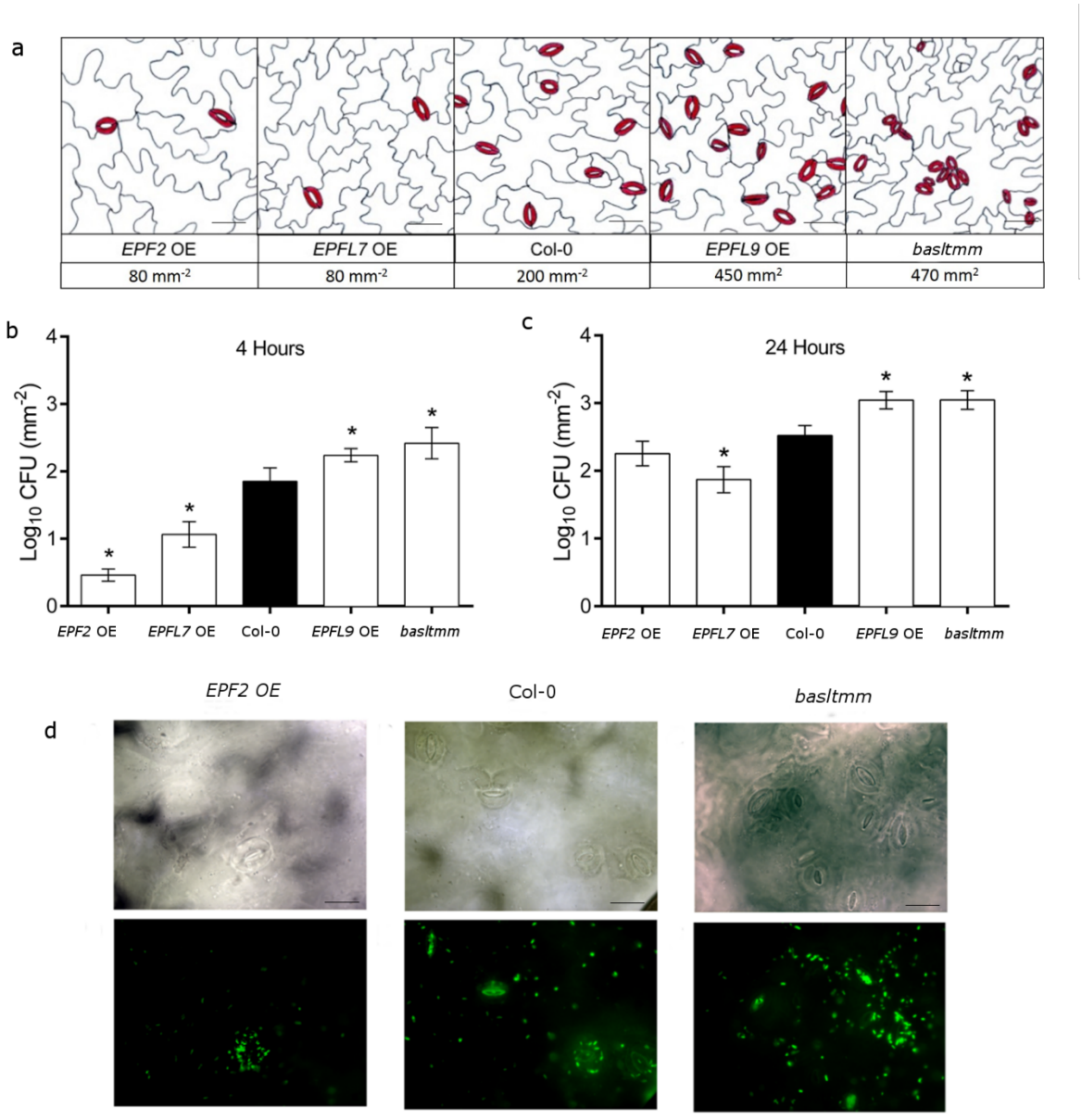

a
EPF2 OE
80 mm-2
EPFL7 OE
80 mm-2
Col-0
200 mm-2
EPFL9 OE
450 mm2
basltmm
470 mm2
b
4 Hours
Log10 CFU (mm-2)
EPF2 OE
EPFL7 OE
Col-0
EPFL9 OE
basltmm
c
24 Hours
Log10 CFU (mm-2)
EPF2 OE
EPFL7 OE
Col-0
EPFL9 OE
basltmm
d
EPF2 OE
Col-0
basltmm

Figure 3.2. The effect of stomatal density on bacterial infection of Arabidopsis. a) Epidermal traces of Arabidopsis mutants with altered stomatal densities and stomatal patterning. Stomata are indicated in red. Genotypes of plants and average stomatal density of mature leaves are indicated below images. b) Colony forming units of bacteria extracted from leaves of *SD* mutants at 4 hours post inoculation with the bacteria *Pseudomonas syringae* (5 plants used for each genotype). c) Colony forming units of bacteria extracted from leaves of *SD* mutants at 24 hours post inoculation with the bacteria *Pseudomonas syringae*. Bars represent SE. *=P<0.05 denoting statistically significant differences from Col-0 plants following unpaired t-tests (5 plants used for each genotype). d) Light microscopy images of leaf surface to show the positions of stomata. Images below show fluorescent images of GFP-tagged *Pseudomonas syringae* 12 hours post inoculation. Mutant genotypes as indicated. Scale bar represents 20 microns.

To assess whether the difference in pathogen colonisation noted in Figure 2a and 2b translated into longer term pathogen resistance and had a physiological effect on plant performance F_v/F_m values were recorded at 24, 30, 36 and 48 hours after dip inoculation with *Pst*DC3000. Dr Hanna Horak assisted with this analysis. Our results showed that at all time points *EPF2 OE* plants with reduced *SD* were performing more efficiently than the Col-0 wild-type (Figure 3a). This difference translated into a visual response at 72 hpi, where Col-0 plants showed significantly more symptoms than *EPF2 OE* plants (Figure 3b).

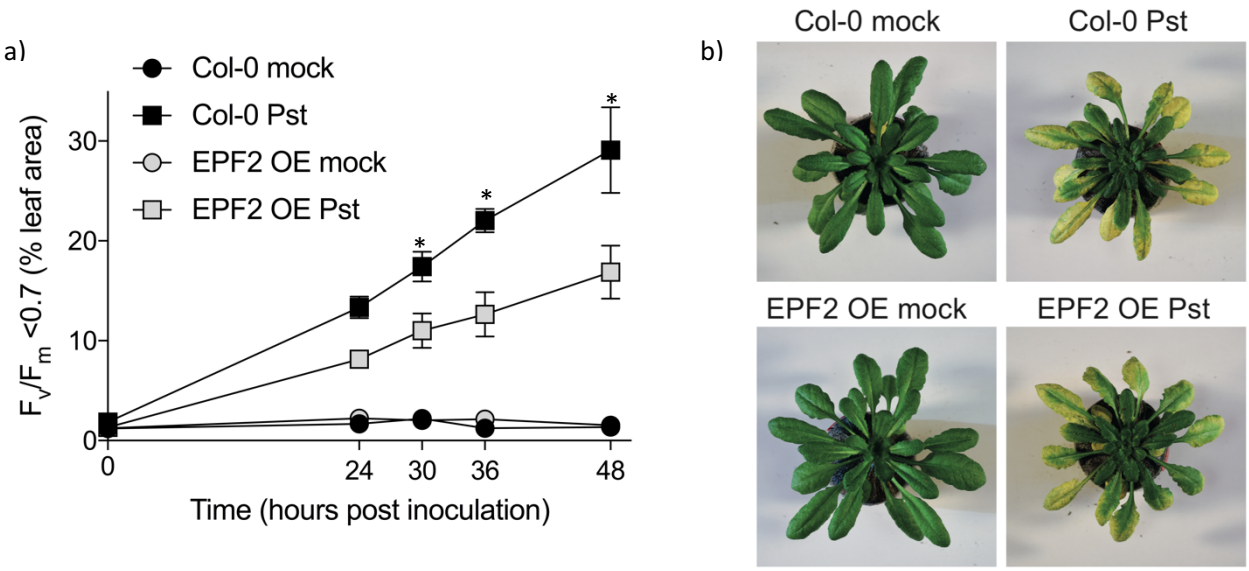


Figure 3.3 A reduction in *SD* enhances plant resistance to dip inoculation with *Pst*. a) F_V/F_M values as a percentage of leaf area of Col-0 and EPF2 OE plants mock treated and treated with *Pst*DC3000 at 4 time points following treatments. b) images of Col-0 and EPF2 OE plants mock treated and treated with *Pst*DC3000 72 hours post inoculation. * denotes statistically significant differences between Col-0 *Pst* treated plants and EPF2 0E *Pst* treated plants (2-way ANOVA with significant genotype*Treatment). Fv/Fm analysis and photos taken by Dr Hanna Horak.

The results presented in Figures 2 and 3 indicate that stomatal density affected the entry of *Pst* into the host *Arabidopsis*. To investigate whether stomatal frequency affects entry of stomatal entering pathogens in different host species, wheat (*Triticum aestivum*) plants that had been genetically manipulated to overexpress an EPF-like gene and had significantly lower *SD* than control plants were challenged with the wheat fungal and stomatal pathogen brown rust (*Puccinia triticina*). Untransformed wheat leaves (cv. Fielder) formed approximately 64 stomata/mm^2, whereas independently transformed lines overexpressing the EPF-like gene formed approximately 43 and 44 stomata/mm^2 on fully expanded leaves of 3 week old plants. Brown rust spores were inoculated onto three wheat lines and left to germinate for 15 hours. Spores and hyphae were stained and visualised under fluorescence microscopy. The leaf *SD* and percentage of spores on each leaf that had formed appressorium over stomata was then counted.

The results showed that the EPF overexpressing lines that had a reduced stomatal density have a lower percentage of germinated spores forming appressoria over stomatal pores (Figure 4). Lines 2.7 and 4.16 with *SDs* of 76% and 74% of controls had between a 13% and 15% reduction in the number of spores forming appressoria over stomata respectively. Thus, infection by this fungal pathogen of wheat appeared to be affected by reduced *SD*, which is in-line with the above results from infection of *Pst* on its *Arabidopsis* host.

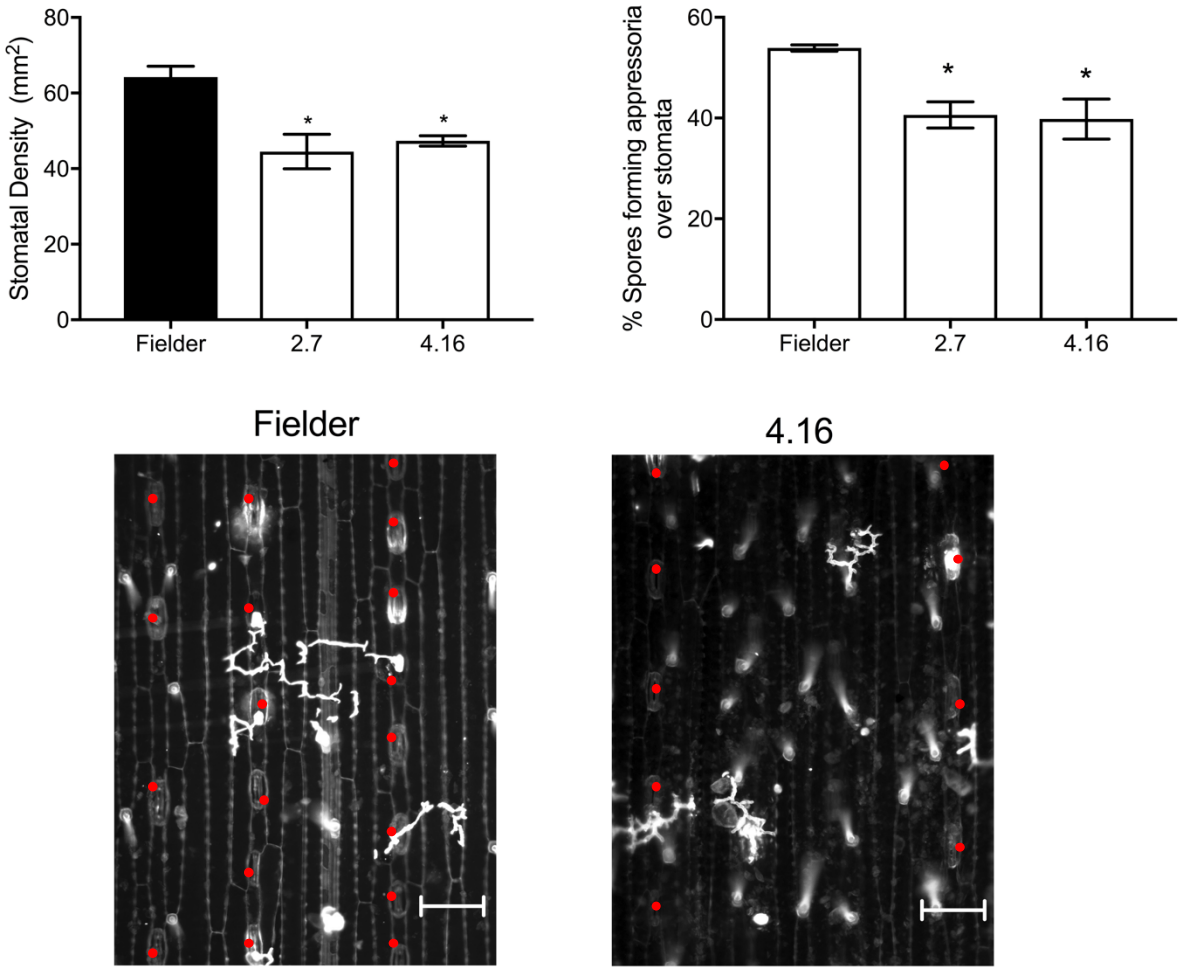


Figure 3.4. The effect of stomatal density on colonisation of Brown Rust on Wheat plants overexpressing an EPF like peptide. a) Mean *SD* of wheat lines. Fielder and the two independently transformed control lines overexpressing an EPF-like peptide (line 2.7 and line 4.16). b) Percentage of germinated Brown Rust spores forming appressoriums over the stomatal cavities 12 hours after inoculation. c) Image of brown rust on the leaf epidermis of Fielder variety and line 4.16. * denotes statistically significant difference in comparison to Fielder variety (T-test, $P<0.05$, $n=5$). Scale bars represent 100µ.

3.2.2 Manipulation of stomatal density through the EPF peptides does not affect post invasive defences

The results from infecting Arabidopsis and wheat experiments with stomatal pathogens indicate that there is a strong relationship between stomatal density and pre-invasive immunity to pathogens entering through the stomata. It is however, possible that the over expression of EPF signalling peptides to either reduce or increase stomatal density, could also have had an unexpected effect on post-invasive plant defences. To determine whether the tested genotypes are affected in post-invasive defences, the altered *SD* lines were challenged with *Hyaloperonospora arabidopsidis* (*Hpa*) WACO9. *Hpa* is resisted through similar salicylic acid (SA)-dependent post-invasive defences as *Pst* (Thomma *et al.,* 1998, Ton *et al.,* 2002), but it does not infect Arabidopsis leaves through the stomata (Coates & Beynon 2010) making it a suitable pathogen to test this hypothesis. As a further test, leaves of the Arabidopsis *SD* lines were syringe infiltrated with *Pst*DC3000, thereby bypassing the stomatal entry barriers.

Neither *Arabidopsis* plants overexpressing *EPF2* (with reduced *SD*) or *EPFL9* (with increased *SD*) were altered in their basal resistance to *Hpa*. Scoring of pathogen infection stage showed that there were no statistically significant differences in the distribution over the *Hpa* colonisation classes between the different genotypes (Figure 5a). Moreover, *Pst*DC3000 syringe infiltrated into the *EPF OE* lines did not reveal statistically significant differences in bacterial titres at 3 dpi (Figure 5b). Taken together, these results confirm that the differences in *Pst*DC3000 colonisation of *Arabidopsis* leaves after spray inoculation (Figures 2b & 2c) was most probably a result of the altered stomatal densities of the transgenic plants, rather than differences in post-invasive defences.

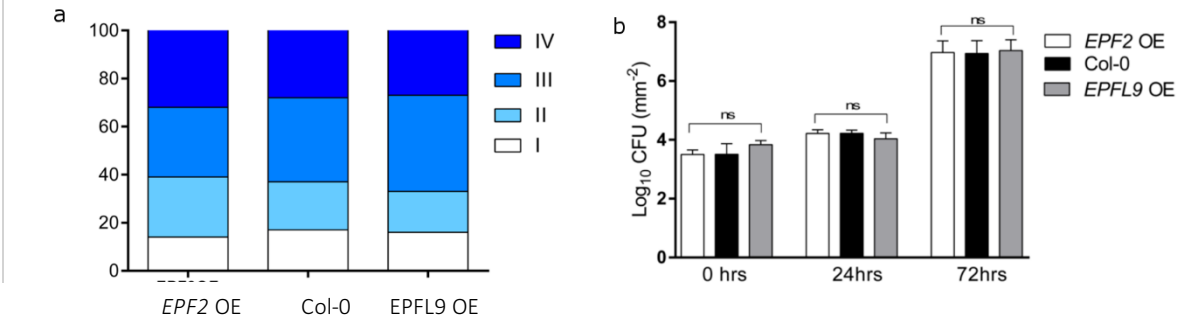


Figure 3.5. Genetic manipulation of stomatal density does not affect resistance to a pathogen that does not enter through the stomata, and does not affect post-invasive resistance to stomatal pathogens. a) Inoculaiton of *EPF2 OE*, Col-0 and *EPFL9 OE* plants with *Hyaloporanospora arabidopsidis* WACO 9 six days after inoculation with conidiospores. Leaves were stained and assigned to four classes of infection; Class 1 representing no visible infection, to Class IV, representing extensive levels of pathogen colonisation. Chi squared test revealed no significant distribution in class distribution between different plant genotypes. b) CFU of *Pst*DC3000 inside plant leaves at 0hrs, 24hrs and 72hrs following syringe infiltration of the bacteria. Bars on graph represent SE. No significant differences in colony forming units were observed between the different plant genotypes ($p<0.05$, n=8 plants, ANOVA test used to compare mutant genotypes with Col-0).

To further examine the effect of overexpressing the EPF signalling peptide on basal levels of signalling hormones known to be involved in defence, extracts of leaves of the stomatal development lines *EPF2OE*, *EPFL9OE* and Col-0 control plants were analysed by UPLC-qTOF.

Salicylic acid (SA) and jasmonic acid (JA) are the most important hormones used in defence against pathogens. It is well known that SA is primarily used to promote responses that resist attack from biotrophic pathogens, whilst JA is effective in mounting defence mechanisms against necrotrophic pathogens or herbivores. ABA is considered an important regulatory hormone in defence against both biotrophic pathogens and necrotrophic pathogens (Ton *et al.,* 2009). Firstly, ABA has been implicated in pre-invasive immunity and is an important signalling hormone responsible for shutting stomatal pores in response to pathogen detection (Zheng *et al.,* 2010). ABA also plays an important role when inducing callose formation around the substomatal cavity following pathogen ingress (Ton & Mauch-Mani, 2004; Flors *et al.,* 2005). The mass spectrometry results revealed variation in levels of these three hormones. There were no significant differences seen in SA, JA or ABA levels when comparing the EPF mutants with Col-0. This finding is somewhat surprising as ABA is also an important drought hormone that can be stored and produced in guard cells (Bauer *et al.,* 2013). Therefore, it is plausible that plants with altered *SD* have an altered capacity to store or produce ABA, considering that these lines may be experiencing drought differently and, therefore, regulate ABA biosynthesis in different ways. However, the UPLC-qTOF quantification of ABA did not reveal significant differences between the genotypes (figure 6). It is possible that differences in guard cell hormone levels might not be detected in whole leaf extracts, and that more detailed or extensive sampling may reveal differences. However, the results in Figure 6 support the notion that the *SD* lines are unaffected in production of SA and JA, which control post-invasive defences against biotrophic and necrotrophic pathogens, respectively. This reinforces the conclusion that the differences in resistance to spray-inoculated *Pst* are caused by variation in *SD* rather than differences in post-invasive defences.

