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Phytohormone Crosstalk via Legume Lectin like-Proteins Regulates Systemic Acquired Resistance and Abiotic Stress Tolerance

Jennifer Horatia Sales

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Vorsitzende: Prof. Dr. Corinna Dawid

Prüfender der Dissertation:

1. Prof. Dr. Jörg Durner
2. Prof. Dr. Brigitte Poppenberger-Sieberer

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Contents

Summary

In order to maximise resource management in the face of abiotic stresses and emerging diseases, we must consider the interplay between different stress responses, and thus different phytohormone signalling pathways. The phytohormones salicylic acid (SA) and jasmonic acid (JA) promote two, mutually antagonistic immune pathways respectively protecting plants from biotrophic pathogens and necrotrophic pathogens or insects. This trade-off largely precludes the exploitation of SA and JA immune components for crop protection, raising the interest in immune signalling components that disrupt SA-JA antagonism. A local pathogen infection primes SA-dependent immunity in systemic tissues. This so-called systemic acquired resistance (SAR) ensures a long-lasting, broad-spectrum disease resistance that is not subject to SA-JA antagonism.

Legume Lectin-like Proteins (LLP1) 1, 2 and 3 are membrane-localised proteins with a predicted carbohydrate-binding domain found in *Arabidopsis thaliana*. Here, the knockout mutants *llp1-1* and *llp3*, and the triple silencing line *RNAi:LLP1-3* were used to investigate the role of LLPs in different stress responses. *LLP1* is responsive to SA, whereas *LLP3* is responsive to methyl jasmonate (MeJA). Although neither affect local defence, both are required individually for normal SAR signalling. Additionally, when all three LLPs are lost then systemic susceptibility is observed. The LLPs are also required for normal responses to high salinity in both seedlings and mature plants. All the transgenic lines show reduced primary root growth in seedlings after salt treatment or MeJA treatment. *RNAi:LLP1-3* plants showed gene expression changes only in the branch of JA associated with the transcription factor ETHYLENE RESPONSE FACTOR 1 (ERF1), and not MYC2. *llp3* phenotypes could be complemented by the expression of transgenic *LLP3* under its native promoter.

Here, I show that two sequence-related LLPs promote SAR through spatially separated functions with JA promoting local SAR signal generation through LLP3. In concert with LLP1, which is important for systemic recognition and propagation of SAR signals, LLP3 promotes both SA-dependent SAR and JA-mediated immunity. Thus, exploitation of LLP-associated signalling cues might allow application of plant innate immune signals to promote plant health.

Zusammenfassung

Angesichts des abiotischen Stresses und neu auftretender Pflanzenkrankheiten müssen wir für vollumfängliche Pflanzenschutzstrategien das Zusammenspiel zwischen verschiedenen Stressreaktionen und damit verschiedene Phytohormon-Signalwege berücksichtigen. Die Phytohormone Salicylsäure (SA) und Jasmonsäure (JA) aktivieren zwei zueinander antagonistische Immunstoffwechselwege, die Pflanzen jeweils vor biotrophen Pathogenen auf der einen Seite oder nekrotrophen Pathogenen und Insekten auf der anderen Seite schützen. Dieser Konflikt schließt die Nutzung von SA- und JA-abhängigen Immunsystem-Bestandteilen für den Pflanzenschutz weitgehend aus, was das Interesse an Verbindungen im Immunstoffwechsel erhöht, die den SA-JA-Antagonismus aufheben. Eine lokale Infektion mit einem Krankheitserreger führt zu einer SA-abhängigen Immunität in systemischen Geweben. Diese so genannte systemisch erworbene Resistenz (Systemic Acquired Resistance, SAR) gewährleistet eine lang anhaltende, breit gefächerte Krankheitsresistenz, die nicht dem SA-JA-Antagonismus unterliegt.

Die Legume Lectin-like Proteins (LLP) 1, 2 und 3 sind membranständige Proteine mit einer vorhergesagten Kohlenhydrat-bindenden Domäne, die in *Arabidopsis thaliana* vorkommen. Hier wurden die Knockout-Mutanten *llp1-1* und *llp3* sowie die Dreifach-Silencing-Linie *RNAi:LLP1-3* verwendet, um die Rolle der LLPs bei verschiedenen Stressreaktionen zu untersuchen. *LLP1* spricht auf SA an, während *LLP3* auf Methyljasmonat (MeJA) reagiert. Obwohl weder LLP1 noch LLP3 die lokale Abwehr beeinflussen, sind beide einzeln betrachtet für die normale SAR-Signalübertragung erforderlich. Zusätzlich wird bei Verlust aller drei LLPs eine systemische Empfindlichkeit beobachtet. Darüber hinaus werden die LLPs für eine normale Reaktion auf hohe Salzkonzentrationen sowohl in Keimlingen als auch in erwachsenen Pflanzen benötigt. *llp1-1*-, *llp3*- und *RNAi:LLP1-3*-Keimlinge zeigen nach Salz- oder MeJA-Behandlung ein reduziertes Hauptwurzelwachstum. *RNAi:LLP1-3*-Pflanzen zeigen weiterhin eine Fehlregulation des ETHYLENE RESPONSE FACTOR1 (*ERF1*), nicht aber des MYC2, was darauf hindeutet, dass die Reaktion von nur einem Zweig der JA-Signalübertragung abhängig ist. Durch die Expression von transgenem *LLP3*

unter seinem nativen Promotor konnten die phänotypischen Defekte der *llp3*-Mutante komplementiert werden.