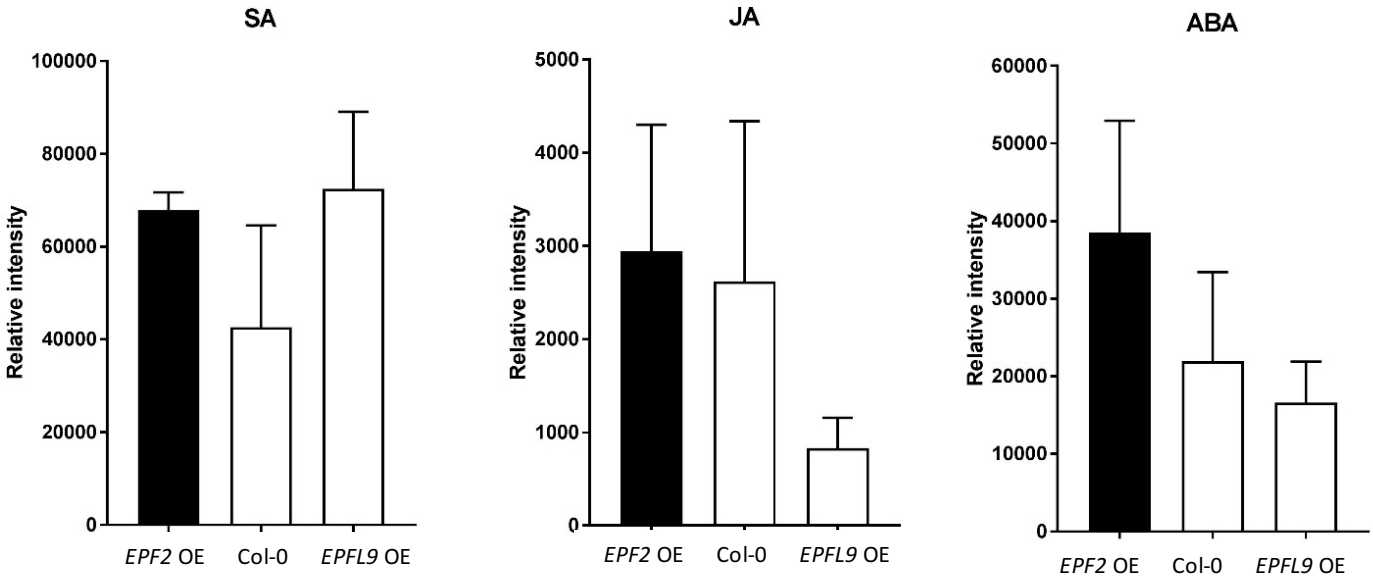


Figure 3.6. Analysis of important defence signalling hormones through the use of UPLC-qTOF mass spectrometry. a) Intensity reading of salicylic acid, b) intensity reading of jasmonic acid c) of intensity reading of abscisic acid (n=4 plants).

3.3 Discussion

In this chapter, it was demonstrated that altering the stomatal density of plants significantly alters levels of plant pre-invasive immunity to stomatal pathogens. It was also shown that the manipulation of *SD* by altering the expression levels of the EPF signalling peptides does not affect a plants ability to defend itself from infection by other means. Taken together these results suggest a potentially long lasting and durable way of reducing pathogen ingress during the early stages of plant infection by stomatal entering pathogens.

The relationship between stomatal density and pre-invasive immunity has received little previous attention. Work by Li *et al.* (2014) has shown that tomato plants grown under elevated CO_2 levels allow significantly fewer bacteria to enter through the stomatal pores following infection with *Pst*DC3000. The authors attributed this to a smaller stomatal pore area caused by stomatal closure under elevated CO_2 and thus a smaller total entry area for the bacteria to exploit. These results are in line with the results presented in this chapter which show that fewer stomatal entry points correlates with significantly fewer bacteria inside the leaf at 4 and 24 hpi.

Wheat plants with reduced *SD* also showed a significant difference in the percentage of germinated spores being able to form appressorium infection structures over guard cells at 12 hours' post inoculation with brown rust spores. In this experiment, *SD* mutants with fewer stomata had approximately 15% fewer appresoria forming over guard cells 12 hours post inoculation. This result suggests that crops with reduced *SD* could have enhanced resistance to a range of important agricultural pathogens that infect leaves via the stomata.

Niks (1990) germinated barley rust spores on plates before inoculating them onto barley seedlings and showed a negative correlation between germ tube length of inoculated spores and final fungal colony size 6 days after inoculation (Figure 7). The wheat EPF-OE lines have significantly fewer stomata than the wild-type variety. Consequently, germ tubes will on average have to grow for

longer, using up more of the spore's resources before locating a stomatal pore and forming an infection structure.

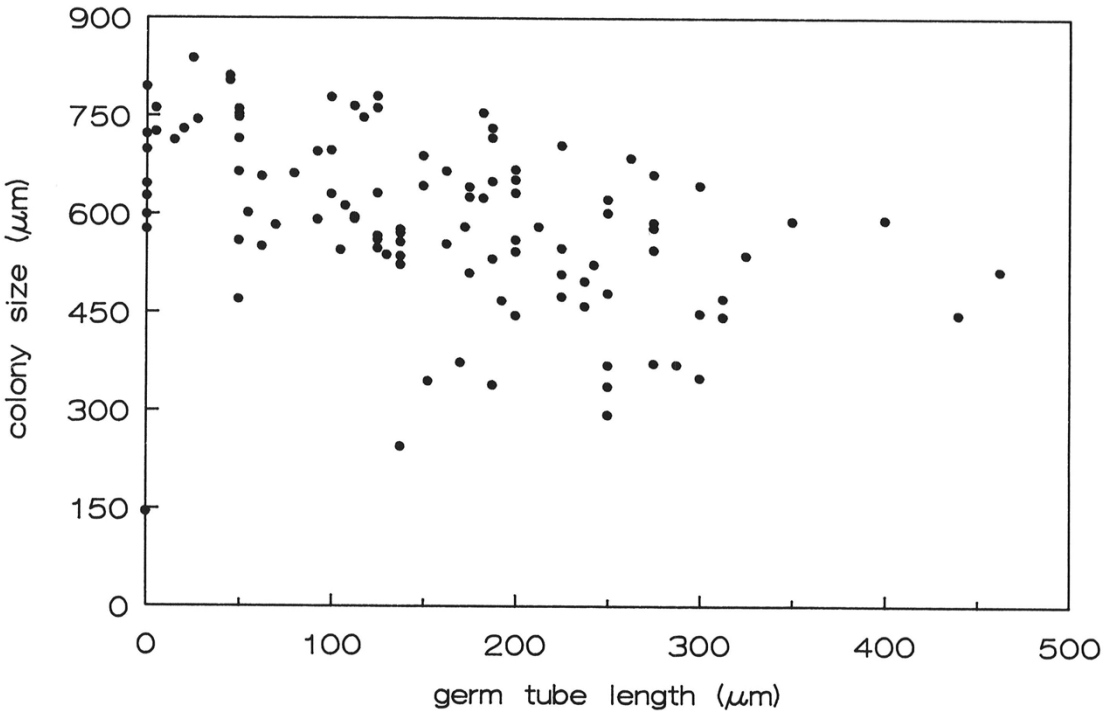


Figure 3.7. Scatterplot of germ tube length and colony size of 107 colonies of *Puccinia hordei*, colony size taken 6 days after inoculation of germ tubes onto seedling barley plants (figure from Niks. 1990)

It would be interesting to see if the longer time taken for the pathogen to gain access to the host translates into the plant being better able to induce important host defences before the pathogen enters the leaf appoplast. This would lead to less successful pathogen infection due to the pathogen using up more resources and the plant having more time to detect the pathogen and induce appropriate host defences. By comparing callose deposition and defence gene induction across a time course after spore inoculation it should be possible to determine if a longer initial infection process changes the rate of host defence induction in reduced *SD* plants.

For further research, it would be interesting to examine the long-term effect of stomatal density on disease/epidemic progression in the wheat mutants. To do this, it would be necessary to explore rust epidemic progression in greenhouses with larger populations of plants in the field.

Previous Arabidopsis experiments have shown that guard cells and mesophyll cells surrounding the sub-stomatal cavity have higher expression of the *FLS2* gene, which encodes a receptor that detects the presence of flagellin during bacterial pathogen attack (Beck *et al.,* 2014). Although the FLS2 receptor is known to play an important role in inducing defences against bacterial pathogens, the results in this Chapter do not support a link between *SD* and inducible plant defences. To further investigate this, it might be necessary to quantify the speed and/or intensity of defence-related gene induction in the stomatal cells following pathogen infection or PAMP application in the stomatal mutants. However, the *SD* lines were not altered in basal resistance against *Hpa*, nor were they affected in *Pst* resistance following syringe-infiltration into the leaves (Figure 5). This strongly suggests that the changes in *SD per se* do not have a wider impact on the responsiveness of post-invasive basal defences.

The feasibility of reducing *SD* in C3 grasses such as barley and rice has already been explored, and reduced *SD* barley mutants show no reductions in yield (Hughes *et al.,* 2017). Here it is shown that reducing *SD* may further increase plant fitness when encountering stomatal pathogens by increasing host pre-invasive immunity. Many devastating bacterial and fungal crop pathogens gain access to their hosts through the stomatal pores. Traditional methods of increasing plant resistance to these pathogens has predominantly focussed on breeding of varieties with single resistance genes that recognize the activity of specific pathogen effectors. This type of qualitative disease resistance (also referred to as race-specific resistance or effector-triggered immunity) typically does not offer durable resistance, since there is strong selection pressure on the pathogen to co-evolve alternative effectors that are no longer recognized by the host, thereby breaking the race-specific resistance. The experiments described in this chapter have explored a physiological defence mechanism that is potentially more durable than race-specific resistance. Although arguably less effective, this type of stomatal resistance may prove longer lasting than race-specific resistance. The results in this chapter suggest that future work to combine enhancements in genetic resistance with physiological barriers

to pathogens (i.e reduced stomatal density) might help the production of crops that express durable and effective disease resistance.

A potentially interesting avenue for future work would be to reduce stomatal development on the stems to reduce susceptibly to the stem rust pathogen. Stem rust is a particularly devastating pathogen causing the stem to collapse and the head of wheat to fall over. Mutants of Arabidopsis in *EPFL6*/*CHALLAH* have an interesting phenotype in that they lack stomata on the stem. Although there are clear physiological growth differences between monocots and dicots, it would be interesting to investigate whether if the *EPFL6* gene has a similar function in monocots. If successful, manipulation of this epidermal patterning factor could prove a useful tool to combat wheat stem rust.

3.4 Conclusions

The experiments described in this Chapter aimed to address whether manipulation of stomatal density through genetic manipulation of the EPF signalling peptides could prove a useful tool for reducing pathogen infection. The broad conclusions of this Chapter are as follows:

- Stomatal density of *SD*-altered Arabidopsis lines correlated with the number of bacteria entering the leaf at the relatively early time points of infection. This relationship was evident up to at least 24 hpi.
- Significantly fewer germinating brown rust spores are able to locate stomata and form infection structures on *SD*-altered transgenic wheat lines with fewer stomata.
- Manipulating *SD* by overexpression of *EPF* genes does not affect basal resistance against the biotrophic pathogen *Hpa*, nor does it affect resistance against pressure-infiltrated *Pst*DC3000. This indicates that *EPF* genes do not influence post-invasive defences against (hemi)biotrophic pathogens.
- Genetic manipulation of *SD* does not significantly alter constitutive levels of the defence signalling hormones SA, JA and ABA.

Chapter 4: The effect of pathogen infection on stomatal formation

4.1 Introduction

In Chapter 3, the effect of stomatal density on early pathogen colonisation was investigated. The results indicated that plants that are genetically manipulated to have fewer stomata exhibit lower levels of infection by stomatal pathogens. Using the interaction between *Arabidopsis thaliana* and *Pseudomonas syringae* strain DC3000 (*Pst*DC3000) as a model system, this Chapter explores the effect of localised pathogen infection on the formation of stomata in newly formed distal leaves. Additionally, this Chapter described the local and systemic signalling pathways controlling this *SD* response.

As outlined in Chapter 1, it is well known that environmental conditions experienced by mature Arabidopsis leaves can influence the epidermal characteristics of newly developing leaves. Local exposure to altered light intensity, CO_2 concentrations and humidity all affect the frequency at which stomata develop in newly formed leaves (Schoch *et al.,*1980; Lake *et al.,* 2001; Thomas, Woodward & Quick, 2004). The chemical nature of the long-distance (or systemic) signals regulating stomatal development remains unknown. Previous studies on the effects of pathogens on epidermal leaf morphology has revealed some interesting results. Experiments focusing on viral infection of Arabidopsis has shown a reduction in stomatal density (*SD*) and stomatal index (*SI*) in leaves forming after viral infection. Further to this, Lake and Wade (2009) showed that powdery mildew (*Erysiphe cichoracearum)* induces an increase in *SD* of 15% on the abaxial leaf epidermis under ambient CO_2 concentrations and an *SD* increase of 38% under elevated CO_2 (Lake & Wade 2009). However, these studies did not report the mechanism by which plants modulate stomatal development following pathogen infection. Moreover, the pathogens used in these studies are non-stomatal pathogens that do not infect via the stomatal pores. As stomatal pores are important entry sites for other pathogens, experiments were designed to investigate the stomatal development response of plants after they had been infected with *Pseudomonas syringae* DC3000 (*Pst*DC3000), a leaf pathogen that requires

stomata for its infection.

4.1.1 Pathogen resistance and stomatal density are both regulated by DNA methylation

Epigenetics refers to the study of heritable or long term changes in gene expression due to a change in the transcriptional potential of DNA that is not caused by alterations to the DNA sequence itself. For example, methyl groups can be added to cytosines at CG, CHG and CHH sequence contexts in pants. DNA methylation alters gene expression by influencing transcription or modifying chromatin structure. The methylation state of DNA can be maintained over mitosis and meiosis, thus provides a medium for inheritance of specific gene expression. DNA methylation patterns can faithfully be transmitted to the next generation, where they can provide a memory of biotic stress and allow a faster and/or stronger defence response to pathogens in progeny form disease plants (Luna *et al.,* 2012). In the case of *Pst*, this is maintained over a stress free generation and improves resistance to a range of pathogens in subsequent generations (Luna *et al.,* 2012). Changes in DNA methylation can direct post-translational modification of histones, which influence the density of chromatin and the expression and/or responsiveness of genes (Kohler & Springer 2017).

Environmental conditions such as low relative humidity can induce RNA-directed DNA methylation, which has been shown to supress stomatal development. The offspring of plants subjected to low relative humidity have a stomatal index that is 27% lower than control plants. This effect was shown to last for three successive generations (Tricker *et al.,* 2012, Tricker *et al.,* 2013). The molecular basis of this developmental control is through the methylation of DNA adjacent to the *SPCH* and *FAMA* gene loci, which encode transcription factors regulating stomatal density (as described in section 1.4). This DNA methylation acts to suppress the expression of these two genes and to reduce stomata number.

As the previous research described above has indicated that DNA methylation can epigenetically affect levels of plant resistance to *Pst*, and the extent of stomatal development, experiments were designed in this study to investigate whether a *Pst*-induced systemic reduction in *SD* has an epigenetic basis.

4.2 Results

4.2.1 Local Infection of mature leaves with *Pst* reduces stomatal density in newly developing leaves

To understand the effect of infection by *Pst*DC3000 on stomatal development three mature leaves of five week-old *Arabidopsis* plants were syringe-infiltrated with *Pst*DC3000. This method of infection forces a known concentration of bacteria into the leaf apoplast. At the time of infection the smallest developing leaves emerging from the apical meristem were marked with a small dot of white paint. The stomatal density and stomatal index of fully expanded mature leaves developing after the marked leaves, was determined from epidermal leaf impressions. The results in Figure 1b clearly show that plants that have been exposed to *Pst*DC3000 develop new leaves that have a lower stomatal density than those plants that were mock inoculated with $MgSO_4$. This experiment was repeated on several occasions, the average reduction in *SD* of leaves developing after *Pst*DC3000 infection across 7 independent experiments ranged from 7% to 20% (Table 1).

Col-0 Mock (Stomata mm^{-2})	Col-0 *Pst*DC3000 (Stomata mm^{-2})	% reduction	P-value
215.6	184.0	14.7	0.0356
198.7	163.3	17.8	<0.0001
284.0	268.0	5.7	0.534
253.0	210.0	20.0	<0.0001
226.7	201.3	11.1	0.0396
198.7	167.3	15.6	<0.0001
258.7	234.0	9.5	0.0023

Table 4.1. Reduction in stomatal density following infection by *Pseudomonas syringae* pv. *tomato* DC3000 across seven independent experiments. T-test comparing mock inoculated plants with infected plants.

In the experiment presented in Figure 1b, the mean *SD* of newly developed leaves of *Pst*DC3000-inoculated was 169 mm^2 compared to 210 mm^2 in newly developed leaves of mock-inoculated plants. There was also a reduction in the epidermal pavement cell density, as a result of which there was no reduction in Stomatal index (*SI*, i.e. the proportion of cells that are stomata). Although there was no significant difference between the size of leaves that developed after *Pst*DC3000 inoculation, the guard cells were slightly larger and showed a significantly longer mean guard cell length (i.e. 20μm rather than 18μm in length; Figure 1d). In contrast to mock-inoculated leaves, leaves that had been infected with *Pst*DC3000 wilted over the weeks following infection. To investigate whether the observed change in stomata density and length occurred as a result of a loss in photosynthetic capacity, rather than a defence-related long-distance signal, an experiment was set up to investigate whether excision of leaves (instead of infection) affects stomatal development in newly developing leaves. Three leaves were excised from uninfected mature Arabidopsis plants. The leaves developing after excision of these leaves were subsequently examined for stomatal density when fully expanded. This control experiment did not reveal statistically significant differences in *SD* as a consequence of leaf removal (Figure 1c). To confirm that the observed reduction in SD following localised *Pst*DC3000 inoculation was a result of a systemic signal rather than systemic movement of bacteria *in planta* or bacterial contamination of the developing rosette during primary inoculation, bacterial titres were quantified in the infected leaves and developing rossete by dilution plating. As is shown in Figure 1e, no colony forming units of *Pst*DC3000 could be detected in extracts from the rosette tissues. Together, these results strongly indicate that infection of mature Arabidopsis leaves by *Pst*DC3000 causes a systemic reduction in *SD*, which causes the development of fewer but larger guard cells and epidermal pavement cells.

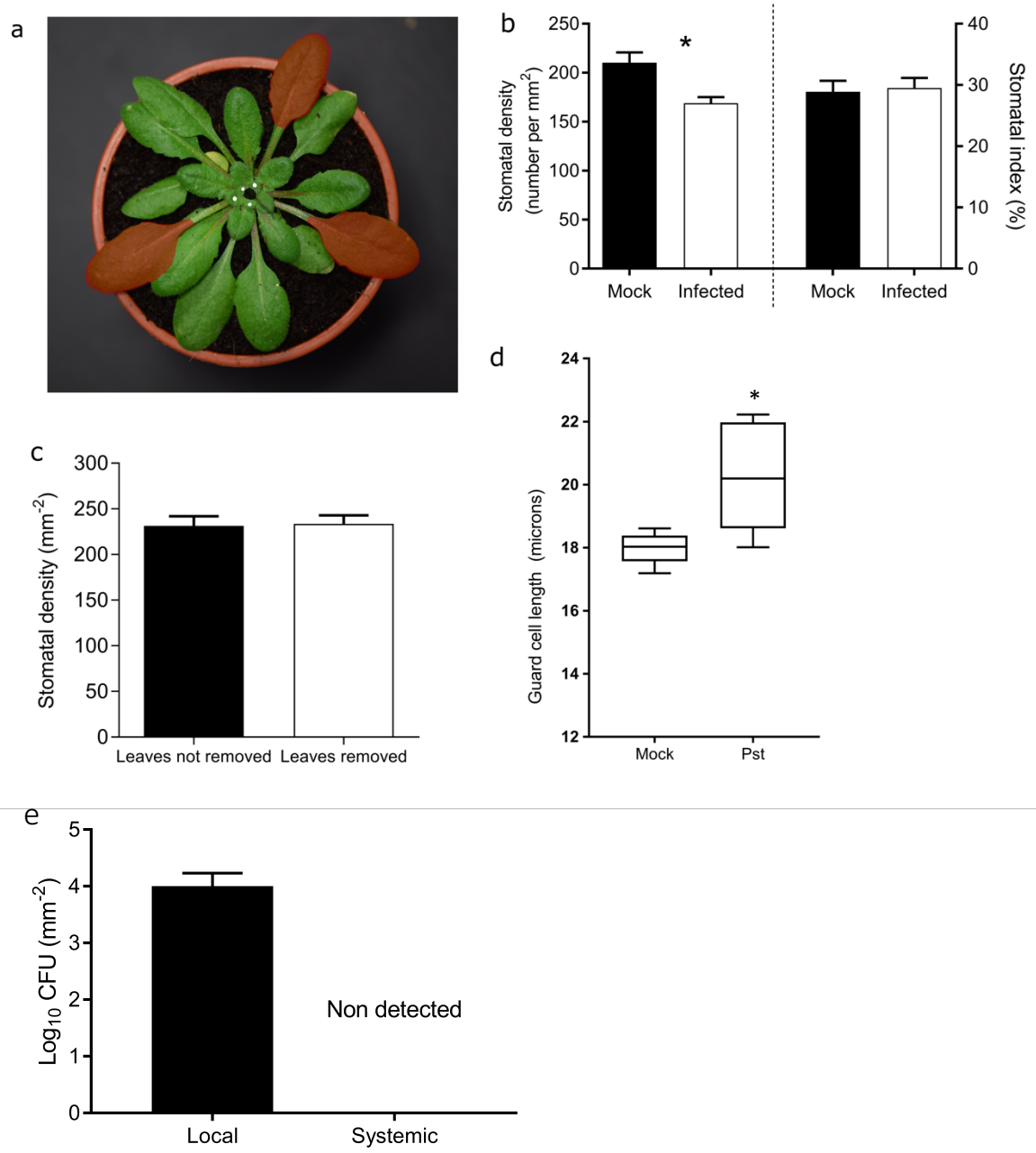

a
b
Stomatal density (number per mm2)
Stomatal index (%)
Mock
Infected
Mock
Infected
c
Stomatal density (mm-2)
Leaves not removed
Leaves removed
d
Guard cell length (microns)
Mock
Pst
e
Log10 CFU (mm-2)
Non detected
Local
Systemic

Figure 4.1. Stomatal density is reduced in leaves that develop following localised infection with *Pst*DC3000 by a systemic signal. a) Illustration of the experimental procedure used to infect Arabidopsis plants. Three mature leaves indicated in red were syringe infiltrated with *Pst*DC3000, mock treated or excised to understand the effect on *SD*. The developing leaves present in the rossete at the time of infection were marked with a small dot of white paint. The black area represents the position of leaves developing after infection that were taken for *SD* counts after bacterial infection. b) Mean *SD* and stomatal index of fully expanded leaves taken after infection or mock treatment. c) Mean *SD* of subsequently developing leaves of untreated Col-0 plants that either had 3 leaves removed or had leaves left intact. d) mean guard cell length taken from fully expanded leaves developing after mock inoculation and after *Pst*DC3000 infection. e) Colony forming units of *Pst*DC3000 detected form the developing rosette and from the infected leaves following syringe infiltration. * significant difference in comparison to mock treatment, T-test ($p<0.05$, $n=8$). Error bars = SEM.

To investigate the nature of the signalling pathways controlling the systemic reduction in *SD* following pathogen infection, the effects of localised elicitor treatment, as well as mutations in previously established defence signalling pathways of Arabidopsis, were assayed. These defence signalling mutants and elicitor experiments were set up in a similar way to those in Figure 1 and the results are described in the following two sections.

4.2.2 Systemic reductions in Stomatal Density and Local Pathogen Defence Signalling

To examine the effect of local pathogen signals on systemic *SD*, three local leaves were infiltrated with chemical defence elicitors triggering local defence signalling at the sites of pathogen attack. The defence signal elicitors that were selected included two pathogen associated molecular patterns (PAMPs). The first PAMP was flg22, a 22 amino acid derived from bacteria flagellin which is known to be an important immunity eliciting signal during bacterial infection that induces short-term stomatal closure (Mellotto *et al.,* 2006) and other downstream defences, including activation of SA-dependent signalling pathways (Zipfel *et al.,* 2004). The other PAMP was lipopolysaccharide (LPS), an integral component of Gram-negative bacterial cell walls. Like flg22, LPS is known to induce the expression of plant antimicrobial metabolites and induce accumulation of pathogenesis-related proteins (Erbs & Newman *et al.,* 2003; Zeilder *et al.,* 2004). In addition, SA was infiltrated into the local leaves to examine the effects of local accumulation of this hormone on the systemic *SD* response, since it is well-documented that SA biosynthesis is upregulated at the local sites of pathogen attack.

As shown in Figure 2a, local *Pst*DC3000 inoculation resulted in a systemic *SD* reduction in newly develop leaves by 18%. Furthermore, local treatment with flg22 and SA reduced systemic *SD* by 22% and 13%, respectively. A T-test confirmed that these *SD* reductions were statistically significant in comparison to the corresponding mock treatments. However, infiltration with LPS failed to induce a statistically significant reduction in *SD*, suggesting that LPS does not trigger this developmental response.

To further examine the involvement of flg22 and SA in the systemic *SD* response, experiments were conducted with mutant lines of Arabidopsis that lack the FLS2 receptor (which detects flg22), and are therefore impaired in their ability to activate a defence response to flg22. Infiltration of *fls2c* mutants with *Pst*DC3000 did not result in a statistically significant reduction in *SD* (Figure 2b). A notable finding from this experiment was that the *fls2c* mutant had a significantly lower *SD* density then Col-0 plants, even when not infected with the bacterium. This unexpected finding will be discussed later in the chapter. However, the results of these experiments clearly indicate that flg22 and the FLS2 receptor play a critical role in the perception of bacterial pathogens leading to the systemic reduction in *SD* seen following pathogen infection, and that SA is involved in transducing the signal.

To investigate the role of SA in the systemic *SD* response the following SA signalling mutants were tested: *sid2-1* plants, which are impaired in their ability to increase SA production following pathogen attack (Wildermuth *et al.,* 2001), *npr1-1* plants, which lack the key regulatory NPR1 protein in the SA signalling response (Cao *et al.,* 1994), and NahG plants, which are depleted in basal and inducible SA accumulation due to the conversion of endogenous SA into catechol by the SA hydroxylase NAHG (Van Wees & Glazebrook 2003). Infiltration of *sid2-1* and NahG mutants with *Pst*DC3000 did not result in a statistically significant reduction of *SD* in the newly developing leaves, further supporting the role of SA in this response (Figure 2b). However, infiltration of *npr1-1* mutants with *Pst*DC3000 did induce a statistically significant reduction in *SD*, which was comparable to that of Col-0 plants. This result suggests that NPR1-induced transcriptional control of downstream defence signalling genes is not required for the developmental response of stomata to *Pst*DC3000. Thus, some but not all local defence signalling components are involved in perceiving the local *Pst*DC3000 infection and generating a systemic signal that suppresses stomatal development in systemic leaves developing after infection.

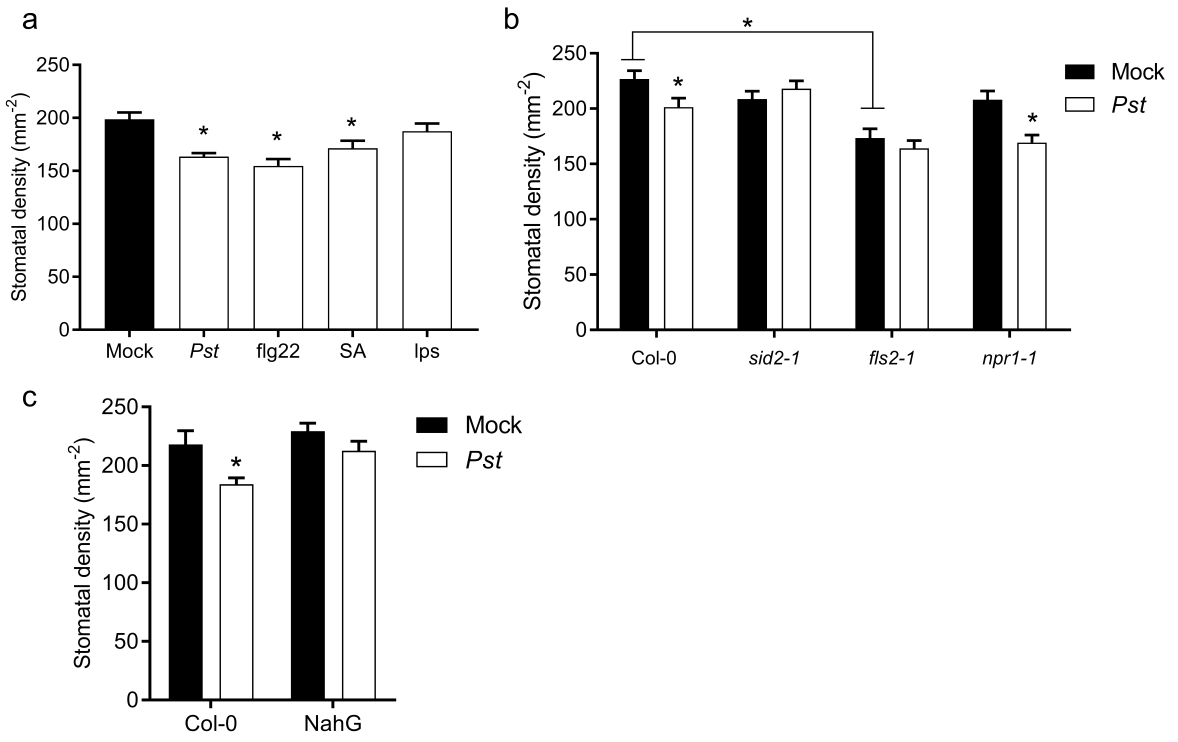


Figure 4.2. PstDC3000-Induced reductions in stomatal density require perception of flagellin by the FLS2 receptor and the accumulation/production of salicyclic acid. a) Mean stomatal densities of Col-0 plants syringe infiltrated with defence elicitors flagellin (flg22), salicylic acid (SA) and lipopolysaccharides (LPS). b) Mean stomatal densities of Col-0, *sid2-1*, *fls2c*, *npr1-1* following mock inoculation and inoculation with *Pst*DC3000. c) Mean Stomatal density of *NahG* and Col-0 control plants after infiltration with *Pst*DC3000. * Indicates significant differences in comparison to mock treatment, T-test ($p<0.05$, n=8). Error bars = SEM. Significant differences in *SD* between genotypes were detected by two-way ANOVA.

4.2.3 Involvement of systemic plant defence signals in the systemic reduction in stomatal density

To identify the possible long distance defence signals emanating from *Pst*DC3000 infected leaves that mediate reductions in *SD,* Arabidopsis mutants impaired in the generation of long-distance defence signals were tested for *Pst*DC3000-induced reduction of *SD*. Three mutants impaired in long-distance signalling during systemic acquired resistance (SAR) were studied. SAR is a systemic response to localised pathogen attack, resulting in a primed immune state that enables a faster and/or stronger induction of defence responses upon secondary challenge by a pathogen (Conrath *et al.,* 2006).

As discussed in Chapter I, azelaic acid (AzA), the lipid transfer protein DIR1 and pipecoloic acid (L-Pip) have been reported to act as systemic SAR signals, or a SAR-amplifying signal in the systemic tissues, respectively. Syringe infiltration experiments were carried out using the *ald1* mutant, which is impaired in the synthesis of L-Pip (Song *et al.,* 2004), the lipid transfer protein mutant *dir1*, which is impaired in long-distance SAR signalling (Maldonado *et al.,* 2002), and the *azi1* mutant, which lacks another lipid transfer protein that is critical for perception of the systemic AzA signal (Jung *et al.,* 2009). Both the *dir1* mutant and the *ald1* mutant retained the ability to reduce systemic *SD* upon infection, suggesting that DIR1 and L-pip are not involved in the systemic *SD* response (Figure 3a, 3c, 3d). Conversely, *Pst*DC3000 infiltration of *azi1* plants, which are insensitive to AzA, failed to induce a statistically significant reduction in stomatal density following infection (Figure 3d). This experiment was repeated 3 times with similar outcomes. Although all three experiments consistently showed a small reduction in *SD,* this effect was never statistically significant in comparison to the corresponding Col-0 controls. This indicates that AZI1 is involved in the *SD* response, but that other other signalling components with similar function have a similar but weaker contribution to the response.

To further examine the role of AzA in the systemic *SD* response, leaves of Col-0 plants were infiltrated with 1 mM AzA (a concentration that is sufficient to trigger SAR; Jung *et al.,* 2009). The result of this experiments is shown in Figure 3b, demonstrating that AzA alone failed to induce systemic SD

reduction. This suggests that AzA is not the systemic signal that brings about the *Pst*-induced reduction in stomatal development in the distal developing leaves. Thus, it is possible that *AZI1* plays a role in mediating the systemic *SD* response through transport of a different signalling molecule than AzA. Alternatively, AZI1 acts independently of its role in the AzA response, for instance through its recently described role as a proline-rich protein (Pitzschke *et al.,* 2016).

Finally, to investigate the role of ABA in the systemic *SD* response, leaves of the *nced3/nced5* double mutant, which is impaired in ABA biosynthesis (Frey *et al.,* 2012), were infiltrated with *Pst*DC3000. As is shown in Figure 3c, this ABA-deficient mutant was still able to mount a statistically significant reduction in *SD* compared to mock-treated *nced3/nced5* plants. Hence, ABA does not play a role of significance in the systemic *SD* response to *Pst*DC3000.

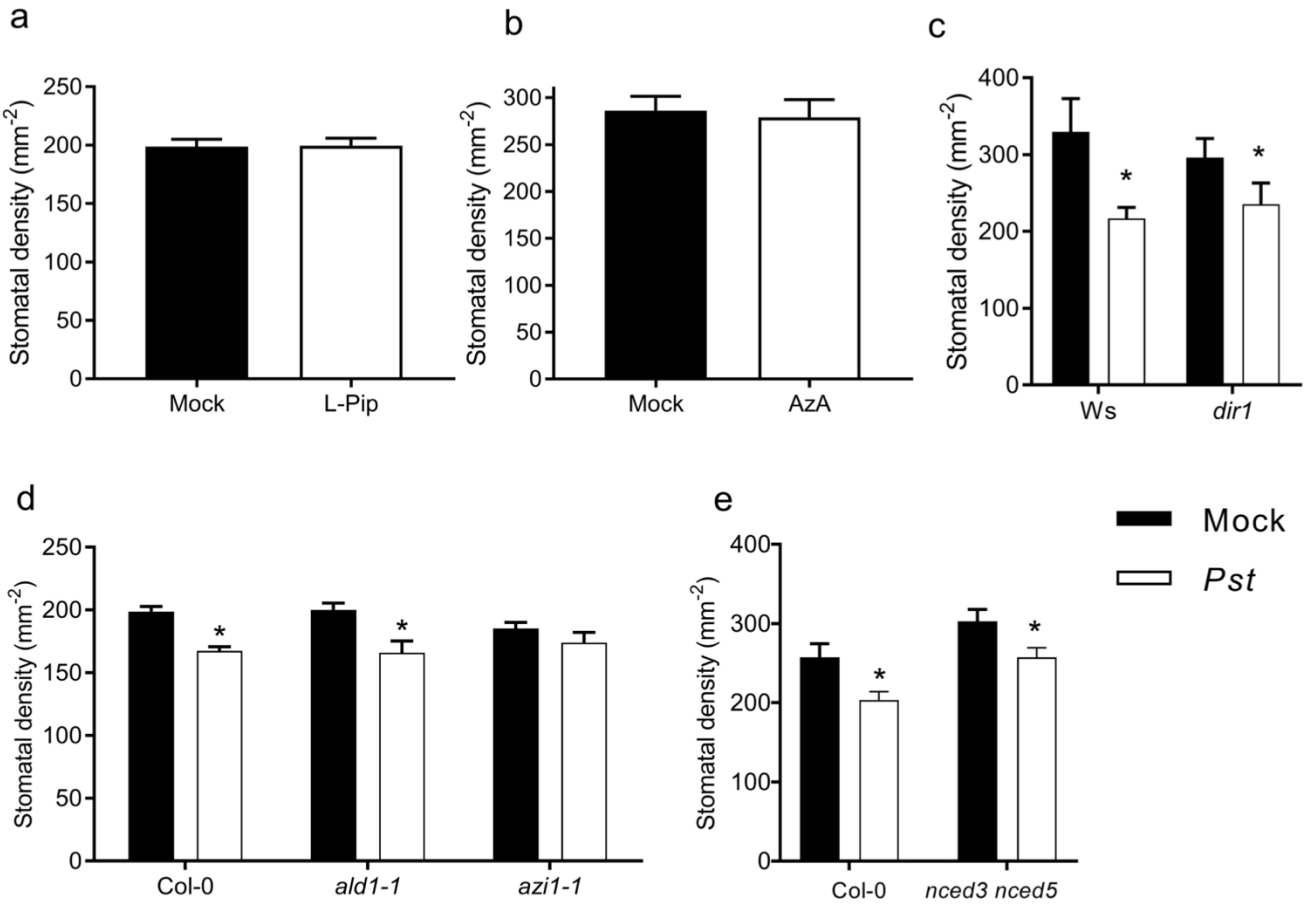


Figure 4.3. Systemic defence signalling and its integration with systemic stomatal reductions. a) Mean stomatal densities of leaves developing after infiltration with pipecolic acid (L-Pip). b) Mean stomatal densities of leaves developing after 1mM azelaic acid (AzA) infiltration. c) Mean stomatal density of distal developing leaves of the systemic signalling and lipid transfer protein mutant *dir1* after *Pst*DC3000 infiltration. d) Mean stomatal density of the systemic signalling mutants *ald1* and *azi1* after *Pst*DC3000 infiltration. e) Mean stomatal density of Col-0 and *nced3/nced5* mutants following *Pst*DC3000 infiltration. * denote statistically significant differences to mock control T-test ($p<0.05$, n=8). Error bars = SEM.

4.2.4 Known stomatal development genes are not differentially expressed following PstDC3000 infiltration

To explore a possible role for stomatal patterning genes in the systemic *SD* response to *Pst*DC3000, RT-qPCR was conducted to profile transcript levels of four genes that are known to be important in regulating the frequency at which stomata develop. *EPF2* and *EPFL9* both encode small signalling peptides (as discussed in Chapter I), whilst *SPEECHLESS* and *MUTE* encode transcription factors, controlling progression of cell divisions through the stomatal lineage (Pillitteri *et al.,* 2007). RNA was extracted from the developing leaf rosette at 24 and 48 hours after infiltration of mature leaves with *Pst*DC3000. No significant differences in *EPF2*, *EPFL9*, *SPEECHLESS* or *MUTE* were observed at either of these time points after infection (Figures 4a & 4b). These results indicate that stomatal regulatory components are not directly involved in the systemic *SD* response to *Pst* infection.