In dieser Arbeit wird gezeigt, dass zwei sequenzverwandte LLPs SAR durch räumlich getrennte Funktionen fördern, wobei JA die lokale SAR-Signalerzeugung durch LLP3 fördert. Zusammen mit LLP1, das für die systemische Erkennung und Übertragung von SAR-Signalen wichtig ist, fördert LLP3 sowohl SA-abhängiges SAR als auch eine JA-vermittelte Immunität. Daher könnte die Nutzung von LLP-assoziierten Signalen die Anwendung von pflanzeneigenen Immunsignalen zur Förderung der Pflanzengesundheit ermöglichen.

Abbreviations

As well as SI units, metric prefixes, and the abbreviations for nucleotides, the following abbreviations were used in this work:

Pst	*Pseudomonas syringae pathovar Tomato*
ABA	Abscisic Acid
ABF	ABA Response Factor
ALD1	AGD2-like Defence Response protein 1
AzA	Azelaic Acid
AZI1	Azelaic acid induced 1
bHLH	Basic Helix-loop-helix
BTH	Benzothiadiazole
CAMTA	Calmodulin-Binding Transcription Activator
CBP60g	Calmodulin-Binding Protein 60g
COI1	Coronatine Insensitive 1
CPK5	Calcium-dependent Protein Kinase
DIR1	Defective in Induced Resistance 1
EARLI1	Early Arabidopsis Aluminium Induced 1
EDS1	Enhanced Disease Susceptibility
EIN3	Ethylene Induced 3
ERF1	Ethylene Response Factor 1
ETI	Effector-Triggered Immunity
ETS	Effector-Triggered Susceptibility
FMO1	Flavin-dependent Monooxygenase 1
G3P	Glycerol 3 Phosphate
GGPPS12	Geranyl Geranyl Diphosphate Synthase 12
HR	Hypersensitive response
IAA	Indole-3-Acetic Acid
ICS1	Isochorismate Synthase 1
ISR	Induced Systemic Resistance
JA	Jasmonic Acid

JA-Ile	JA-Isoleucine
JAZ	Jasmonate Zim Domain
LLP	Legume Lectin-like Protein
MED25	Mediator 25
MeJA	Methyl Jasmonate
MeSA	Methyl Salicylate
MPK	Mitogen-activated Protein Kinase
NHP	N-Hydroxypipecolic acid
NLR	Nucleotide Binding Leucine-rich Repeat
NO	Nitric Oxide
N-Oglc-NHP	*O*-glycosylated N-Hydroxypipecolic acid
NPR1	Non-expressor of Pathogenesis Related genes 1
NUDT7	Nudix Hydrolase 7
ORA59	Octadecanoid-Responsive Arabidopsis 59
P/M/DAMP	Pathogen/Microbe/Damage Asscoiated Molecular Pattern
PAD4	Protein Arginine Deiminase 4
PDF1.2	Plant Defensin 1.2
PDLP	Plamodesmata-Localising Protein
Pip	Pipecolic Acid
PP2C	Protein Phosphatase 2C
PR	Pathogenesis-Related
PRR	Pattern Recognition Recpetor
PTI	PAMP-Triggered Immunity
RBOHD	Respiratory Burst Oxidase Homologue protein D
RLK	Receptor-like Kinase
RLP	Receptor-Like Protein
RNAi	RNA interference
ROS	Reactive Oxygen Species
SA	Salicylic Acid
SAG	SA 2-O-β-d-glucose
SAMT	SA Methyl Transferase

SARD4	SAR Deficient 4
SGE	SA Glucose Ester
SnRK	SNF-Related Kinase
TNL	TIR-Like NLR
TPS24	Terpene Synthase 24
VOC	Volatile Organic Compound
VSP2	Vegetative Storage Protein 2

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1. Introduction

Food security is still one of the major challenges faced by humanity in the modern age, especially in the face of changing climates. Climate change brings in its wake changes to water supplies, soil salinity and extreme temperatures, as well as changing the geographical distribution of plant pathogens. All of these can have devastating effects on harvests, particularly amongst the poorest regions of the world. Food insecurity can bring about the tipping point for violence and instability in societies, and exacerbate the risk of political unrest and conflicts (Holleman *et al.*, 2017). Factors such as pathogens, weeds, and abiotic stress accounted for preharvest crop losses of 35% of possible biological output in 2018, which is an increase of around 3-fold from 50 years ago (Mesterházy *et al.*, 2020). This therefore presents an urgent need for understanding of crop stresses and development of novel methods for improving crop health and yields.

In order to protect themselves from stress encountered on a daily basis, plants have evolved a repertoire of defence responses. As they lack the dedicated immune cells and complex homeostatic systems that are found in animals, it has been necessary for plants to develop alternative strategies of dealing with these stressors. The exact nature of these different stress responses, and the complexity of the interaction network between them, has yet to be fully resolved. An important aspect of this is the action of phytohormones and their associated signalling pathways.

1.1 Biotrophic vs necrotrophic defence

Among the stresses faced by plants, a significant portion can be associated with disease caused by microbial pathogens. Pathogens can be differentiated from other forms of microbes that are associated with plants because they have an overall detrimental impact on the fitness and eventual reproductive success of the host, rather than a positive or neutral effect, and are further categorised based on their mode of nutrition (*Table 1*)(de Souza *et al.*, 2016). Biotrophic pathogens can be defined as organisms, primarily bacteria or fungi, that rely on access to living host tissues to acquire their nutrition (Spanu and Panstruga, 2017). This can be contrasted with necrotrophic pathogens, which induce the death of the host tissue before they

are able to utilise it for food (Lewis, 1973). In general, biotrophic pathogens initially infect cells of the epidermis, and then infiltrate deeper into the tissue via structures known as haustoria (although this is not always the case) and use these to infiltrate the living cells. Consequently, biotrophic pathogens tend to have a relatively specific host range. Necrotrophic pathogens such as *Alternaria brassicicola* and *Botrytis cinerea,* on the other hand, use various toxins to induce cells' death, and normally have a much wider host range (Mengiste, 2012). It appears that these modes of nutrition have evolved from progenitors that utilised saprotrophy, in which the microbe is able to derive its nutrition from dead plant cells without having had a causal role in their death (Martin *et al.*, 2016).

Table 1: Overview of the differences between biotrophic, hemibiotrophic and necrotrophic pathogens (Lemarié et al., 2015; Moore et al., 2020).

Feature	Biotrophs	Necrotrophs	Hemibiotrophs
Culture	Can't be grown in axenic culture	Easy to grow in axenic culture	Can be grown in axenic culture
Host Range	Narrow host range	Wide host range	Normally narrow host range
Effect on host cells	Does not normally kill host cells. Cell death occurs if host recognises pathogen and induces HR.	Kills host cells as quickly as possible.	In first stage of life cycle keeps cells alive, then switches to induction of cell death.
Mechanism of pathogenesis	Uses secretion systems to inject effectors into cells	Degrades cell wall with lytic enzymes and toxins	Uses secretion systems to inject effectors into cells, secretes limited amounts of lytic enzymes
Area of replication	Intercellular	Inter- or intracellular	Inter- then possibly intracellular, depending on the species
Associated defence signalling pathways	SA-dependent pathways via NPR1	JA- and ET-dependent pathways via ERF1	SA-dependent pathways via NPR1 or JA- and ET-dependent pathways via ERF1, depending on the pathogen species

This evolutionary path did not only stop at biotrophic and necrotrophic pathogens but has also led to a range of intermediate possibilities. These are known as

hemibiotrophic pathogens, which utilise both these strategies of nutrient acquisition at different stages of their lifecycles. During the first stage, the pathogen invades and colonises the plant tissue using biotrophy to keep its nutritional source alive. Once it has reached a critical level, it switches to a necrotrophic strategy, and intentionally induces cell death. It is still debated whether in the earlier stages of colonisation hemibiotrophs can be considered to use similar virulence mechanisms to biotrophs, or if the pathogen is simply using stored energy reserves before switching to the initiation of host cell death. This problem arises initially from the original designations of "plant parasites", and how they were associated with obligate or facultative lifestyles (i.e. whether or not they could be maintained in axenic culture) (Thrower, 1966), where the delineation between the two lifestyles is relatively clear cut. However, with modern methods and when attempting to apply this to bacterial or oomycete pathogens, the designation becomes somewhat more complex. This had led to the attempt to classify pathogens by other means, such as by carbohydrate-active enzyme gene content (Hane *et al.*, 2020). Nevertheless, for the rest of the introduction an assumption shall be made that the hemibiotrophic pathogens do indeed make a concrete switch between lifestyles in order to include previous literature around the topic, and that early-stage hemibiotrophs and biotrophs can be considered as the same.

The key pathogen studied in the following work is *Pseudomonas syringae* pathovar *tomato (Pst* or DC3000*)*, a hemibiotroph (Cuppels, 1986). As a species, *P. syringae* causes disease in a range of economically important crops. However, each strain shows relatively high host specificity (Cardan *et al.*, 1999). *Pst* is especially useful as a model pathogen as it is able to infect and cause disease in both tomato and *Arabidopsis* (Whalen *et al.*, 1991*).* It infects aerial portions of the plant, such as the leaves, and favours environmental conditions including high humidity and moderate temperatures. *Pst* generally enters the plant through wounds or open stomata, and is able to spread apoplastically during the biotrophic phase of its life cycle, before switching to necrotrophy, which instigates cell death and results in necrotic lesions (Figure 1). Approximately 5% of the *Pst* genome is comprised of virulence genes (Buell *et al.*, 2003), including different secretion systems and toxins. These allow it to disrupt both PAMP-Triggered Immunity (PTI) and Effector-Triggered Immunity