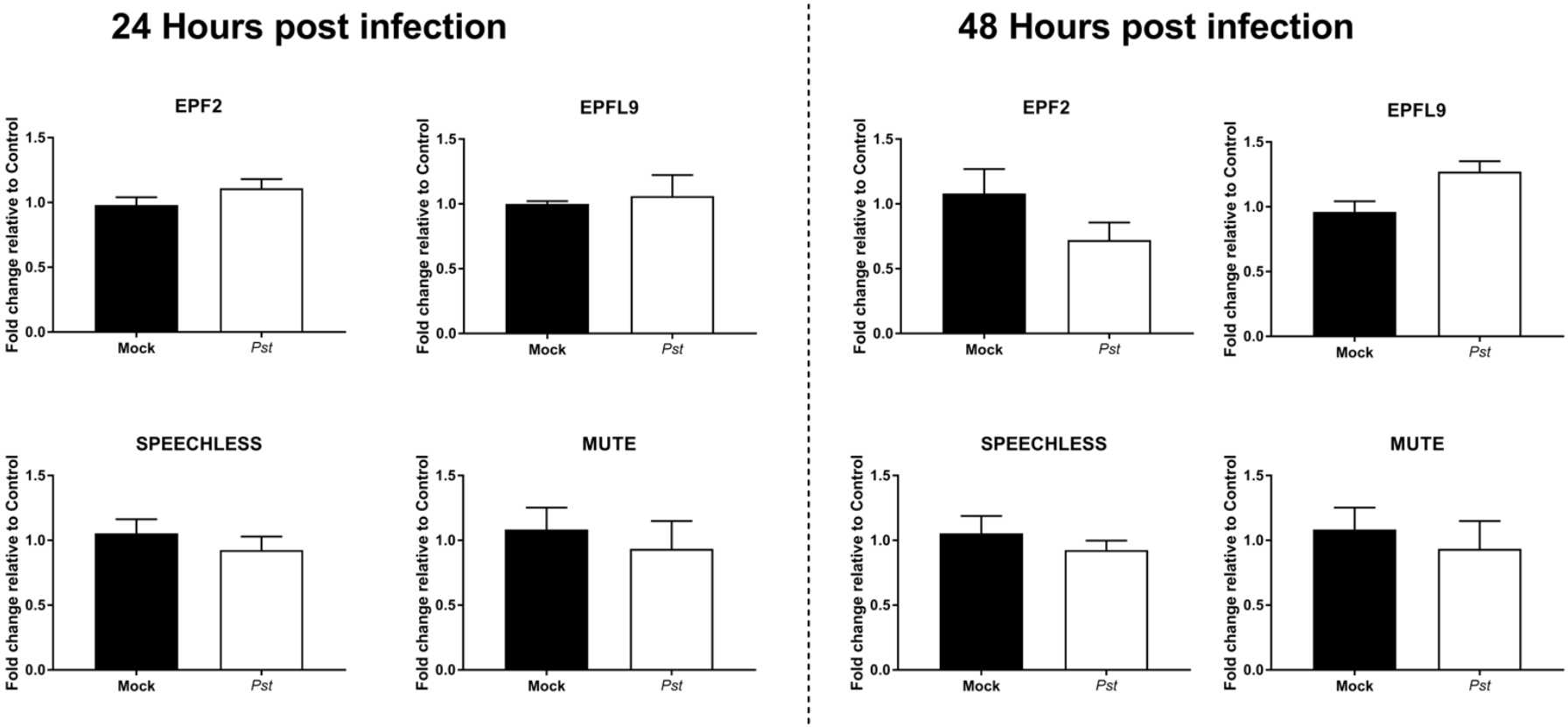


Figure 4.4. Expression levels of stomatal development genes following *Pst*-infection. Rt-qPCR of RNA extracted from developing leaves in the rosette centre following infection of mature leaves with *Pst*DC3000. Expression levels of the stomatal development genes *EPF2*, *EPFL9*, *SPEECHLESS* and *MUTE* at a) 24 hours and b) 48 hours' post inoculation with *Pst*DC3000.

4.2.5 Investigating the role of epigenetics in the maintenance of a systemic reduction in *SD*

To examine the role of DNA methylation in the systemic *SD* response, two Arabidopsis mutants with altered levels of genomic DNA methylation were selected. These were the *ros1* mutant, which has increased levels of DNA methylation due to reduced vegetative DNA demethylation activity (Kanno *et al.,* 2004), and the *npre1* mutant, which is impaired in RNA-directed DNA methylation. Both mutants were infiltrated with *Pst*DC3000 and assayed for systemic *SD* reduction in comparison to the Col-0 wild type. Results from these experiments revealed no impact by either the *ros1* mutation or the *npre1* mutation, suggesting that DNA methylation does not play an important role in the systemic SD response.

Since DNA methylation is not the only mechanism through which plants can alter gene expression plants that had undergone eight successive generations of either *Pst*DC3000 infection or mock treatment were analysed for a *SD* phenotype. Analysis of mature leaves from these lines revealed no differences in *SD* (Figure 5a, 5b & 5c). Taken together these results show no evidence for DNA methylation being responsible for the reduction in *SD* seen after *Pst*DC3000 infiltration, nor is there any evidence to suggest this *SD* phenotype is epigenetically maintained as a long-term response.

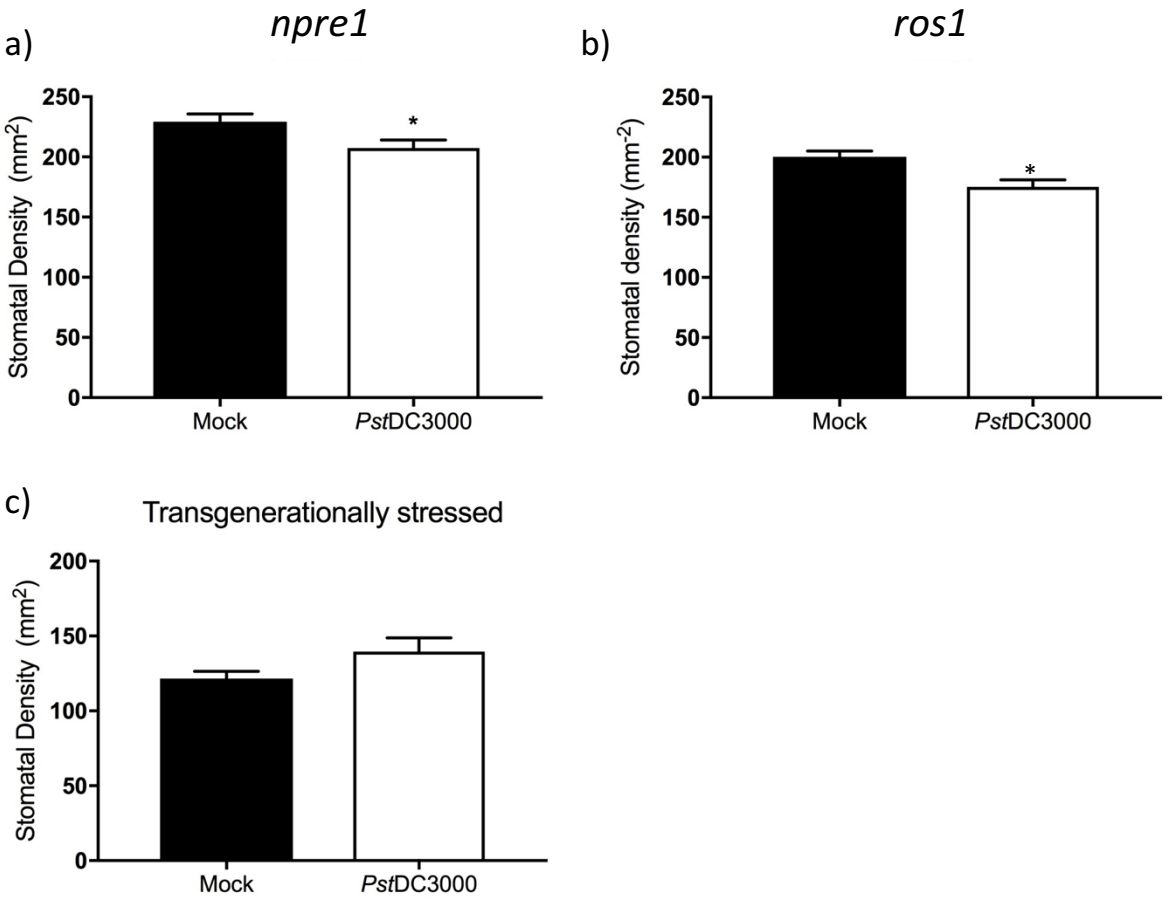


Figure 4.5. Investigating the effect of DNA methylation mutants and transgenerational stress in Pst induced reduction in *SD*. a) Mean *SD* of *npre1* leaves developing following *Pst*DC3000 infiltration of mature leaves. b) Mean *SD* of *ros1* leaves developing following PstDC3000 infiltration. c) Mean *SD* of Col-0 plants that had undergone 8 generations of repeated disease exposure by *Pst*DC3000 or 8 generations of mock inoculation. This population of plants was kindly provided by Dr Lisa Smith. * denote statistically significant differences T-test ($p<0.05$, n=8). Error bars = SEM.

4.3 Discussion

The results presented in this Chapter have demonstrated that local infection of Arabidopsis by the foliar leaf pathogen *Pst*DC3000 results in a systemic *SD* reduction in leaves that develop after infection. Evidence from these experiments showed that *Pst*DC3000 infection causes the induction of a systemic signal transferred from the local infected tissue to the distal systemic developing leaves in the centre of the leaf rosette. This signal induces the formation of larger guard and epidermal cells, hence controlling cell expansion, cell elongation and cell cycle regulatory genes, rather than influencing the ratio between stomatal cells and epiderminal cells (i.e. SI). Indeed, infiltration of mature leaves with *Pst*DC3000 resulted in reduced *SD* on newly developing leaves, but did not result in a change in *SI*. This outcome indicates that the plant is producing fewer, but larger epidermal cells, which was confirmed by the observed increase in mean guard cell length. Thus, it appears that the reduction in *SD* after bacterial infection may occur via an increase in epidermal cell size. The lack of change in *SI* contrasts with the response seen when *Arabidopsis* leaves are exposed to different light levels and different CO_2 concentrations where we see both a change in *SD* and *SI* (Lake *et al.,* 2001) (Figure 6). The results from the *Pst*DC3000 infiltration experiments in this Chapter also contrast with the results of Murray *et al.* (2015), who showed an increase in *SI* in plants infected with a viral pathogen and a reduced *SD*. This indicates that the stomatal development response to plant disease varies between pathogens.

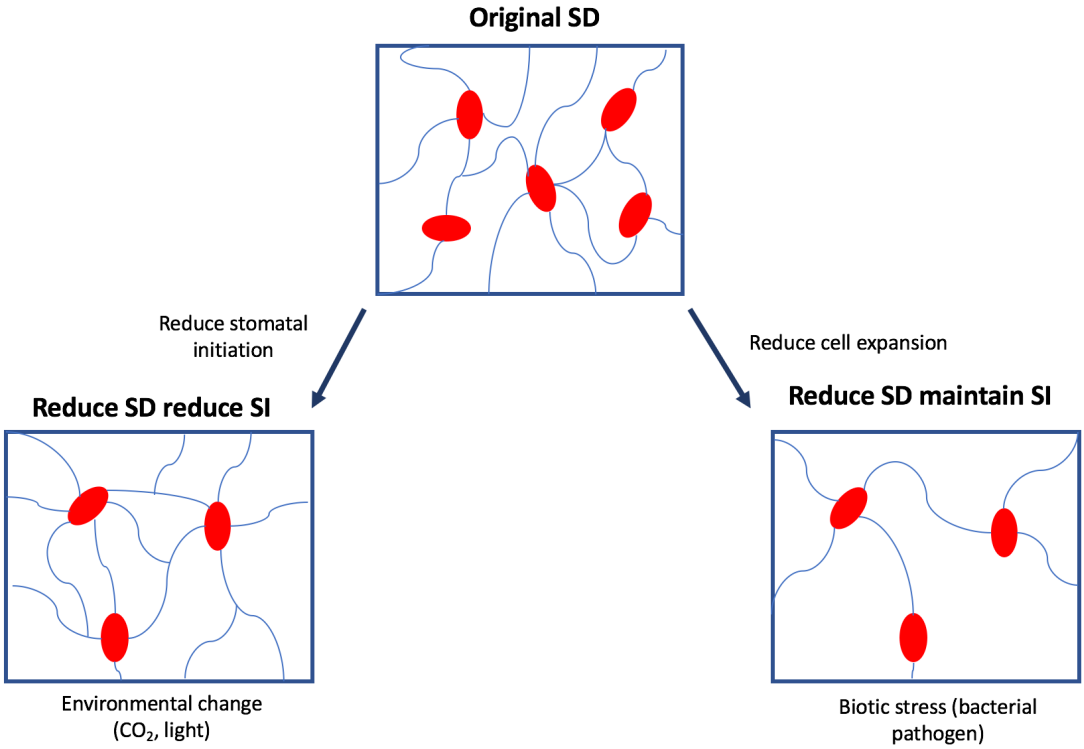


Figure 4.6. Illustration of possible strategies used by Arabidopsis plants to reduce stomatal density in developing leaves. Left-hand branch illustrates epidermal development of *Arabidopsis* leaves following exposure to abiotic stimuli such as high atmospheric CO_2 concentration or low light intensity; a reduction in stomatal density by controlling epidermal cell fate decisions creates more epidermal pavement cells and fewer guard cells. Right-hand branch illustrates epidermal development following infection with a bacterial pathogen; a reduction in both stomatal density and epidermal pavement cell density resulting in fewer and larger stomata and epidermal pavement cells, possibly caused by increased cell expansion and/or a reduction in the number of cell cycles.

The response of several Arabidopsis genotypes with defects in local and systemic defence signalling provided further insights into the molecular and biochemical mechanisms controlling the systemic *SD* response to *Pst*DC3000 infection. Pathogen infiltration experiments with mutants impaired in local defence signalling evens upon pathogen infection revealed that detection of flagellin by the FLS2 receptor, SA production and SA accumulation are all required for the systemic *SD* reduction response. SA has a well-documented role in the induction of several host defences during pathogen infection, even being implicated in the induction of stomatal closure following bacterial pathogen attack (Zheng *et al.,* 2010). Experiments with mutants impaired in systemic SAR signalling indicated requirement for the lipid transfer protein AZI1. However, many additional components known to mediate SAR, including signalling molecules, like L-pip, AzA, the bacterial PAMP LPS, and the transcriptional defence regulatory protein NPR1, were not critical for pathogen-induced reductions in stomatal development.

AZI1 is a member of a family of lipid transfer proteins and is critical for the regulation of systemic acquired resistance (SAR) through its probable interaction with azelaic acid (AzA) (Jung *et al.,* 2009). Although experiments in this chapter showed that AzA is not required for the systemic reduction in *SD* observed, *azi1* plants did not show a significant reduction in SD following *Pst*DC3000 infection but a small (non-significant) reduction in *SD* was reproducibly observed in systemic leaves of this mutant following infection. This could be explained by functional redundancy between members of the lipid transfer protein (LTP) family and suggest that other LTPs may be involved in the response. LTPs are encoded by a large gene family, within this the AZI1 gene is part of a tandem array of four related LTPs on chromosome 4 in Arabidopsis plants (ensembl.org). The SD response to local *Pst*DC3000 infection was also examined in another LTP mutant, *dir1-1*. DIR1 also plays a prominent role in the systemic induction of SAR (Maldonado *et al.,* 2002). However, bacterial infiltration caused a wild-type *SD* response in the *dir1-1* mutant, indicating that DIR1 is not involved in the systemic reduction in *SD* observed. Thus, the LTP AZI1, and possibly other related LTPs encoded by the same gene family, are involved in the systemic *SD* response to *Pst*DC3000, although it appears that AzA itself is not the systemic signal. Since the AzA precursor 9-oxo nonanic acid (ONA) has also been proposed to act as a

systemic signal (Wittek *et al.,* 2014), it is possible that AZI1 mediates the transport of ONA or a related lipid signalling molecule. Alternatively, AzA alone is not sufficient to induce the systemic response and it may require a parallel signal to mediate the full response.

Several lines of evidence in the literature point to a link between plant disease and alterations in epidermal leaf morphology. For example, expression of bacterial virulence genes (effectors) in *Arabidopsis* has been shown to result in extreme stomatal clustering and increased stomatal development (Meng *et al.,* 2015). In this Chapter, it was shown that plants lacking the FLS2 receptor develop leaves with significantly reduced *SD* in comparison to Col-0 plants. This evidence points to a convergence of the stomatal development pathway and defence signalling/responses in plants. This could perhaps be mediated through the differential activation of a common mitogen-activated protein kinase (MAPK) cascade. Regulation of the stomatal development pathway through the ERECTA receptor and induction of defence signalling through the FLS2 receptor both utilise the same MAPK and MAPK kinase (MKK) signalling components. Induction of MKK4/5 and MAPK3/6 supresses the formation of stomata (Wang *et al.,* 2007), whilst flg22 activates MPK3/6. Furthermore, MKK4/5 knockout plants are impaired in defence induction by the FLS2 receptor (Asai *et al.,* 2002). It therefore seems possible that differential regulation of this MAPK signalling cascade upon pathogen infection is responsible for enforcing changes in SD caused by pathogen infection. However, as noted above, this does not explain the lack of difference in SI following *Pst* infection.

The question remains as to whether MAPK signalling mutants are affected in the systemic SD response to *Pst*DC3000. Indeed, no evidence could be found in this study for the involvement of the EPF signalling peptides in the *SD* response to PstDC3000. Although EPFs are thought to act upstream of the MAPK signalling cascade, their transcript levels appeared to be unaltered following infection. However, EPFs are activated by proteolytic cleavage and are only expressed in a very small transient manner during development. Thus, a change in the transcript level may not necessarily reflect a

change in activity, which may be more difficult to detect. Further work is required before the involvement of the EPF signalling pathway in the pathogen induced reduction in *SD* can be ruled out.

Epigenetic regulation of SD has been reported to control stomatal development in response to humidity (Tricker *et al.,* 2012, Tricker *et al.,* 2013). In this Chapter, experiments were carried out to investigate the involvement of DNA methylation in pathogen-induced *SD* reductions. The results of these experiments did not support a role for epigenetic regulation of SD in response to *Pst*DC3000 infection. The DNA methylation mutants *ros1* and *npre1* both showed a reduction on *SD* upon infection with *Pst*DC3000 in a similar manner to controls, whilst wild-type plants that had undergone 8 subsequent generations of bacterial infection exhibited similar stomatal development as wild-type plants from a line that had been exposed to 8 stress-free generations. Although regulation by other types of DNA methylation (e.g DDM1-dependent DNA methylation at the pericentromeric regions) cannot be ruled out, these results suggest that systemic changes in leaf stomatal development upon localised *Pst*DC3000 inoculation are not under epigenetic control.

It is possible that Arabidopsis and other plant species have evolved this mechanism of reducing *SD* to stomatal pathogens as an adaptive mechanism to restrict infection when exposed to an environment with high disease risk. Indeed, the results presented in this Chapter 3 show that plants with fewer stomatal pores are more resistant to bacterial and fungal infection. It would be worthwhile to investigate this stomatal development response in other plant species following infection with stomatal pathogens.