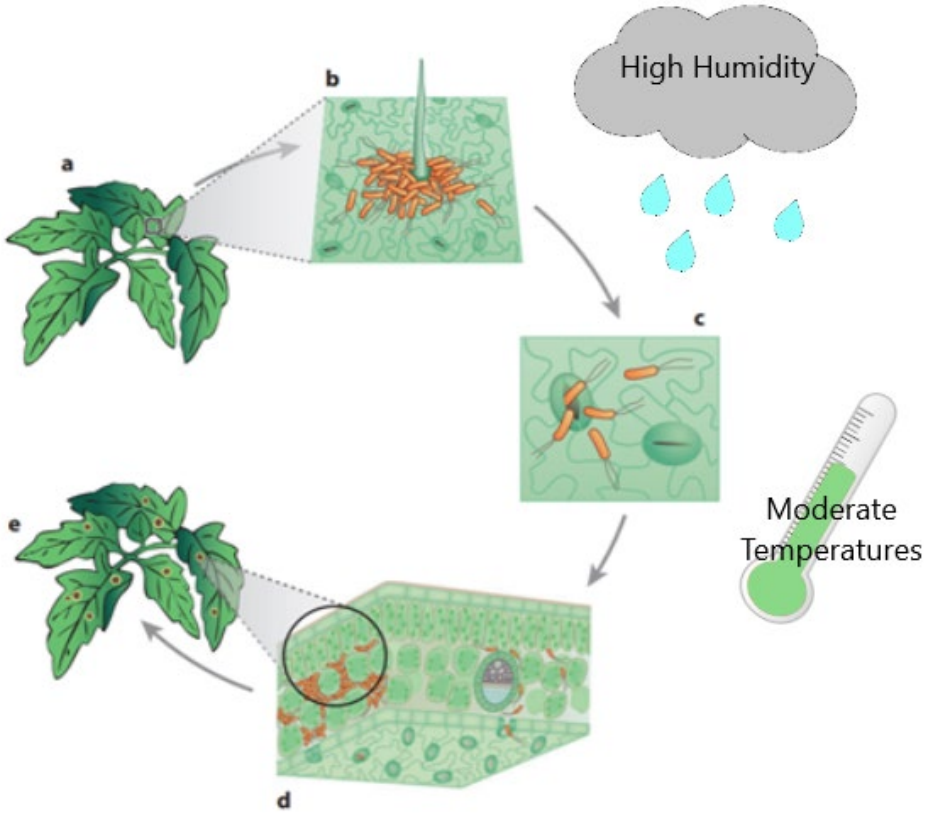

Figure 1: The lifecycle of *Pst*. *Pst* infects only the aboveground tissue, but as the pathogen does not survive easily on the leaf surface, it must find open stomata in order to enter the plant (a,b,c). Once in the apoplast, *Pst* reproduces while deriving nutrition from living cells (d). During that time, it may activate PTI or ETI, depending on whether the interaction with the plant is compatible. At a critical concentration, it switches to necrotrophy and causes cell death and the formation of necrotic lesions (e). Adapted from Xin and He 2013.

(ETI) (see below), although it is still unknown how this disruption is related to nutrient acquisition by the bacteria (Xin and He, 2013).

As already alluded to, both the mechanism of infection and thus the host defence responses against *Pst* may not be so well defined as was originally hoped, as new research breaks down the boundaries between the clearly defined signalling pathways and defence mechanisms against biotrophs and necrotrophs.

The traditional view of biotrophic vs necrotrophic defence is that the former relies on programmed cell death induced by SA signalling pathways while the latter encourages cell survival via Jasmonic Acid (JA) signalling in an effort to deny nutritional sources to the pathogens (see below) (Glazebrook, 2005).

1.1.1 PTI and ETI

Plants rely on different layers of signalling during biotrophic immunity. The initial step in prompting a specific type of defence response depends upon whether the pathogen is "compatible" with the host or not. If after the host-pathogen interaction the pathogen is successful in inducing disease, then the interaction was "compatible". If the plant is able to successfully defend itself against the pathogen and ward off any deleterious effects, then the interaction was incompatible. The balance between these outcomes starts to be decided when the host recognises either a Pathogen-Associated Molecular Pattern (PAMP) or Microbe-Associated Molecular Pattern (MAMP), which often take the form of oligosaccharides or glycoproteins, such as the flagellin molecule found on the surface of many bacterial species (Boller and Felix, 2009). These are recognised by PAMP Recognition Receptors (PRRs) on the surface of the plant cells. PRRs tend to be either receptor-like kinases (RLKs) or receptor-like proteins (RLPs), and both are transmembrane proteins with a ligand-binding ectodomain (Boutrot and Zipfel, 2017). These receptors can be further categorised, for example by whether they have lectin domains (Singh and Zimmerli, 2013). Sometimes they also require accessory or adaptor proteins that are also found in the cell membrane (Zipfel, 2009). Activation of these receptors will generally initiate so-call PAMP-Triggered Immunity, or PTI (Jones and Dangl, 2006; Zipfel, 2009; Zipfel and Robatzek, 2010). Because PAMPs are often conserved between bacterial strains, being able to recognise them provides the plant with an efficient way of protecting itself against multiple possible diseases. PTI can also be induced if the plant recognises molecules from its own cells which have been released after damage or pathogen-induced necrosis, which are known as Damage-Associated Molecular Patterns, or DAMPS (Choi and Klessig, 2016). These can take a variety of forms, including peptides, polysaccharides, and extracellular ATP. Whether the initiation of PTI comes from PAMPs, MAMPs, or DAMPs, the downstream signalling pathways are very similar, and involve processes such as defence gene upregulation, reactive oxygen species (ROS) production, callose deposition, Ca^{2+}influx and Mitogen Activated Protein Kinase (MPK)3/6 activation (Choi and Klessig, 2016). If pathogens are not able to suppress this layer of host defence, then they will not be able to cause disease.

As plant pathogens still exist, they must have found a mechanism by which to overcome the base layer of PTI. This is primarily orchestrated by the injection of effector proteins into the plant cells via type three secretion systems (Alfano and Collmer, 2004; Xin and He, 2013; Galán *et al.*, 2014). These effector proteins are then able to suppress the basal PTI response and allow the pathogens to spread within the host unnoticed, in a state known as Effector Triggered Susceptibility (ETS) (Jones and Dangl, 2006).

However, through the evolutionary arms race, plants have evolved to recognise these effector proteins even once they have made it to within the cytosol. They do this via disease resistance (R) proteins, typically intracellular nucleotide-binding domain and leucine-rich repeat proteins (NLRs), which detect the effector molecules and then induce Effector-Triggered Immunity, or ETI (Jones and Dangl, 2006). The two main types of NLRs found in plants are categorised depending on their N-terminal domain

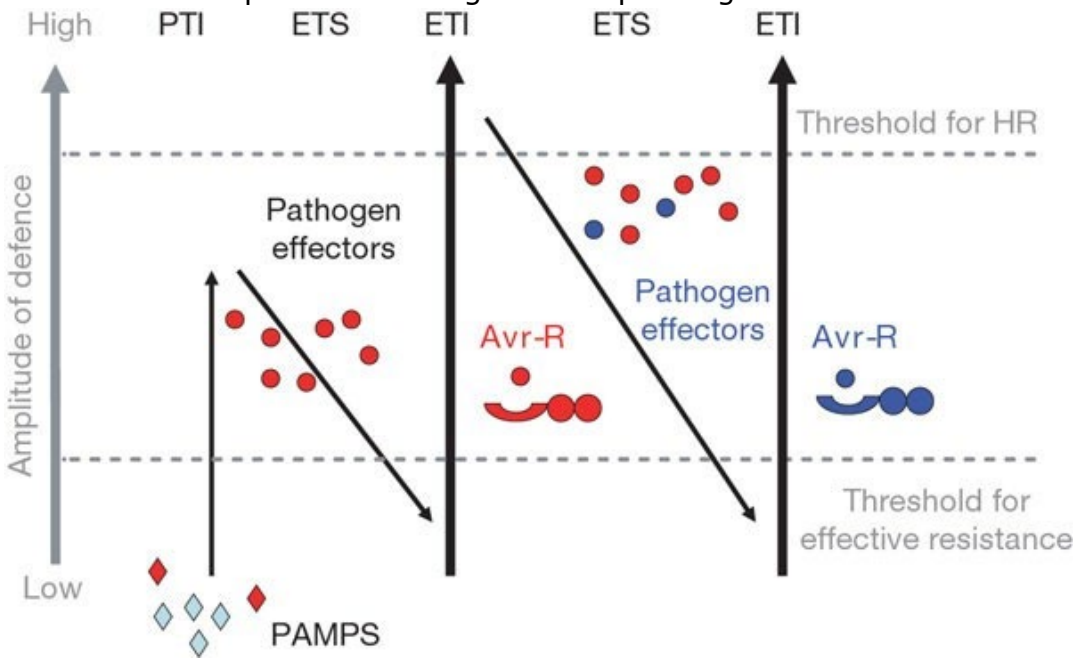

Figure 2: The zig-zag model of plant immune defence. After recognition of PAMPs from the surface of pathogens, a weak PTI response is initiated. To overcome this, pathogens inject effector proteins into the host cells to repress defence, leading to ETS. If the host has a receptor for the specific effector, then a stronger ETI defence response will occur, and lead to a hypersensitive response and cell death to prevent the spread of the pathogen (adapted from Jones and Dangle 2006).

architecture, either as coiled-coil (CC)-NLRs or as Toll-interleukin 1 receptor domain (TIR) NLRs, also known as TNLs (Zhang *et al.*, 2017*a*). TNLs induce immune responses through the regulator Enhanced Disease Susceptibility 1 (Heidrich *et al.*, 2011). The cycle of pathogen and host evolving resistance to one another continues, and results in the zig-zag model of plant innate defence (Figure 2: Jones and Dangl, 2006).

ETI is a relatively stronger immune response than PTI, and often results in a rapid, localised pattern of programmed cell death known as the hypersensitive response (HR). Because (hemi)biotrophic pathogens rely on living tissues in order to obtain their nutrition, theoretically if they are surrounded by dead cells they will no longer be able to reproduce and spread to uninfected parts of the plant, thus containing the disease (Goodman *et al.*, 1994; Van Loon, 1997).

Although they respond to different stimuli, the extent of the overlap between the cascades induced by PTI and ETI is becoming more apparent (Kadota *et al.*, 2019). Signalling components such as Ca^{2+} and MPK cascades are initiated in both responses, and both ultimately utilise outcomes such as the generation of ROS and antimicrobial compounds (Tsuda *et al.*, 2013; Yu *et al.*, 2017). The phosphorylation of RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD), which is required for ROS synthesis, was also identified as a point of convergence between the pathways (Kadota *et al.*, 2019). The strength and temporal output from each system is different, with ETI showing a more robust and long-lasting response, indicating that the mechanism of activation still has an important role in the final outcome (Kadota *et al.*, 2019).