4.4 Conclusions

Local infection of Arabidopsis by the bacterial leaf pathogen *Pst*DC3000 induces a systemic reduction in *SD* in the newly developing leaves. Detection of flagellin by the FLS2 receptor, SA production and SA accumulation are required for this response. The LTP Azi1, which is known to have an important role in the systemic regulation of SAR (Jung *et al.,* 2009), is also involved in the *SD* response to *Pst*DC3000 infection. Furthermore, under un-stressed conditions, the presence of the FLS2 receptor has a major effect on stomatal development. The mechanism by which FLS2 influences stomatal development remain unknown. It is possible that *Arabidopsis* and other plants reduce their *SD* following pathogen infection as an adaptive mechanism to reduce infection through the stomatal pores in environments imposing high levels of biotic stress.

The general conclusions of the is chapter are as follows;

- Syringe infiltration of mature leaves with *Pst*DC3000 results in a 10-20% reduction in stomatal density on leaves developing after infection
- Infection leads to the formation of larger guard and epidermal cells, possibly by controlling pavement cell expansion, cell elongation or cell cycle genes, rather than controlling EPF or SPCH gene expression.
- The FLS2 receptor, SA biosynthesis and SA accumulation are required to induce a systemic reduction in *SD*.
- The lipid transfer protein Azi1 may play an important, yet unknown, role in the systemic *SD* response.
- Systemic reductions in stomatal development following pathogen infection are unlikely to involve ABA, L-Pip, DIR1 and LPS.
- The FLS2 receptor has a role in regulating stomatal development in the absence and presence of infection.
- There is no evidence to suggest that changes in *SD* are regulated epigenetically.

- Reductions in *SD* are not maintained transgenerationally following pathogen stress.

4.5 Future work

Although the research conducted in this chapter has answered outstanding questions about the effect of bacterial infection on stomatal development, further experiments could be conducted to improve our understanding of this response. It seems plausible that a common MAPK/MAPKKK signalling cascade could provide a convergence point between the response to pathogen detection by the FLS2 receptor and the control of stomatal density. This could be tested using a range of *Arabidopsis* MAPK/MAPKKK signalling mutants. Although these mutants are often compromised in growth, these experiments could provide further insight into this response.

It remains to be demonstrated whether SAR is induced under the experimental conditions used to assay the systemic *SD* response. This can be tested by determining whether similarly treated Arabidopsis plants are capable of mounting a stronger, faster and more efficient defence response to a secondary pathogen inoculation or elicitor treatment.

Chapter 5 –role of the guard cell-specific *CYP86A2/ATT1* gene in plant responses to biotic and abiotic stress.

5.1 Introduction

Whilst maintaining the theme of stomatal immunity the experiments described in Chapter 5 did not investigate the interaction between stomatal density and pathogen infection. The work described here explored the hypothesis that cells of the stomatal complex, as the first barriers to entry from stomatal pathogens, could have enhanced innate immune response to bacterial pathogens. Unlike mammals, plants do not have mobile cells to defend against infection, instead plants rely on the innate immunity of each individual cell. This chapter explores the hypothesis that specialised cells in plants can produce antimicrobial compounds that are effective in resisting bacterial infection.

Due to the colonisation strategies used by pathogens, not all cells are equally likely to come into contact with pathogens. The guard cells that surround stomatal pores are particularly important as the stomata represent a weak point in the plants epidermis, and a natural opening into the host. Guard cells and the mesophyll cells surrounding the underling stomatal cavity, are thought to be primed with highly sensitised cellular machinery that detects the presence of bacterial pathogens (Beck *et al.,* 2014). Stomata are not passive entry points. Upon detection of pathogenic bacteria or fungi stomata rapidly shut, restricting or slowing the entry of the pathogen into the plant (Melloto et al., 2006). While our understanding of how stomata close to limit pathogen ingress is rapidly expanding, little is known of the role of specific antimicrobial metabolites synthesised by guard cells and adjacent mesophyll cells used to combat pathogen infection.

Recently, gene trapping experiments have shown stomatal specific expression pattern for the *ATT1* gene (Galbiati *et al.,* 2008, Francia *et al.,* 2008). This gene has been previously reported to confer resistance to the pathogenic bacteria *Pseudomonas* syringae (*Pst*) (Xiao *et al.,* 2004). *ATT1* encodes CYP86A2, a cytochrome p450 mono-oxygenase enzyme, which is a member of a large family of enzymes that are known to catalyse the degradation of fatty acids. A mutation in the gene leading to

lack of expression results in a five-fold increase in bacterial growth when plants are infected by dip inoculation. In this inoculation protocol, bacteria were applied to the leaves in solution and were left to colonise the plant naturally though the stomata. Interestingly this reduction in bacterial growth was not noted when plants are syringe-infected, an inoculation protocol that bypasses the stomata by forcing the suspended bacteria through the epidermis into leaf apoplast (Xiao *et al.,* 2004). This suggests that the increase in bacterial growth observed when *ATT1* is absent, could be a component of pre-invasive or early acting defences, rather than a defence mechanism effective against the bacterium after the pathogen has already colonised. The experiments described in this Chapter investigates the role of *ATT1* in defence against biotic and abiotic stress. The results revealed an unexpected role for *ATT1* in mediating drought tolerance, and began to investigate how *att1* mutants achieve drought tolerance.

5.1.2 Previous research into ATT1

The effect of the *att1* mutation in response to infection by *Pst* has been previously explored. Using the avrpto-luc reporter line of *Pseudomonas syringae* pv. *phaseolicola* (which is a non-host pathogen on Arabidopsis; Xiao *et al.* (2004)) showed that in the absence of *ATT1*, luciferase activity was up to 10-fold higher than in wild-type plants 12 hours after inoculation, suggesting that the *att1* mutant may be more susceptible to infection. The large induction of the *AvrPto* gene in null mutants gave the name to the *ATT1* gene (i.e. Aberrant induction of Type Three gene) as *AvrPto* is secreted through a type three secretion system by the bacteria. It is thought the *AvrPto* effector protein of *Pst* competes for the plant FLS2 receptor complex and blocks flagellin binding, thereby triggering effector-triggered susceptibility (ETS; Xiang *et al.,* 2008). Thus, *AvrPto* expression enhances the ability of *Pst* to infect Arabidopsis (Hauck *et al.,* 2003; Xiang *et al.,* 2008). This assay was done using *P. syringae pv. phaseolicola*, which is a non-host pathogen. Consequently, the increased luciferase activity does not necessarily indicate increased colonization, but indicates increased induction of the avrPto gene promoter (Hauck *et al.,* 2003).

Following dip inoculation of *att1* plants with *Pst*DC3000 Xiao *et al.* (2004) reported a small increase in bacterial growth *in planta* 2-4 days after infection; this difference in bacterial growth was however not seen when plants were inoculated by syringe infiltration. Since these experiments were conducted, the *ATT1* gene has been shown to be expressed almost exclusively in the guard cells of mature plants (Figure 1a) (Galbiati *et al.,* 2008). Taken together, these results suggest that the increase in bacterial growth observed following dip inoculation of *att1* plants (an infection method that allows bacteria to infect through the stomatal pores), may be a component of pre-invasive early-acting defences.

5.1.3 *ATT1* and its related genes

Cytochrome P450s are a superfamily of genes found in both prokaryotes, eukaryotes, viruses and archaea. Both in plants and animals cytochrome P450s are known to catalyse hydroxylation and epoxidation of medium and long chain fatty acids, a process whereby a hydroxyl or an epoxy group is added to a fatty acid molecule (Salaun & Helvig, 1995). It is thought that these enzymes have distinct substrate preferences and distinct differences in the chain position, at which they modify fatty acids. In plants, fatty acids and fatty acid modifying enzymes, are particularly important during the production of the plant cuticle, which provides a waterproof barrier on the plant aerial surfaces.

Fatty acids are also important signalling molecules in both plants and animals. For example, plants lacking the lipoxygenase LOX1 are impaired in their ability to close their stomata in response to bacterial stimuli. LOX1 is also expressed in guard cells and catalyses the deoxygenation of fatty acids. It is thought that LOX1 products are important components of the signalling pathway used to induce stomatal closure in response to pathogen attack (Montillet *et al.,* 2013).

ATT1 is a member of a large family of 5 cytochrome P450s known as the CYP86A family, a group known to play important roles in the hydroxylation of fatty acids at the ω-position. CYP86A family members share a high degree of sequence similarity (Duan & Schuler 2005) (Figure 2b). Both the ATT1 (CYP86A2) and CYP86A8 have both been implicated in the production of the cutin monomers

that are incorporated into the waxy cuticle that covers the plant epidermis. Indeed, CYP86A2 mutants have been shown to produce 30% less cutin monomers, and their cuticle has a thicker and spongier appearance under magnification (Wellesen *et al.,* 2001, Xiao *et al.,* 2004).

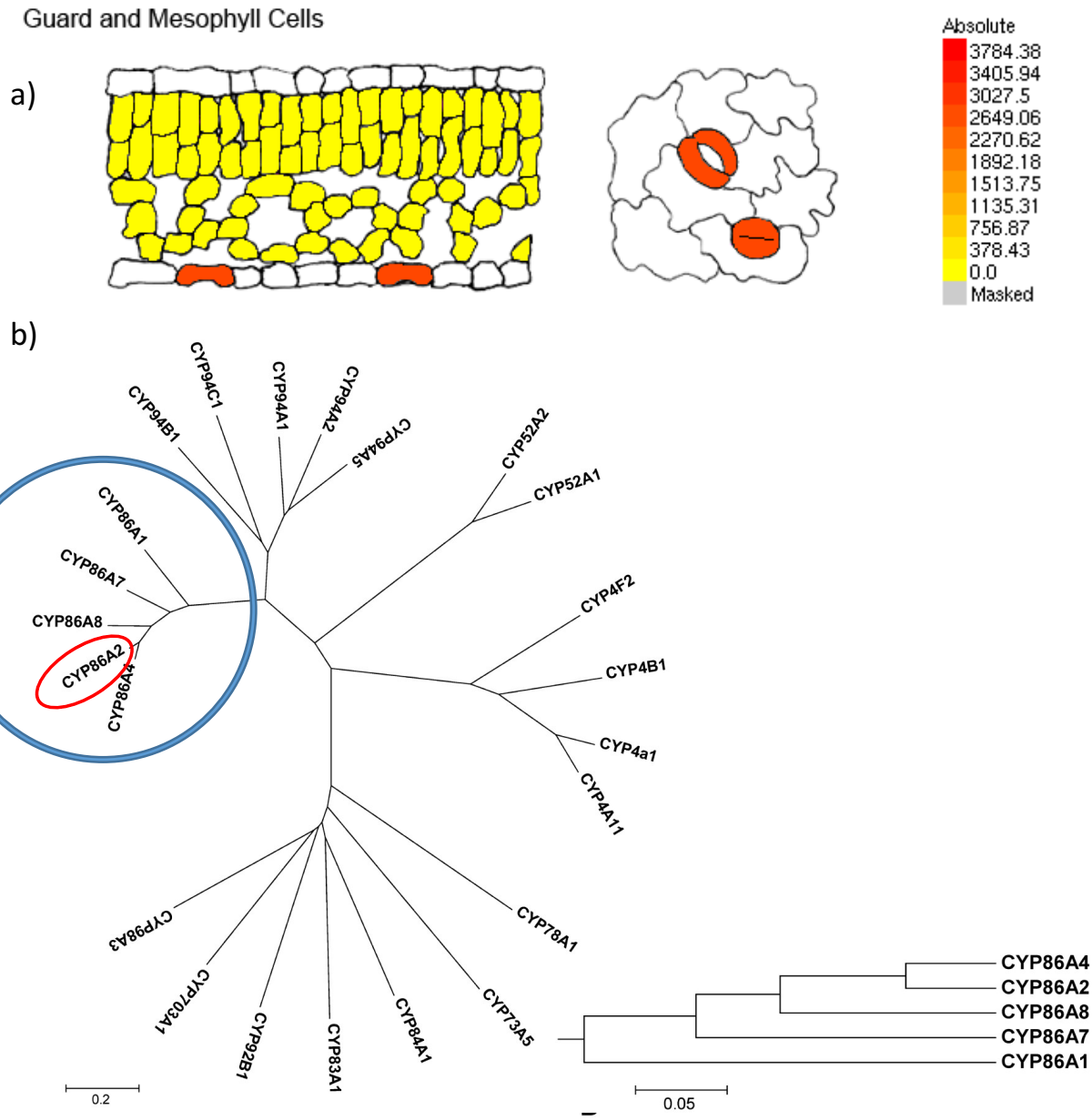


Figure 5.1. The gene expression pattern of *ATT1/CYP86A* and other closely related genes in the Arabidopsis CYP family. a) EFP browser diagram depicting known expression levels of the *CYP86A2/ATT1* gene. Diagram shows transient expressions of *ATT1*, data derived from Yang *et al.* (2008) microarray experiment with protoplasted guard cells and mesophyll cells. Expression is confined to the guard cells. b) Phylogenetic trees for fatty acid *ω*-hydroxylases (Duan & Schuler, 2005).

5.1.4 The relationship between cuticle mutants and epidermal cell patterning

Cuticle mutants have previously been reported to have several interesting phenotypes, and studies using Arabidopsis have revealed that cuticle wax mutants have altered epidermal cell patterning and exhibit differences in their ability to cope with drought conditions. Studies of *cer-g* mutants (that lack various fatty acid elongase condensing enzymes thought to regulate wax production) has revealed that mutants often have abnormal stomatal patterning. The CER mutations can result in double and sometimes triple stomatal complexes (i.e. formed adjacent to one another), both not normally seen in wild-type plants (Zeiger & Stebbins, 1972).

Studies of *att1* mutants have revealed that detached leaves lose two-fold more water than Col-0 plant under both light and dark conditions (Xiao *et al.,* 2004), this suggests that plants are losing more water through the cuticle or that their stomata are less able to close. These experiments did not however quantify water loss from the plant through the cuticle and the stomata separately.

5.1.5 Physiological responses to drought

One of the most interesting findings reported in this chapter is the discovery that despite their reported enhanced water loss, *att1* mutants are less susceptible to drought. It is thus important to discuss this topic in this introduction.

During water limiting conditions plants have a number of inducible mechanisms to resist the effects of drought. These mechanisms can be split into three categories; water conservation, water acquisition and dehydration tolerance. Each of these drought responses are discussed in turn below.

Water conservation - It is estimated that 95% of all water lost from the plant is through the stomata. As a result, closure of the stomatal pores is one of the most important factors controlling water conservation upon the onset of drought (Lawson & Blatt, 2014). Abscisic acid (ABA) is the predominant signalling hormone used to close stomata in response to drought. Synthesised in the roots, guard cells and mesophyll cells under drought conditions, ABA generates a number of

metabolic changes within the guard cells causing the loss of cell turgor pressure (as discussed in the introduction, section 1.2). Thus stomatal closure is one of the most effective mechanisms of conserving water in the plant.

As well as closing stomata under drought ABA induces physiological changes that help the plant to conserve water. ABA also down regulates leaf hydraulic conductance through decreasing the permeability of leaf vascular tissue allowing plants to maintain water within the plant for longer under drought conditions (Kudoyarova *et al.,* 2011).

Water acquisition - Water acquisition is another way to deal with drought conditions, instead of restricting water loss, a plant can increase its capacity to find water in the soil. One mechanism is to create a hydrophobic epidermis on the areas of mature root system, this makes it more difficult for water to enter the plant through the mature root and results In the majority of water entering the plant thrugh the newly developed root hairs that have proliferated in previously untapped areas of soil (Taiz & Zieger, 2010).

Dehydration tolerance – Out of the three mechanisms of drought tolerance discussed dehydration tolerance is perhaps the least well understood. Increased survival of plants due to dehydration tolerance is expressed by lower mortality rates under low plant internal water status (Blum & Tubersa, 2018). In Arabidopsis 356 genes have been shown to be differentially regulated by dehydration (Narusaka *et al.,* 2002). Many of these genes encode transcription factors which induce enzymes that detoxify the products of oxidative stress, genes that encode enzymes that degrade proteins or genes that encode proteins that alter the structure of cell walls.

5.1.6 Chapter aims

The experiments in this Chapter have addressed the role of the Arabidopsis *ATT1* gene in plant responses to pathogen inoculation and drought stress. Using *att1* null mutants, different biotrophic and necrotrophic pathogens and drought exposure, the following aspects were investigated:

- The influence of *ATT1* on leaf epidermal physiology and function.
- The role of *ATT1* in plant resistance against biotrophic and necrotrophic pathogens.
- The role of *ATT1* in drought tolerance.

5.2 Results

5.2.1 Phenotyping the *att1* mutant

The *att1* mutant has two interesting phenotypes. As reported previously, when infected with the bacterial pathogen *Pseudomonas syringae* strain DC3000, mutants lacking ATT1 appear more susceptible to bacterial infection. In the current study, it was found that *att1* mutants also exhibit a drought phenotype. Mutants lacking a functional gene are more tolerant to drought (Figure 3a). The focus of this results section is to understand how the mutation in *att1* results in both pathogen and drought tolerant phenotypes.

Two *Arabidopsis att1* mutant alleles were used in this research. These were obtained from SALK; and were T-DNA insertion mutants. Sites of insertion were checked by PCR (Figure 2). Lack of a band in Figure 2b indicated the presence of a tDNA insertion interrupting then *ATT1* gene, where the product is too large for PCR amplification. Lack of ATT1 transcript in the two mutants was confirmed by RT-PCR.

The first experiments conducted aimed to phenotype *att1* plants, cell counts from epidermal peels revealed there were no significant differences in the stomatal density of *att1* plants in comparison to wildtype plants (Figure 3c). There were also no differences in the ratio of stomatal cells to epidermal pavement cells (Stomatal index) (Figure 3c). Measurements of mean guard cell length showed that *att1* plants had a significantly smaller mean guard cell length than wildtype plants (Figure 3d). Epidermal traces of cell patterning show a good comparison of guard cell size between the two genotypes (Figure 3e). Although no direct measure was made to the eye *att1* mutants appeared to have more tessellated epidermal pavement cells than Col-0 plants.