1.1.2 Salicylic Acid in defence

The other key commonality between the PTI and ETI pathways is that they both strongly rely on signalling through the salicylic acid (SA) cascade (Vlot *et al.*, 2009). This pathway is also important in SAR (see below). SA is a beta hydroxy phenolic acid that is synthesised in relatively low amounts by either the isochorismate or phenylalanine ammonia-lyase pathways (Ding and Ding, 2020). Early research associating SA with biotic defence showed endogenous increases in the hormone after viral infections in tobacco and cucumber (Malamy *et al.*, 1990; Métraux *et al.*, 1990).

It was also associated with the HR lesions typically seen after ETI, indicating an important role for SA in this system (Radojičić *et al.*, 2018). However, SA levels are still heightened in mutants that do not form lesions, such as *suppressor of npr1-1*, indicating that cell death is not required to initiate SA biosynthesis, and higher SA levels alone do not initiate an HR response (Li *et al.*, 2001).

For the initiation of SA signalling, the phytohormone must not only be biosynthesised, but also recognised. The main receptor that activates SA-mediated signalling is NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) (Wu *et al.*, 2012; Manohar *et al.*, 2015). Along with TGA transcription factors it initiates defence gene expression and resistance against pathogens (Fan and Dong, 2002) and its absence leads to increased susceptibility (Delaney *et al.*, 1995). In plants where *NPR1* is overexpressed, cell death after bacterial infection is attenuated, and in the *npr1-1* mutant it is enhanced (Rate and Greenberg, 2001), indicating that SA regulates cell death during the HR response through NPR1. There is also an overall increased susceptibility to pathogens in *npr1,* and a decrease in the accumulation of certain pathogenesis-related (PR) proteins, most notably PR1 and PR5 (Glazebrook *et al.*, 1996; Cao *et al.*, 1997). The effect on gene expression comes from the nuclear localisation of NPR1 after an increase in cellular SA levels (Kinkema *et al.*, 2000; Maier *et al.*, 2011). Once in the nucleus, NPR1 is thought to act as a cofactor along with members of the TGA transcription factor family, forming a transcriptional complex that can bind to *activation sequence-1* (*as-1*)-like motifs found in the promoters of *PR* genes (Strompen *et al.*, 1998; Zhou *et al.*, 2000; Després *et al.*, 2003). However, although it orchestrates SA signalling, NPR1 also helps to finetune the output of these signals by having an additional role in negatively regulating SA biosynthesis, specifically through *ISOCHORISMATE SYNTHASE1* (*ICS1,* also known as *SID2*) (Zhang *et al.*, 2010). NPR1 can also respond to signals from jasmonate pathways so has been touted as a key signalling regulator in a broad range of biotic defence responses (Spoel *et al.*, 2003; Dong, 2004). MeJA-induced defence in Induced Systemic Resistance (ISR) was blocked in *npr1* plants, indicating both that a jasmonate signal is required for ISR, but also NPR1 is capable of responding to more than one phytohormone, although this response may result in the initiation of more than one downstream process (Pieterse *et al.*, 1998). However, NPR1's

important functions have also made it a target for some pathogens. Necrotrophic *B. cinerea*, for example, uses an SA-mimicking exopolysaccharide to induce signalling through NPR1, and potentiate its own pathogenesis in tomato (El-Oirdi *et al.*, 2011).

The two other SA receptors, NPR3/4, although analogues of NPR1, conversely function as redundant transcriptional corepressors of SA-induced gene expression (Zhang *et al.*, 2006; Shi *et al.*, 2013; Ding *et al.*, 2018). They too are able to form complexes with TGA transcription factors, but this time to repress defence gene expression (Ding *et al.*, 2018). Gain-of-function *npr4* mutants showed constitutive SA pathway repression, indicating that NPR4 acts as a bona fide SA receptor, rather than an E3 ligase that regulates the degradation of NPR1 as had previously been suggested (Fu *et al.*, 2012). This may also account for the genetic redundancies between NPR3 and NPR4, with one being able to function in the place of the other (Kuai *et al.*, 2015). SA can also positively support its own regulation by inhibiting transcriptional repression by NPR3/4 when it is present at high levels (Ding *et al.*, 2018). NPR3/4 are also able to bolster downstream signalling in jasmonate cascades, both via upregulation of JA-dependent genes, as well as JA biosynthesis. This possibly occurs by NPR3/4 promoting the degradation of JASMONATE ZIM DOMAIN (JAZ) proteins (Liu *et al.*, 2016). This may function as a mechanism to prevent the spread of necrotrophic pathogens in the dead cells left in the HR lesion.

Although NPR1 and its homologues have an undeniable role in defence, it appears that they may be dependent on temporal and spatial factors. SA levels and susceptibility to pathogens are known to fluctuate during the circadian cycle. It appears that NPR1 may have a direct influence on the regulation of clock genes including *TIMING OF CAB2 EXPRESSION 1 (TOC1)* and *LATE ELONGATED HYPOCOTYL (LHY)* (Zhou *et al.*, 2015*a*). This suggests that not only is plant immunity dependent on circadian rhythms, but *vice versa* the circadian clock is also under the influence of the plant immune status. The spatial aspect must also be taken into account, with *npr3* mutants showing increased resistance to *P. syringae* in flowers and not leaves (Shi *et al.*, 2013). These intricacies begin to hint at the complex levels of regulation that are needed for normal defence responses.