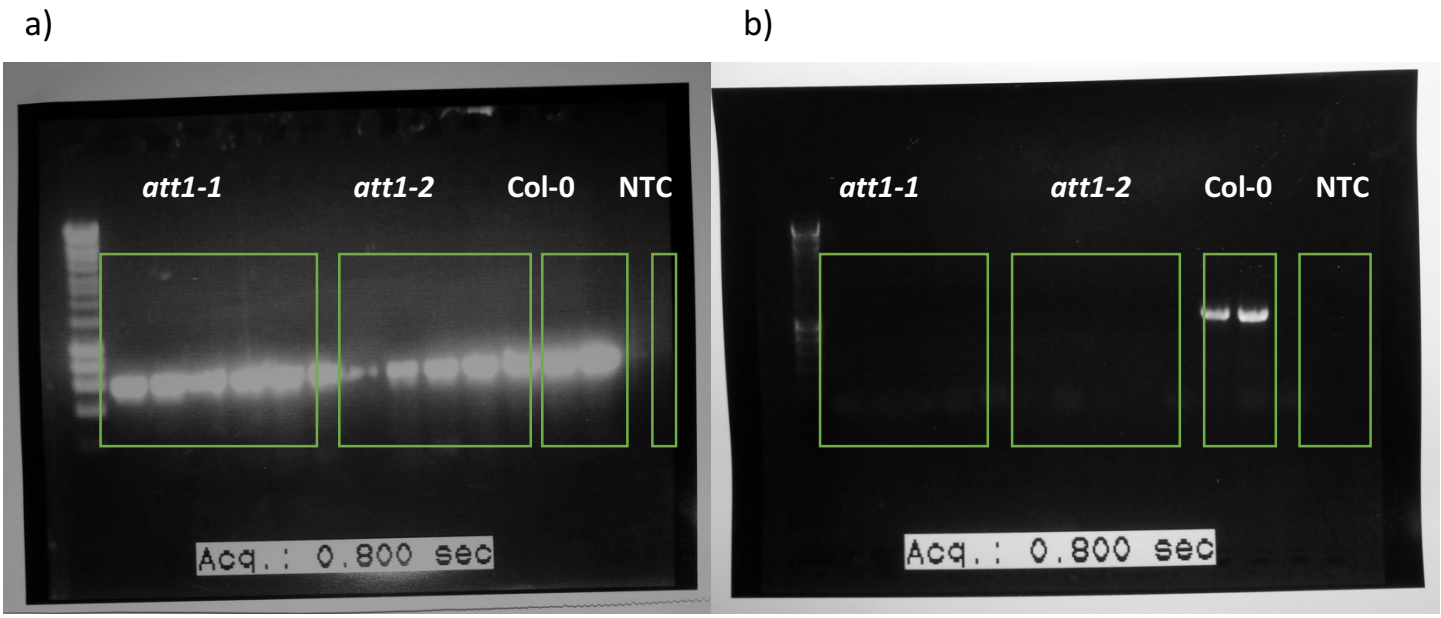
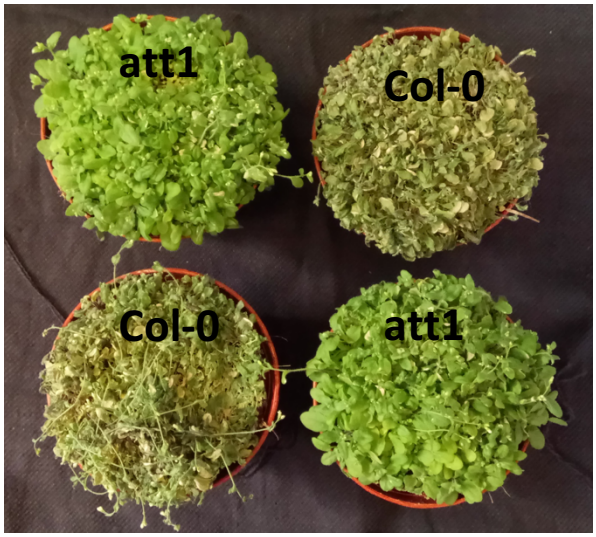
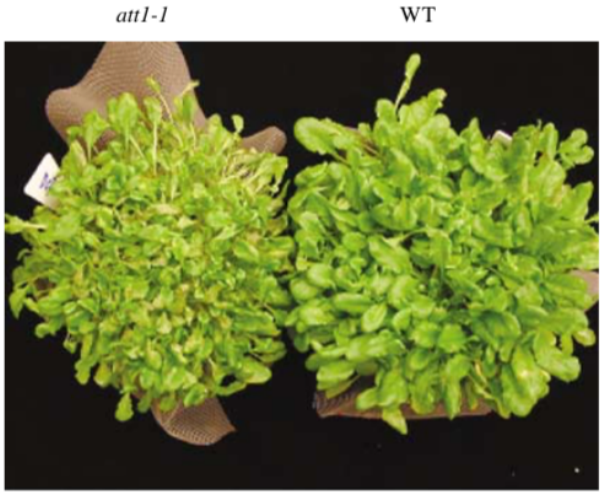


Figure 5.2. PCR verification of T-DNA insertions in *ATT1* gene SALK lines. From right to left showing 12 samples, 1-5 show DNA extracts of *att1-1* plants, 6-10 show DNA extracts of *att1-2* plants 11-12 show DNA extracts from Col-0 plants and 13 and 14 show no template controls (NTC) for each primer pair. DNA fragments amplified using *ATT1* specific primers homologous to regions upstream and downstream of the T-DNA insertion or the NOX1 control primer. a) shows PCR with NOX1 primer controls, well 2-6 *att1-1*, wells 7, 9-11 *att1-2*, well 13-14 Col-0, wells 15 NTC. b) wells 2-6 *att1-1*, wells 7-11 *att1-2*, wells 12-13 Col-0 and well 14 NTC. Absence of a band indicates the presence of the T-DNA insertion.

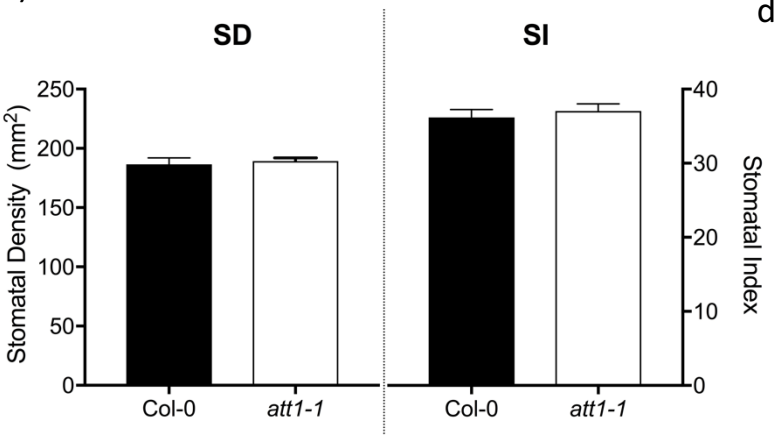
a)


att1
Col-0
Col-0
att1

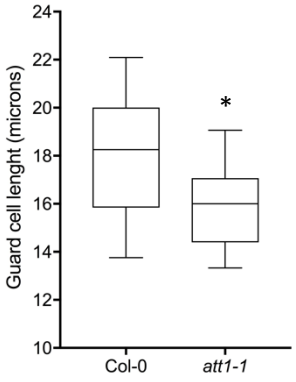
b)


att1-1
WT

c)


SD
SI
Stomatal Density (mm2)
Stomatal Index
250
200
150
100
50
0
40
30
20
10
0
Col-0
att1-1
Col-0
att1-1

d)


Guard cell lenght (microns)
24
22
20
18
16
14
12
10
*
Col-0
att1-1

e)

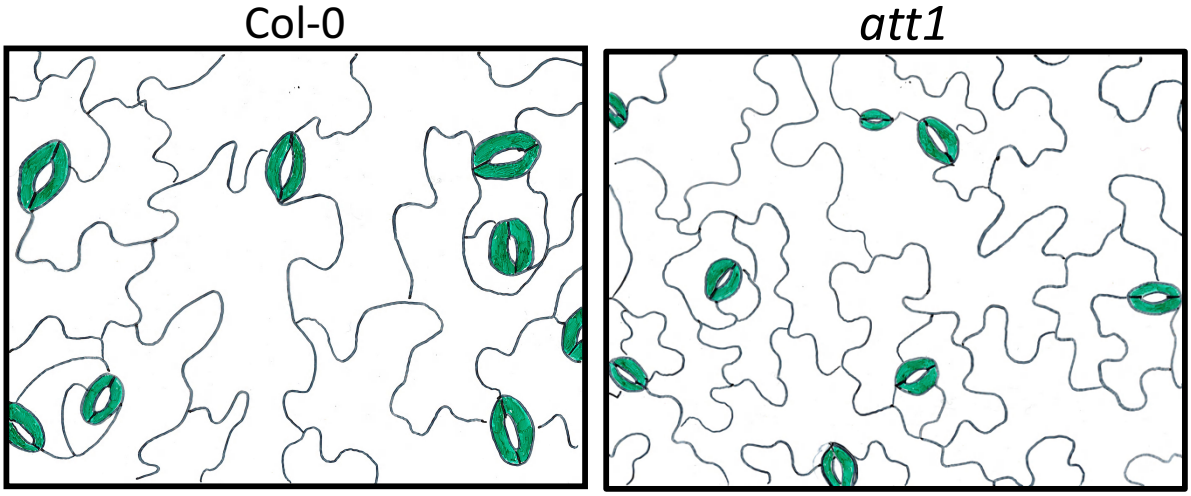

Col-0
att1

Figure 5.3. Comparison of defence, drought and epidermal patterning phenotypes between Col-0 and *att1* mutants. a) Comparison of Col-0 and *att1-1* plants following a 6 day drought treatment, images show att1-1 plants were more drought tolerant b) Image taken form Xio *et al.,* (2004) showing that *att1* plants appear to be more susceptible to infection by *Pseudomonas syringae*. c) Mean stomatal density and stomatal index of Col-0 and *att1-1* plants, d) Mean guard cell length of Col-0 and *att1-1* plants. e) epidermal traces of Col-0 and *att1-1* plants. * denotes statistically significant differences (t-test, $p<0.05$, $n=54$)

5.2.2 *att1* mutant guard cells are responsive to flg22 treatment

To assess the responsiveness of *att1* guard cells to pathogen attack Col-0 and *att1* plants were treated with 400nm of the flagellin peptide, a concentration known to induce stomatal closure (Melotto *et al.,* 2006). Epidermal peels were subsequently removed from the abaxial surface of mature flagellin treated leaves and stomatal apertures were measured under light microscopy. In both genotypes flagellin treatment resulted in a significant reduction in stomatal aperture at both 30 minutes and 90 minutes after application (Figure 4b & 4c). The percentage mean reduction in apertures of Col-0 and *att1-1* stomata were at 30 minute 15% and 22% respectively, indicating that ATT1 Is not required for stomatal closure. Under mock treated conditions *att1-1* plants exhibited significantly smaller stomatal aperture than col-0 plants (figure 4b). This is consistent with the finding that *att1-1* plants have significantly smaller mean guard cell length, and therefore smaller apertures than Col-0 plants (Figure 3d).

At 90 minutes after flagellin treatment stomatal apertures were significantly smaller than at the 30-minute time point. At this time point there was no statistical difference in mean stomatal apertures between flagellin treated *att1* plants and flagellin treated Col-0 plants (Figure 4c). Thus, although the *att1* stomata were smaller in this experiment they appear to shut to a similar extent in response to flagellin.

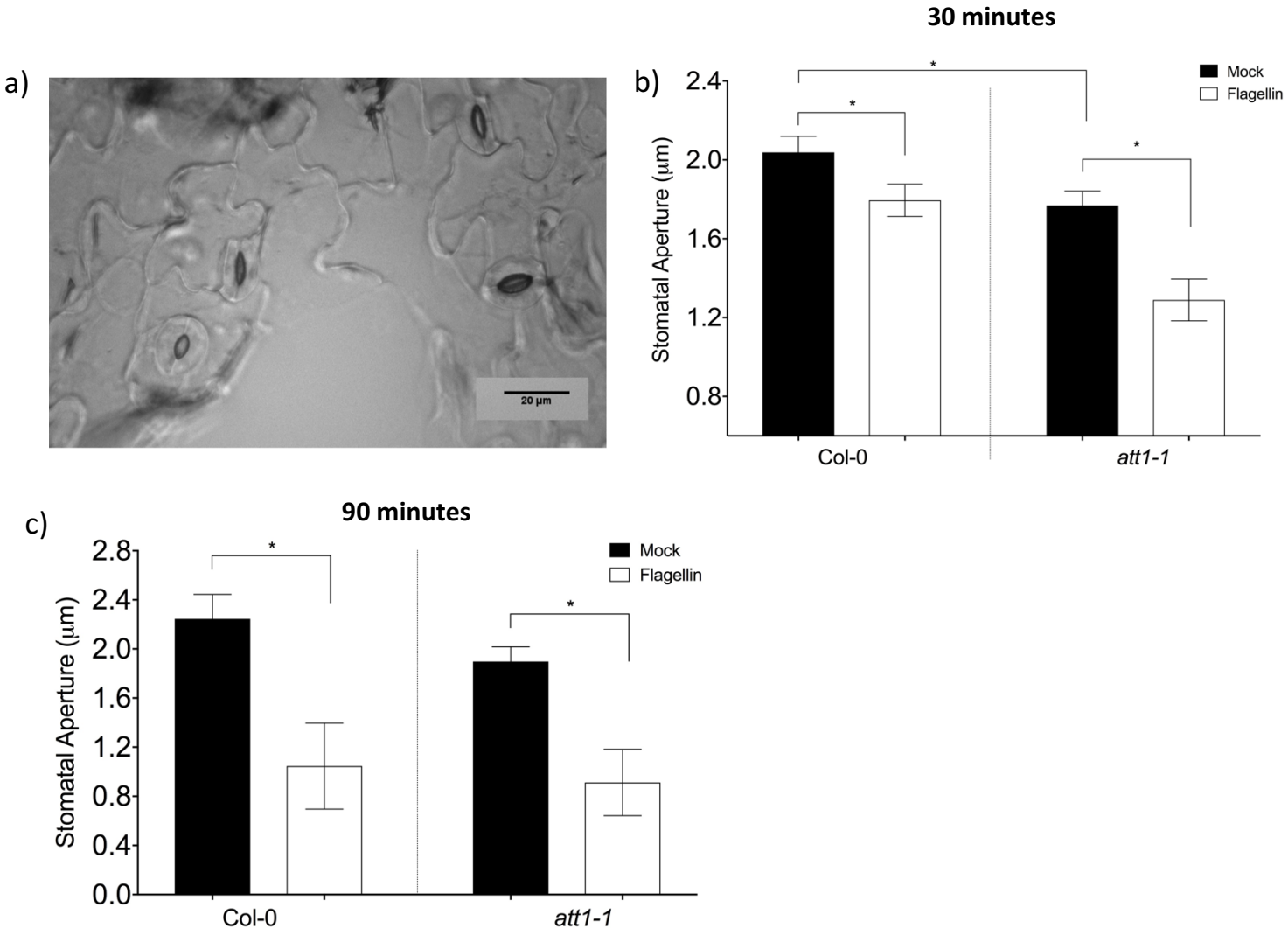


Figure 5.4. Stomatal response to treatment with 400nM flagellin. a) Example of images taken to measure stomata apertures. b) Mean stomatal aperture, comparing between mock treated plants and flagellin treated plants 30 minutes after flagellin application. c) Mean stomatal aperture, comparing between mock treated plants and flagellin treated plants 90 minutes after flagellin application. * denotes statistically significant differences (t-test, n=50 stomata, p=<0.05), error bars represent SE.

5.2.3 Assays to examine the susceptibility of *att1* mutants to pathogen infection

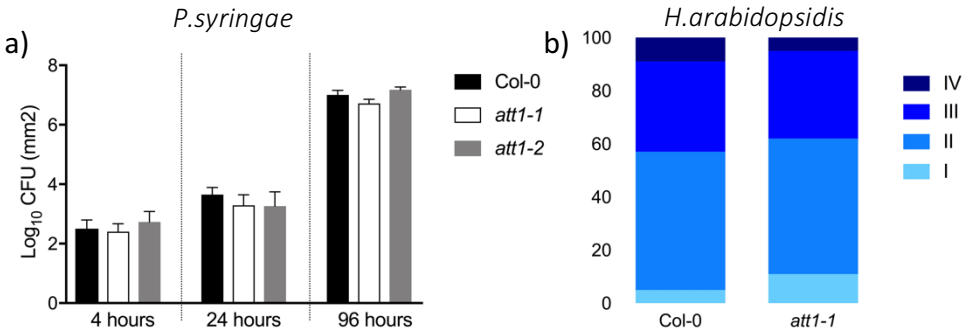
To further examine the enhanced susceptibility to *Pst*DC3000 phenotype reported in the literature, *att1* plants were challenged with a variety of biotrophic, necrotrophic and stomatal pathogens. This enabled us to further characterise the defence phenotype seen in *att1* plants. Dip inoculation of *att1-1*, *att1-2* and Col-0 with *Pst*DC3000, followed by extraction and quantification of bacteria colonising the interior of the leaf over a time series allowed the comparison of the effectiveness of both the pre-invasive and post invasive defences of Col-0 plants and *att1* plants.

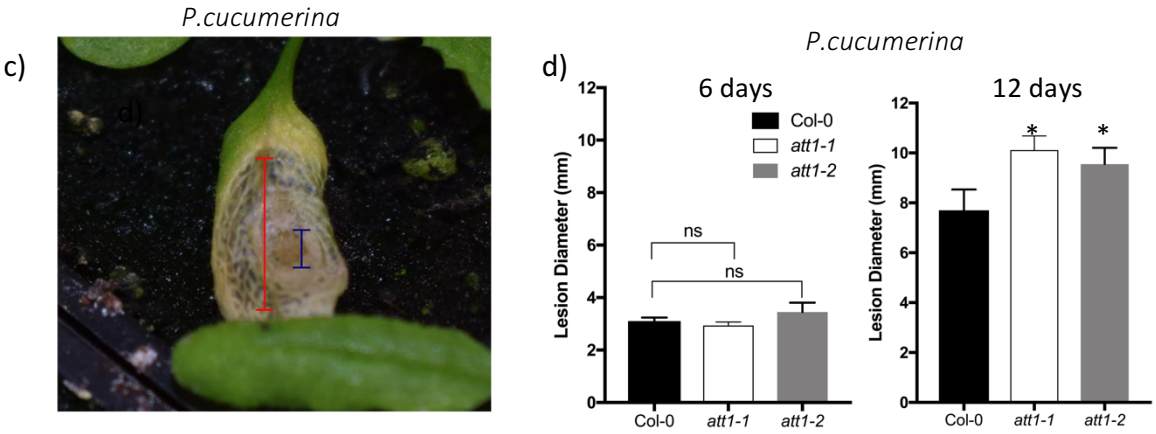
Extraction of bacteria from leaves at 4 hours post inoculation gives a good proxy for the effectiveness of pre-invasive defences. Surprisingly, no differences in the quantity of bacteria entering the leaf during the early stages of infection could be detected between the two *att1* lines and the Col-0 plants (Figure 5a). Moreover, in contrast to Xiao *et al.* (2004), there were no significant difference in the number of bacteria colonising the leaf at both 24 and 96 Hours post inoculation (Hpi). This experiment was repeated 4 times and similar results were obtained on each separate occasion. These results show that the *att1* mutants are unaffected in pre- and post-invasive defences against *Pst*DC3000. However, at 5 dpi with *Pst*DC3000, *att1* lines developed significantly more disease symptoms compared to Col-0 plants (Figure 5e), which is in line with the results reported by Xiao *et al.* (2004).

Inoculation with Hpa, which does not enter thought the stomata, did not reveal a difference in colonisation at 6 dpi between wild-type and *att1* lines after scoring of over 50 leaves from each genotype for an infection class distribution across genotypes (Figure 5b). Again, that supports the idea that *att1* mutants are not affected in post invasive resistance to (hemi)biotrophic pathogens such as *Pst* and *hpa*.

Drop inoculation of mature Col-0 and att1 plants with the necrotrophic pathogen *Plectospaeraella cucumerina* revealed significantly larger disease lesions at 12 dpi compared to wildtype plants (figure 5c & 5d). These results suggest that *att1* plants are more susceptible to necrotrophic pathogens.

Thus, the experiments reported here do not support the previously published role for ATT1 in enhancing defences to either stomatal or non-stomatal (hemi)biotrophic pathogens, but reveal a potential role in defence against (non-stomatal) necrotrophic pathogens. The results also show that *att1* mutant plants develops more severe disease symptoms to *Pst*DC3000 than Col-0 after inoculation by *Pst*DC3000, indicating that *att1* plants are impaired in pathogen tolerance rather than pathogen resistance.


a)
P.syringae
Log10 CFU (mm2)
0
2
4
6
8
4 hours
24 hours
96 hours
Col-0
att1-1
att1-2
b)
H.arabidopsidis
100
80
60
40
20
0
Col-0
att1-1
IV
III
II
I


c)
P.cucumerina
d)
d)
P.cucumerina
6 days
12 days
Col-0
att1-1
att1-2
ns
ns
*
*
Lesion Diameter (mm)
0
2
4
6
8
10
12
Col-0
att1-1
att1-2

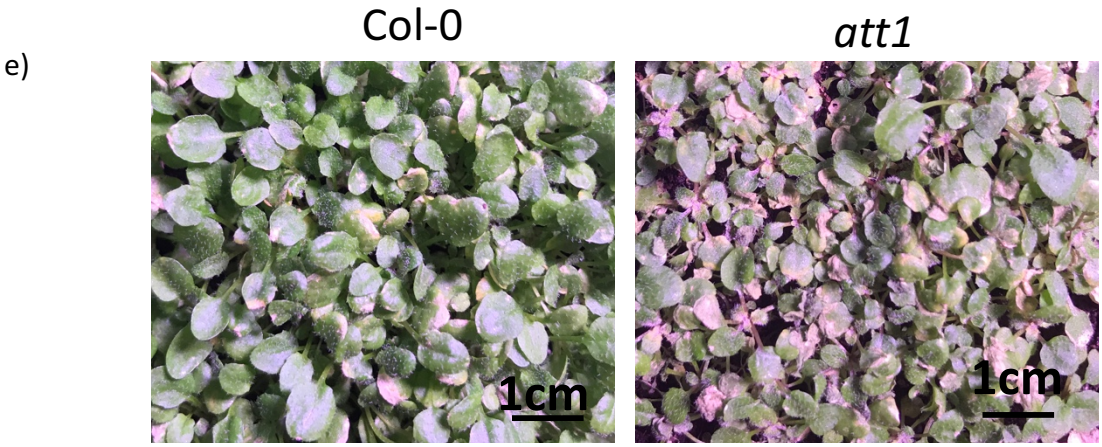

e)
Col-0
att1
1cm
1cm

Figure 5.5. Assays comparing physiological responses of *att1* and Col-0 plants to infection. a) Dip inoculation of genotypes with *Pseudomonas syringae*, graph shows log CFU of bacteria extracted from the leaf 4, 24 and 96 hours post infection. b) *Hyaloporanospora arabidopsidis* infection of Col-0 and *att1-1* plants six days after inoculation with conidiospores. Leaves were stained and assigned four classes of infection; Class 1 representing no visible infection, to Class IV, representing extensive levels of pathogen colonisation. Chi squared test revealed no significant distribution in class distribution between different plant genotypes. c) Image showing the lesions created by infection with the necrotrophic pathogen *Plectosphaerella cucumerina*, red line represents area of active pathogen infection d) Lesion diameters of *Plectosphaerella cucumerina* infection on Col-0, *att1-1* and *att1-2* plants at 6 and 12 days after drop inoculation. e) images comparing the visual infection symptoms of *Pst*DC3000 spray inoculation of Col-0 and *att1* plants 5 days after infection. * represent significant differences from 0 hour mock treated controls (t-test, $p<0.05$, n=3).

5.2.4 *ATT1* Transcription is induced by bacterial inoculation

To understand whether *ATT1* transcription was induced by bacterial infection mature Col-0 plants were dip inoculated with either a mock solution, *Pst*DC3000 or *Pst* AvrRpt2. Whole leaf tissue samples for RT-qPCR analysis were collected at 0, 4 and 24 hpi. Results from this experiment show that *ATT1* is specifically induced by both bacterial strains compared to the mock treatment at 4 hpi. At 24 hpi, there was again no induction of *ATT1* under mock treatment. Interestingly, at 24 hpi, *ATT1* transcript levels were significantly repressed in the leaves infected with *Pst*DC3000 compared to the mock control, whereas there was still a statistically significant induction of *ATT1* in leaves inoculated with the avirulent *Pst* avrRpm1 strain (Figure 6).

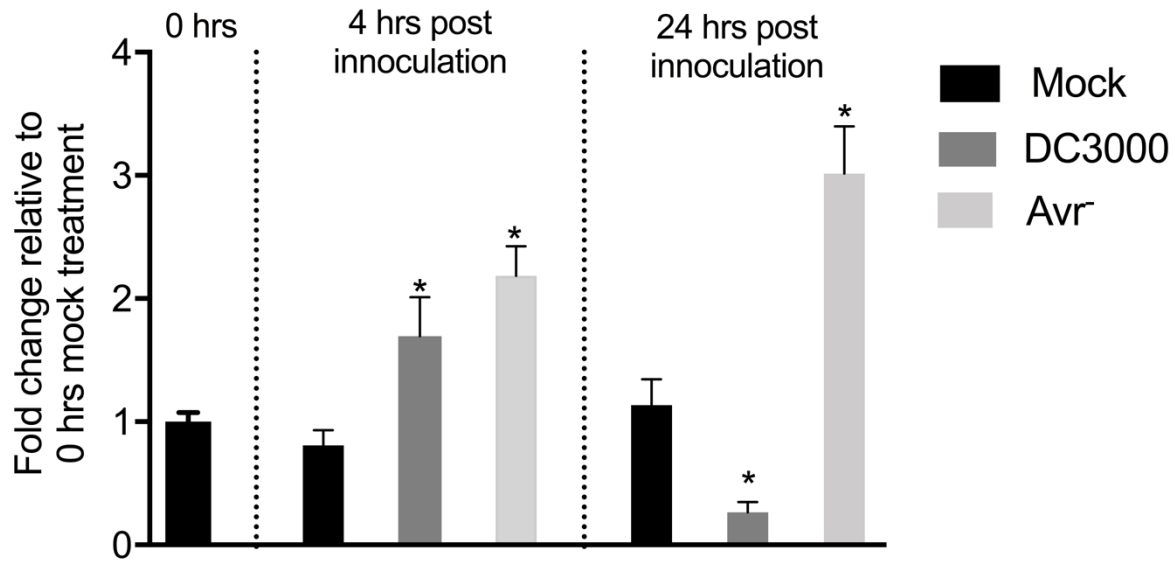


Figure 5.6. RT-qPCR analysis of *ATT1* transcript levels in mature Col-0 leaves following dip inoculation with mock solution, dip inoculation with *Pst*DC3000 and dip inoculation with *Pst* avrRpm1 at 0, 4 and 24 hpi. * represent significant differences from 0 hour mock treated controls (t-test, $p<0.05$, n=3).

5.2.5 Hormone profiling of *att1* mutant

The *att1* genotype has previously been reported to alter resistance to *Pst*DC3000 infection (Xiao *et al.,* 2004). The experiments reported in this Chapter did not revealed any difference in bacterial colonisation, but did suggest differences in the symptoms seen in *att1* plants following *Pst*DC3000 infection (i.e. plants lacking the ATT1 showed more severe disease symptoms). It was also found that *att1* formed larger infection lesions caused by the necrotrophic pathogen *P. cucumarina* than Col-0 plants. It was therefore decided to investigate whether there were any differences in signalling hormone levels within the mutant plants. Mature leaves were collected from 4 plants of each genotype and hormone abundance was quantified by UPLC qTOF MS.

The two major defence hormones salicyclic acid (SA), primarily responsible for defence from biotrophic pathogens, and jasmonic acid (JA), primarily responsible for defence against necrotrophic pathogens showed large variability and no significant differences or trends in hormone abundance could be drawn between Col-0 and *att1* lines. There was, however, a significantly higher abundance of the drought hormone ABA in both *att1* lines (Figure 7). Results show on average *att1* lines contain over twice as much ABA as Col-0 plants. ABA is known to play a role in defence against pathogens (Ton *et al.,* 2009) but it is more commonly associated with abiotic stress responses, particularly in enhancing tolerance to drought conditions.

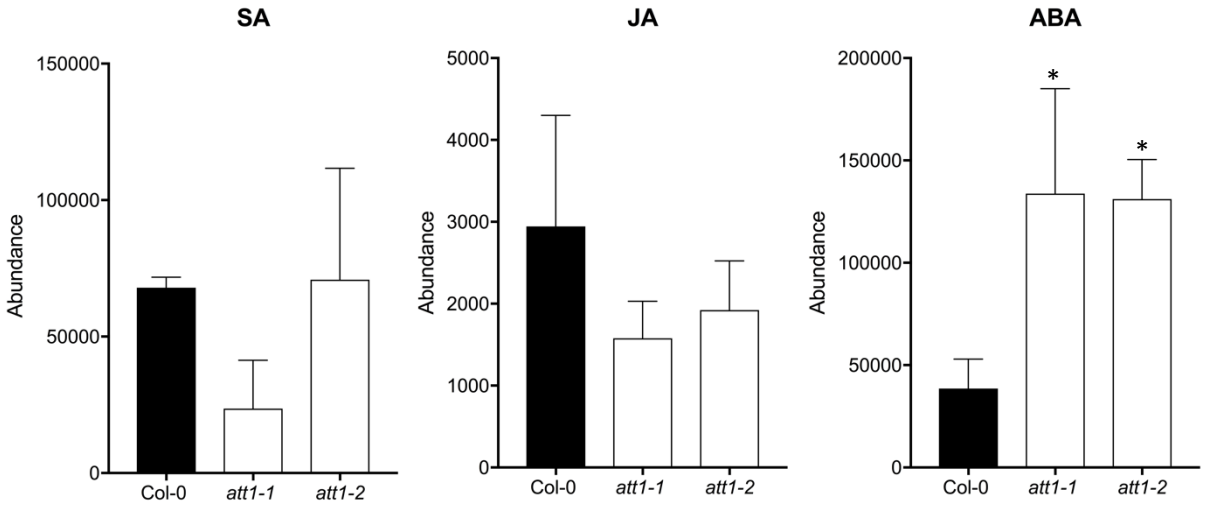


Figure 5.7. Salicylic acid, jasmonic acid and abscisic acid horomone profiling of Col-0, *att-1* and *att1-2* mutants using ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC qTOF MS). a) Salicylic acid abundances in Col-0, *att1-1* and *att1-2* plants b) Jasmonic acid abundances in Col-0, *att1-1* and *att1-2* plants c) Abscisic acid abundances in Col-0, *att1-1* and *att1-2* plants. * represent significant differences from 0 hour mock treated controls (ANOVA, $p<0.05$, n=4 plants).

5.2.6 ATT1 null mutants are drought tolerant

To examine the response of *att1* null mutants to drought conditions, water was withheld from 6 week old *Arabidopsis* plants during a terminal drought experiment. Over the course of the drought experiment four plants of each genotype (Col-0, *att1-1* and *att1-2*) were studied and a number of physiological parameters were used to monitor drought stress. To assess water loss the leaf temperature was observed using a thermal imaging camera; the Licor LI6400 was used to measure gas exchange and carbon assimilation, whilst photosynthetic performance was measured by fluorescence using a Flir Pen. Further to this leaf conductance was examined both through the cuticle and through the stomata.

At day 1 of the drought experiment thermal imaging of the leaves showed no obvious difference in surface leaf temperature between Col-0 plants and the *att1* null mutant lines suggesting that plants were losing water at similar rates. IRGA measurements also detected no difference in assimilation rate between the three lines tested indicating that there was not a difference in photosynthetic rate. However, under well-watered conditions both *att1-1* and *att1-2* lines had significantly higher levels of transpiration through stomatal pores when measured by IRGA (Figure 8a). The photosynthetic efficiency of photosystem II was also not significantly different between genotypes (Figure 8d).

Following 4 days without watering the leaf surface temperature and assimilation rate were comparable between the genotypes (Figure 8b). When comparing IR thermal images between day 1 and day 4 it was apparent that average leaf temperatures are slightly higher than those seen on day 1, indicating all plants were transpiring less. In contrast to day 1 there were no statistical differences between the stomatal conductances of the genotypes as assessed by IRGA. Col-0 plants did not alter stomatal conductance between day 1 and day 4 of drought. *att1* lines however showed a significantly reduced stomatal conductance rate on day 4 when compared day 1. On day 4 there were also no significant differences in photosynthetic rate between Col-0 and the *att1* lines at day 4 (Figure 8d).

After 6 days without watering all plants were showing drought symptoms. Col-0 plants in particular had wilted to a much greater extent than *att1* plants. Surface leaf temperature of both genotypes were hotter after 6 days than at either days 1 or 4, indicating substantially less transpiration from all the plants in the experiments.

In summary Col-0 plants substantially reduced assimilation and stomatal conductance levels between days 4 and 6, as expected for plants under drought stress. However, at this time point *att1-1* and *att1-2* plants were significantly less affected by drought than the Col-0 plants. Comparisons between assimilation rates and stomatal conductance between Col-0 and *att-1* plants showed that *att1-1* were able to maintain significantly higher assimilation rates and stomatal conductance after 6 days of drought. Whilst not statistically significant *att1-2* plants also showed increased assimilation and stomatal conductance values compared to Col-0. It was also apparent that *att1* plants were cooler than Col-0 plants (Figure 8c) suggesting that *att1* was transpiring more than Col-0 plants.

Fv/Fm values were measured across the drought period to assess when the drought began to affect the efficiency of Photo system II (PSII) (an early indicator of stress). Col-0 Fv/Fm levels dropped below *att1-1* and *att1-2* levels on days 4, 5 and 6 of drought. *att1* lines maintained higher Fv/Fm values during the drought period indicating higher photosynthetic efficiency (Figure 8d), indicative of plants under lower levels of stress.

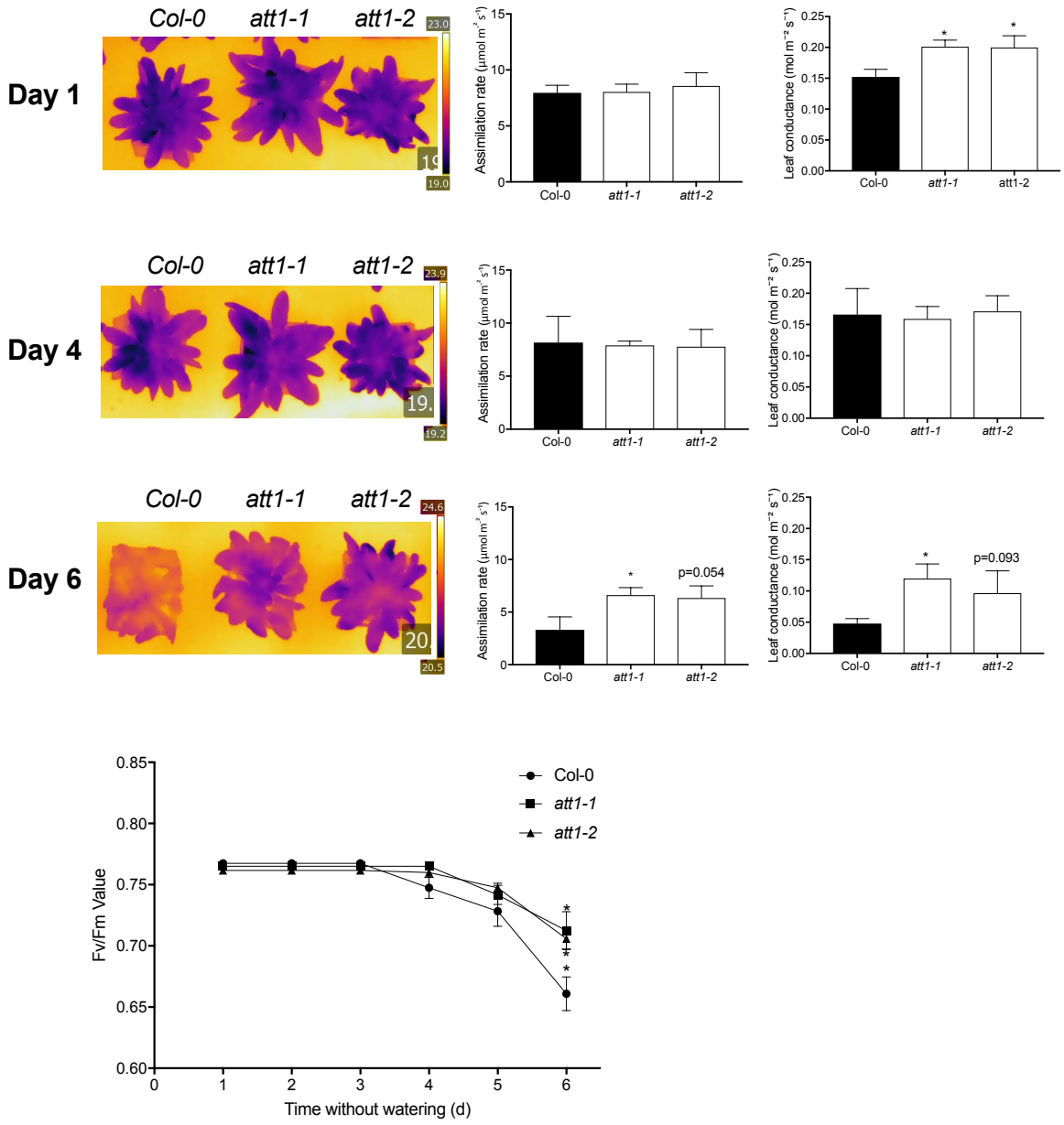
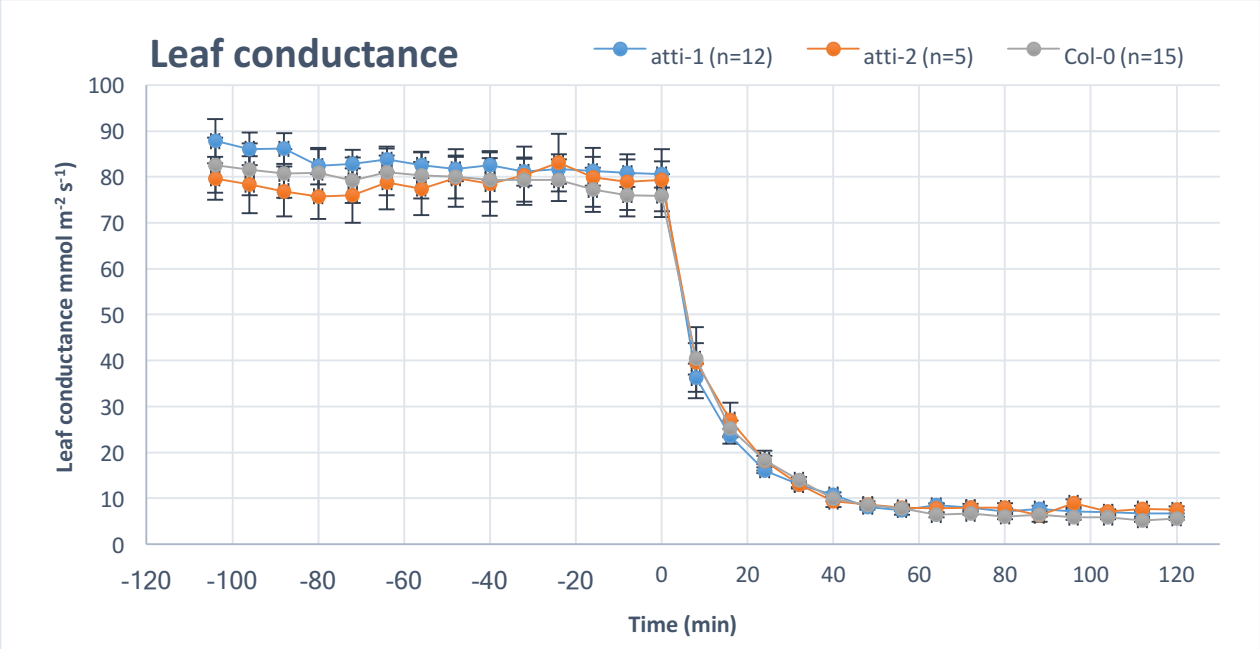


Figure 5.8. Plants lacking the ATT1 are more tolerant to drought conditions. 6 day drought experiments comparing Col-0, *att1-1* and *att1-2* plants. a) Day 1 of drought treatment, representative thermal images of Col-0, *att1-1* and *att1-2* plants (left). Assimilation rate of lines (middle) and stomatal conductance values (right). b) Day 4 of drought treatment. c) Day 6 of drought treatment. d) Daily reading of photosystem II efficiency (Fv/Fm), readings taken using a Flir pen in light adapted conditions. * denotes statistically significant differences (t-test, n=4, p=<0.05), error bars represent SE.