1.1.3 EDS1

EDS1 is a nucleocytoplasmic, lipase-like protein that serves as a key regulator in SA-dependent signalling (Bhandari *et al.*, 2019). It is necessary for both normal basal resistance and TNL-mediated ETI, with most TNLs and at least one CC-NLR requiring EDS1 to propagate their signals (Parker *et al.*, 1996; Aarts *et al.*, 1998; Feys *et al.*, 2001; Wiermer *et al.*, 2005). In *Arabidopsis*, EDS1 functions by forming either homodimers or heterodimers with other proteins, namely phytoalexin deficient4 (PAD4) and senescence-associated gene101 (SAG101) (Feys *et al.*, 2001, 2005; Rietz *et al.*, 2011). These complexes are molecularly and spatially distinct, with EDS1 homodimers localised to the cytoplasm, EDS1-SAG101 heterodimers localised to the nucleus, and EDS1-PAD4 heterodimers being found in both of these compartments (García *et al.*, 2010). The complexes also have non-redundant functions in innate immunity (Zhou *et al.*, 1998; Rietz *et al.*, 2011; Wagner *et al.*, 2013). The functions of EDS1 and PAD4 differ depending on whether they are dimerised or not. Both proteins are required but not in a complex for TNL-induced cell death, but direct interaction is required for basal immunity, which was also associated with induction of SA-mediated immunity (Rietz *et al.*, 2011). The heightened SA levels then signal back into EDS1-PAD4 regulation, forming a positive feedback loop (Cui *et al.*, 2017). The role of EDS1 in innate immunity is so strong that overexpression causes autoimmunity resulting in HR-like lesions and cell death (Cui *et al.*, 2017). However, the EDS1-dependent signalling alone has some different downstream outputs from normal pathogen induced immunity, such as not being required for MPK3/MPK6 activation that is important in PTI and ETI.

EDS1 also has a role in SA- and NPR1-independent innate immunity, as there are greater levels of susceptibility in *eds1-2 sid2-1* double mutants compared to the individual single mutant lines (*sid2* being defective in SA biosynthesis) (Li *et al.*, 2001; Bartsch *et al.*, 2006; Cui *et al.*, 2017). Also, ETI mediated by the CC-NLRs RPM1 and RPS2, which respectively recognise the effector proteins AvrRpm1 and AvrRpt2 (Yu *et al.*, 1993; Grant *et al.*, 1995), can be induced in the absence of either EDS1 or SA (Venugopal *et al.*, 2009). This allows EDS1 to compensate for defects in SA-signalling, and involves FLAVIN DEPENDENT MONOOXYGENASE1 (FMO1) and a member of the Nudix hydrolase family NUDT7 (Bartsch *et al.*, 2006). On the other

hand, high SA accumulation can compensate for increased susceptibility in *eds1* (Clarke *et al.*, 2001). This may have occurred because of a high evolutionary pressure by pathogens on the SA-mediated response, and so allows each defence sector to protect the other (Venugopal *et al.*, 2009; Cui *et al.*, 2017). It may also be to allow SA and EDS1 to coordinate the responses within and around the HR lesion to control cell death around the infection foci, depending on which EDS1 complexes are present (Straus *et al.*, 2010).

EDS1-PAD4 complexes may present a junction not only at which biotrophic immunity is initiated, but also one at which JA pathways can inhibit them. MPK4 is able to simultaneously repress the SA sector through EDS1-PAD4 while promoting JA signalling (Petersen *et al.*, 2000). This allows increased defence against *A. brassicicola* at the expense of biotrophic immunity. Conversely, EDS1 can repress the JA-activating function of MPK4, and so presents a potentially important node in SA-JA crosstalk (see below) (Wiermer *et al.*, 2005).

1.1.4 Jasmonic acid in defence

JA is critical for defence against necrotrophic pathogens, herbivory, and some types of abiotic stress, as well as Induced Systemic Resistance (ISR). In plant defence, jasmonate signalling refers to pathways involving JA, its methyl ester (MeJA), or its isoleucine conjugate JA-Ile, which are all derivatives of a class of fatty acids (Ruan *et al.*, 2019). Their biosynthesis can occur via three different pathways, including from α-linolenic acid and hexadecatrienoic acid (Chini *et al.*, 2018), all of which occur in the chloroplast, peroxisome and cytoplasm. ABC transporters control the subcellular distribution of JA and JA-Ile which, as the bioactive form, requires transport into the nucleus. JA is also transported into the apoplast for intercellular signalling (Ruan *et al.*, 2019). Under control conditions, JA signalling is held in a base state by jasmonate Zinc finger Influorescence Meristem (ZIM)-domain (JAZ) proteins, which constitutively repress the expression of JA-sensitive genes (Pauwels and Goossens, 2011). When JA-Ile binds to its receptor, CORONATINE INSENITIVE 1 (COI1), it is able to associate with the SKP1 protein and the Cullin protein to form an active SCF-type E3 ubiquitin ligase (SCF^{COI1}). This complex then promotes the degradation of the JAZ proteins, allowing the JA-induced genes to be expressed, and

thus initiating the signalling cascade (Yan *et al.*, 2007; Chini *et al.*, 2007; Thines *et al.*, 2007). More recently a study suggested that actually COI1 forms a complex directly with the JAZ proteins, and transports them to the 26S proteasome for degradation (Sheard *et al.*, 2010). After the JAZ proteins have been degraded, gene expression is controlled by a number of different transcription factors, from classes including MYB, NAC and WRKY. For the purpose of this thesis, we will concentrate on two key transcription factors: MYC2 and ERF1.