5.2.7 att1 mutants do not loose significantly more water through the cuticle

To determine whether the significantly higher leaf water conductance in *att1* genotypes as measured by IRGA was due to increased water conductance through the cuticle or through the guard cells, cuticle conductance was compared between Col-0 and *att1* mutants. Experiments were conducted in a sealed chamber measuring whole leaf conductance. At time point 0 leaves were cut from the plant, this should induce full stomatal closure across the leaf. Any remaining water loss is therefore assumed to be via the cuticle. Between time 0 and time 120 minutes after leaves were cut from the plants leaf conductance was measured by IRGA. During this time no significant differences in leaf conductance levels between Col-0 and *att1* mutant lines could be detected indicating that *att1* plants do not have significantly enhanced cuticle conductance to water (Figure 9a and 9b).



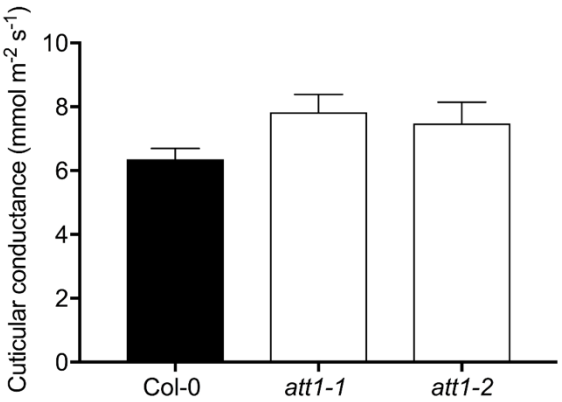


Figure 5.9. Leaf conductance of Col-0 plants, att1-1 and att1-2 mutants through the cuticle. a) Leaf conductance values throughout the course of the experiment. Time 0 represents cutting of the leaf causing stomatal closure. b) average circular conductance following stabilisation of conductance values after stomatal closure. T-test revealed no significant differences between Col-0 and *att1* lines. Dr Hanna Horak assisted with the design and analysis of experiments presented in this Figure.

5.3 Discussion

In this results section, plants lacking expression of the *ATT1* gene were shown to have different responses to both biotrophic and necrotrophic pathogens. It was also shown that *ATT1* plants are more resistant to drought conditions. Despite being previously reported to have resistance to *Pst*DC3000 infection, here it is shown that the stomatal closure response and bacterial titre inside the plant is not altered between *att1* mutants and Col-0 plants. However, *att1* plants are less tolerant to infection, displaying a greater extent of disease symptoms upon infection with *Pst*DC3000 and other pathogens.

5.3.1 ATT1's role in during pathogen infection

It was expected before undertaking this research that CYP86A2 (encoded by the *ATT1* gene) played an important role in modifying long chain fatty acids important for the stomatal closure seen in *Arabidopsis* in response to pathogen infection. Although long chain fatty acids are important components of signalling pathways responsible for closing stomata in response to bacterial infection (Montillet *et al.,* 2013) our results do not suggest that CYP86A2 is responsible for any components of stomatal closure in response to pathogen infection. Experiments applying the flg22 peptide (known to induce stomatal closure) showed that stomatal closure was not compromised in two *att1* mutant alleles. Indeed, our results suggested that *att1* guard cells are able to shut the pore at least as effectively as controls, a result that is discussed further below and may be explained by the smaller size of the guard cells rather that the lack of ATT1 (Raven, 2014).

The results presented in this chapter, in some cases, contradict research previously published on the *ATT1* gene function. For example, spray inoculation failed to show any difference in bacterial numbers inside the leaf at the early time points of infection. This result contradicts the findings of Xiao *et al.* (2004), who reported a small but significant increase in the number of bacterial entering Col-0 plants at early infection time points. At later time points (4 days post infection) the current study failed to

detect a significant difference in bacterial numbers within infected leaves. These results are again in disagreement with Xiao *et al.* (2004), who report a small but significant increase in the number of bacteria within *att1* plants. It was noted, however, that *att1* plants developed significantly more disease symptoms at 5 dpi compared to Col-0 plants, a finding that is more in line with the results of Xiao *et al.* (2004). Assays with *Hpa*, another biotrophic pathogen that does not colonise through stomatal pores, revealed wild-type levels of basal resistance in *att1* plants. Hence, in our experimental system, *att1* mutants are unaffected in pre- and post-invasive defences against (hemi)biotrophic pathogens.

Our results suggest that rather than altering resistance to *Pst* infection the *att1* mutations affect tolerance to the pathogen. Instead of affecting the ability of plants to successfully defend against *Pst* entry and replication, plants lacking ATT1 have a lower tolerance to the effects of the pathogen infection, and thus, show more disease symptoms than wild-type plants. Indeed, disease symptoms do not always correlate with host resistance levels. Experiments using ethylene mutants have shown that the disease symptom development does not always correlate with the number of bacteria within the plant. Bent *et al.* (1992) demonstrated an important role for ethylene in mediating the disease symptomology of *Pst*DC3000 infection. The ethylene-insensitive mutant *ein2* showed significantly less disease symptoms that wild-type plants, even though the bacteria proliferated to similar levels as in leaves of wild-type plants. Hence, mutations in *ATT1* and *EIN2* do not affect basal resistance to *Pst*DC3000, but have opposite effects on the tolerance of the plant to *Pst*DC3000 infection. Whether the disease phenotype of *att1* mutants is related to ethylene signalling requires further investigation.

When trying to understand the role of CYP86A2 it is important to consider the location of *ATT1* gene expression. *ATT1* is the only gene exclusively expressed in the guard cells of mature *Arabidopsis* reported to have a significant effect on plant immunity. During infection by bacterial pathogens it was found that *ATT1* mRNA transcripts are induced, indicating potential role during pathogen infection.

As previously reported FLS2 receptors expression is higher around the guard cells and the sub-stomatal cavity. An enzyme specifically expressed at the guard cell which restricts the expression of the AvrPto effector would ensure that the FLS2 receptor continues to detect and induce defences against the pathogen. More pertinent perhaps is the transcript pattern/levels of CYP86A2 over the early stages of pathogen infection. Analysis of *att1* mRNA transcripts by northern blot analysis performed by Xiao *et al*., tried to address this question. Northern blot analysis indicated that *ATT1* gene expression was induced non-specifically by both infiltration of buffer mock treatments, infiltration of *Pst*DC3000 and non-host strains of the bacteria *P.s.phaseolicola*. However what was clear from the results is that induction of the *ATT1* gene persisted in the mock treatments. By contrast *ATT1* mRNA transcript levels declined following *Pst*DC3000 treatment.

Here through the use of qPCR, an alternative method of estimating gene expression levels than northern blotting, rather than a decline, induction of the *ATT1* gene was observed 4 hours post infection with *Pst* strains DC3000 and avirulent strain Avr^{-}. At 24 hours post infection however it was found that *Pst*DC3000 suppressed *ATT1* expression upon infection, but the avirulent strain does not have the ability to suppress *ATT1* expression following infection.

Assays with the necrotrophic pathogen (*P.cucumarina*) that does not enter through the stomata revealed that *att1* plants are more susceptible to infection from this pathogen. The altered resistance to biotrophic and necrotrophic pathogens may indicate an alteration in the level or response to defence hormones such as ethylene known to trigger defence against biotrophic and necrotrophic pathogens. Another possible explanation for *att1* plants being more susceptible to the necrotrophic pathogen is the fact that *att1* mutant's cuticle lacks 30% of the cutin monomers found in Col-0 plants (Xiao *et al.,* 2004). Indeed, the cuticle has been reported to be an important resistance factor against necrotrophic pathogens (Laluk & Mengiste 2010). However the cuticle, although lacking cutin monomers is reported to be thicker (but perhaps less dense) than the cuticle of Col-0 plants.

Mass spectrometry of uninfected leaf tissue was used to measure the abundance of biotic and abiotic response hormones within uninfected leaves of *att1* and Col-0 plants, and revealed the effect of the ATT1 mutation on basal hormone levels. *att1* leaves do not significantly differ in the abundances of the two-main defence signalling hormones SA and JA. ABA, was however, significantly more abundant with *att1* null mutants containing approximately 2-3 fold higher levels of ABA than Col-0 plants. This could provide at least in part an explanation for the plant response to disease.

The interaction between ABA and other defence hormones is complex. As discussed during the introduction ABA has several reported roles depending on the stage of colonisation of the pathogen (Ton *et al.,* 2009). In the literature, it is widely accepted that SA and ABA work antagonistically. Thus if ATT1 has higher quantities of ABA, SA levels or responses might be supressed. However, SA levels appeared variable and differences were not apparent from the mass spectrometry analysis.

One limitation of the mass spectrometry analysis prevented in this Chapter is that it only gives an understanding of hormone abundance within uninfected plants. In future, it would be useful to quantify defence hormone abundances following infection with both biotrophic and necrotrophic pathogens to determine if plant inducible defences are affected by the *ATT1* gene.

5.3.2 ATT1's role during drought

An entirely novel phenotype of drought tolerance is reported in this Chapter for the *att1* mutant plants.

ATT1/CYP86A2 activity is associated with the production of the waterproof cuticle and might therefore be expected to affect water loss. Mutants develop with a cuticle that contains 30% less cutin monomers, however the cuticle is thicker and spongier than Col-0 plants. Xiao *et al.* (2004) who report that excised *att1* leaves wilt quicker than Col-0 leaves due to the reduction in cutin monomers in the cuticle. It is therefore surprising that our experiments showed that *att1* lines are more tolerant to drought than Col-0 plants. Terminal drought experiments showed that *att1* plants maintained normal photosynthetic rates for 1 day longer than Col-0 plants and were more tolerant to water limiting conditions than Col-0 plants, a phenotype that would not be associated with plants that had significantly higher cuticle permeability, as reported by Xiao *et al.* (2004).

Gas exchange analysis showed that plants lacking ATT1 are able to sustain higher levels of gas exchange throughout the drought treatment always having higher or equivalent level of transpiration compared to Col-0 plants. Experiments measuring water loss through the cuticle also showed that, although there was a trend in *att1* plants to having slightly increased cuticular conductance, there was no significant increase in water loss through the cuticle in these mutants. Taken together these results indicate that *att1* plants are more resistant to drought for reasons other than restricting water loss through the stomata, and the altered structure of the cuticle known in *att1* plants is not significantly affecting water loss through the plants epidermis. Inline with these findings are those of Isaacsons *et al.* (2009) who found no association between the cutin content of tomato fruit (*Solanum lycopersicum*) cuticle and transpiration rates.

As experiments revealed no reduction in water loss through the cuticle it seems appropriate to hypothesise that *att1* mutants are losing more water under well-watered conditions for physiological reasons other than cuticle permeability. One hypothesis would be that *att1* mutants are more

drought tolerant due to the induction of stress defence mechanisms that cope with water deficit to a greater degree than in Col-0 plants. Given the finding that *att1* plants contain significantly higher levels of the drought stress hormone ABA it is possible that higher levels of ABA in ATT1 plants allow the induction of water conservation and water acquisition mechanisms earlier than in Col-0 plants. Giving support to this hypothesis is the finding that *att1* mutants reduce guard cell conductance significantly between day 1 of drought and day 4, whereas Col-0 plants do not redude stomatal conductance until between days 4-6. This suggests that *att1* plants may sense water limiting conditions before Col-0 plants.

There are a multitude of physiological responses induced by the drought hormone ABA that could potentially give rise to a more drought resistant phenotype. For example, ABA is known to induce root branching and root growth (Harris, 2015). This allows the plant to explore previously untapped areas of soil to search for areas of water. To test this hypothesis there are several physiological parameters that could be measured. For example comparing root:shoot ratio between different genotypes plants would give a good indication of whether resource allocation to root growth and water acquisition differs between the two genotypes. However, no obvious differences in plant size were noted during the course experiments reported here.

Another mechanism to increase the uptake of water is to create a hydrophobic epidermis on the areas of mature root system, this makes it more difficult for water to enter the plant through the mature root and results In the majority of water entering the plant through the newly developed root hairs that have proliferated in previously untapped areas of soil (Taiz & Zieger, 2010). In the aerial parts of the plant, ABA is also known to have a significant role in inducing water conservation measures. ABA can regulate leaf hydraulic conductance through decreasing the permeability of leaf vascular tissue allowing plants to maintain water within the plant for longer under drought conditions (Kudoyarova *et al.,* 2011). Other possible reasons for enhanced drought tolerance might be that before undergoing drought stress *att1* plants contain proportionally more water than Col-0 plants. A

simple way of testing this would be to measure leaf water content of mutants and Col-0 plants and ATT1 plants both before and during drought treatment.

5.4 Conclusions

Plants lacking *ATT1* produce more of the abiotic stress hormone ABA than Col-0 plants. This has implications not only for drought tolerance, but also for plant responses to pathogens. Higher levels of ABA in *att1* mutant plants may allow for early induction of water conservation and water acquisition mechanisms than Col-0 plants.

Work from this chapter has explored the physiological and biochemical effect of ATT1 on pathogen responses and plant responses to water limitation. The conclusions from the work are as follows.

- Null mutants in *ATT1* have significantly smaller guard cells than wild type plants but have the same stomatal density and stomatal index.
- ATT1 does not affect the extent of stomatal closure in response to flagellin and does not affect plant resistance to biotrophic pathogens. However, att1 mutants are more susceptible to necrotrophic pathogens.
- ATT1 plants have significantly higher ABA levels than Col-0 plants but are not affected in basal levels of SA or JA.
- ATT1 mutants show tolerance to water limiting conditions. This drought tolerance is not achieved by reduced water loss through the stomata or cuticle.
- High ABA levels may allow the plant to sense and respond to water limiting conditions faster, inducing physiological mechanisms to conserve, or acquire water before Col-0 plants can respond.

5.6 Future work

To truly understand the role of the *ATT1* gene, and its product (CYP86A2) there are a number of important experiments that should be undertaken.

Firstly, both the substrate and the product of the CYP86A2 enzyme remain unknown. To understand the role of the gene it is important to explore which fatty acid(s) the encoded enzyme catalyses the production of. It may be possible to study thus by heterologously expressing *ATT1* gene in *E.coli* and overexpress a tagged version of the enzyme which can be purified. Then possible substrates could be tested

Although hormone levels of uninfected *att1* plants and Col-0 plants were studied the effect of the *att1* mutation on inducible defences following pathogen infection were not. As well as hormone profiling *att1* mutants that are not infected It would be interesting to again examining hormone abundances by mass spectrometry following infection by either biotrophic or necrotrophic pathogens as well as examining genes known to be respond to SA or JA defences. Results show that *att1* expression is inducible upon pathogen infection, however it is not known if induction of the *ATT1* gene affects transcription of other defence genes.

To follow up on the drought experiments it would be interesting to measure a number of physiological parameters of *att1* mutants to try gain a better understanding of why these plants are more tolerant to drought conditions. For example measuring root:shoot ratio of plants would give a better understanding of resource allocation between growth and water acquisition. Further to this it would be interesting to examine root architecture, root branching, root hair production, and hydraulic conductance of *att1* plants during drought stress.

Chapter 6: Conclusions and future work in the field

Since the discovery of stomatal immunity in 2006 our understanding of the interactions between plants and pathogens occurring around the guard cells has improved greatly. Through a number of independent studies, we now have a relatively good understanding of the mechanisms used to close stomata in response to bacterial infection (discussed in section 1.3). There are however a number of topics that still require further research to better our understanding of the field. It is known that application of a number of PAMPs such as chitin, LPS and flagellin all trigger stomatal closure when detected by the plant. Current studies have investigated the mechanisms by which stomata close in response to flagellin or bacterial pathogens. It remains to be discovered whether stomata close using the same mechanisms in response to flagellin as they do to other PAMPs. Indeed it is not yet clear how similar the components are mediating stomatal closure in response to biotic or abiotic stimuli.

Although our understanding of how guard cells close stomata in response to pathogens has developed greatly we still do not fully understand the mechanisms used by pathogens to detect and locate the presence of guard cells. It is known that bacteria detect and respond to chemical stimuli emitted by lettuce leaves grown in light conditions, but not under dark conditions to locate the presence of stomata (Kroupitski *et al.,* 2009). However, we still do not know which chemical or metabolic signatures bacteria are using to locate guard cells. Furthermore there is little conclusive evidence as to how fungal pathogens find and infect through stomatal pores in many species of plants.

Although there is evidence to suggest that fungal rust pathogens in grasses recognise topographical changes in the cellular architecture of the epidermis to detect the location of guard cells, this has only been researched in grass species. In grass species, these topographical changes may be easier to detect than in dicot species due to grasses having epidermal cells arranged in files on the leaf surface, in non-grass species that do not have uniform cellular layouts it seems possible that other species of

fungal pathogens that infect different host species may detect the presence of guard cells in other ways.

If we were to fully understand the chemical and physiological cues detected by pathogens to direct them to stomatal openings it would open new possibilities in manipulating plant biology to create novel and durable resistance to pathogen attack. For example, the genes controlling epidermal cell patterning, cell differentiation and cuticle development around the guard cells are reasonably well characterised. If we understood the exact topographical changes detected by individulal fungal pathogens we could potentially manipulate plant epidermal patterning to prevent pathogens from recognising and locating the presence of stomata. Not knowing exactly how stomatal pathogens colonise reveals our lack of basic understanding of pathogen physiology, and highlights the need for basic physiological studies that appear to have fallen out of favour in recent research.

Whilst the short-term response (stomatal closure) to pathogen infection through stomatal closure has been well studied, the long-term response identified here (alterations in stomatal density) remains to be fully investigated. There are a few studies that previously highlighted interesting links between stomatal development, pathogen infection and components of pathogen signalling (Lake *et al.,* 2009, Meng *et al.,* 2015, Murray *et al.,* 2016). The results presented here significantly advance our knowledge of the effect of bacterial infection on stomatal development, and reveal components of the signalling pathway used to modulate stomatal development in response to bacterial infection. While this study shows an adaptive response to bacterial pathogen infection in *Arabidopsis*, and shows shared components of pathogen signalling and stomatal formation there are many unanswered questions.

For example it is important to understand if this stomatal development response is conserved in other dicot and monocot plant species following pathogen infection. Further studies on other species of plants are need to answer such questions.

The systemic signals responsible for inducing a reduction in stomatal density following pathogen infection also remain to be discovered. It will be difficult to distinguish the systemic signals responsible for reducing stomatal density from those used to prime distal tissue against further pathogen infection. Indeed results presented here suggest that it is possible that the signal for reducing stomatal density is shared with the systemic signals used to biochemically prime plants against infection.

In addition to stomatal immunity, is the almost completely unsearched area of immunity at hydathode pores (sometimes called water stomata or water pores). Structurally similar to stomatal pores hydathode pores connect the xylem vasculature with atmosphere and can be used to expel excess water during conditions of limited transpiration (warm soils, humid conditions and during the dark). Like stomatal pores hydathodes are exploited by several species bacterial pathogens, these entry points allow pathogens access to the xylem and act as a portal for systemic infection of the plant. It is known that exudates from hydathodes are enriched in pathogen response proteins (Grunwald *et al.,* 2003) indicating an important role for defence at the hydathode pore.

Finally cell specific defences of guard cells of stomatal and possibly hydathode pores could provide an interesting avenue for further research. Unpublished microarray data enriched in the guard cell fraction reveals significant enrichment in expression of pathogen response genes in guard cells (Hunt & Gray unpublished). It is known that guard cells have the ability to synthesise a number of complex molecules and hormones. The *ATT1* guard cell expressed gene has been shown to have significant effect on pathogen response in this study. It therefore seems likely that guard cells or sub-stomatal cavities have the potential to synthesise antimicrobial molecules that could be an important component of defence upon primarily contact with pathogens.

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