MYC2 is a basic helix-loop-helix (bHLH) transcription factor encoded by the *JIN1* gene (Kazan and Manners, 2013). It initiates transcription by recruitment of the mediator complex via the MED25 subunit (Chen *et al.*, 2012). As well as activating downstream JA signalling, it also upregulates transcription of *JAZ* genes, and thus negatively feedbacks upon MYC2 signalling, allowing for a greater level of finetuning under stress (Chini *et al.*, 2007; Liu *et al.*, 2019). Downstream MYC2 signalling acts to promote defence against herbivory while repressing necrotrophic immunity (Zhai *et al.*, 2013). This response is characterised by upregulation of VEGETATIVE STORAGE PROTEIN 2 (VSP2) and down regulation of PLANT DEFENSIN 1.2 (PDF1.2) (Lorenzo *et al.*, 2004). MYC2 may also be positively involved in ISR, as it is required for systemic responses against *Pst* after a root treatment with *P. fluorescens* (Pozo *et al.*, 2008). These multiple outcomes highlight the sensitivity needed in decoding MYC2 downstream responses, depending on its interaction partners.

ERF1 (ETHYLENE RESPONSE FACTOR 1) is induced by JA signalling via ETHYLENE INSENSITIVE 3 (EIN3), and is associated with another ERF protein, OCTADECANOID -RESPONSIVE ARABIDOPSIS 59 (ORA59) (He *et al.*, 2017). Overexpression is associated with increased necrotrophic defence against pathogens such as *B. cinerea* and upregulation of the marker gene *PDF1.2* (Berrocal-Lobo *et al.*, 2002). JA biosynthesis is increased after infection with *A. brassicicola*, but this has not yet been explicitly linked to ERF1 signalling (Van Wees *et al.*, 2003; Antico *et al.*, 2012). The ERF1 and MYC2 signalling pathways are mutually antagonistic, with stronger necrotrophic defence against *A. brassicicola* in the *myc2* mutant (Song *et al.*, 2014; Howe *et al.*, 2018).

JA is involved in tolerance against a wide range of abiotic stresses, including high salinity (Golldack *et al.*, 2014; Qiu *et al.*, 2014; Kazan, 2015; Riemann *et al.*, 2015). Treatment with NaCl induces both JA biosynthetic genes and the activation of JA signalling in a MYC2-dependent, abscisic acid (ABA)-independent manner (Jiang and Deyholos, 2006; Valenzuela *et al.*, 2016). Depending on the plant species and circumstances, JA can have either a positive or negative effect on salt tolerance. It was recently shown that the MYC2 branch of JA signalling negatively affects salt tolerance via a repressive role in proline biosynthesis. In that instance MYC2 is activated by the MPK3/MPK6 cascade, and can then feedback upon itself (Verma *et al.*, 2020). Salt stress also causes an upregulation of *JAZ* genes in *Arabidopsis* roots in a COI1-dependent manner. This leads to changes in differentiation of meristematic cells and results in an inhibition of root cell elongation (Valenzuela *et al.*, 2016). Thus, the MYC2 branch of JA signalling positively regulates root shortening in response to high salinity. When considering drought tolerance, JA/MYC2 have a lesser role, being primarily required for stomatal closing and root hydraulic conductivity in this context, both of which are ABA-dependent (Creelman and Mullet, 1995; Munemasa *et al.*, 2007; Sánchez-Romera *et al.*, 2014).

On the other hand, ERF1 is also strongly upregulated by both high salinity and drought stress (Cheng *et al.*, 2013). When ERF1 was overexpressed, plants showed a higher tolerance for both stresses. ERF1 is therefore thought to act as an integration point between JA and ABA signals during abiotic stress.

The presence of these different JA-controlled pathways means that when considering the role of JA signalling in relationship to other defence pathways, different types of interaction must be accounted for.

1.2 Systemic Acquired Resistance

As well as inducing local immune reactions, PTI and ETI also trigger a distal immune response in parts of the plant that have not previously come into direct contact with the pathogen. This phenomenon is known as Systemic Acquired Resistance, or SAR (Vlot *et al.*, 2008, 2020; Spoel and Dong, 2012; Conrath *et al.*, 2015). It comprises three key stages:

I. pathogen perception and signal generation in the local tissue,
II. the movement of a mobile signal, and
III. the recognition of this signal and upregulation of defence in the distal tissues.

SAR shows distinct signalling pathways from the other form of biotic induced resistance, known as Induced Systemic Resistance (ISR), which occurs after contact with beneficial microbes in the rhizosphere (Vlot *et al.*, 2020). It also differs from "priming", which represents a transcriptional potential for faster immune responses rather than a direct upregulation of defence proteins in systemic tissues (Conrath *et al.*, 2015). SAR provides protection against a broad range of pathogens (Fu and Dong, 2013; Vlot *et al.*, 2017). It has been experimentally shown to last between three to ten days, and can be inherited by a plant's progeny that has had no direct exposure to the initial causal agent (Luna *et al.*, 2012). One of the critical differences between SAR and ISR is that SAR occurs after contact with pathogens in the phyllosphere, and so the systemic signal must be both generated and recognised in leaves, while ISR signals originate in the roots. The other difference that has been generally accepted is that while ISR relies on JA signalling, SAR pathways is mediated via two main pathways; one based on SA, the other relying on the non-protein amino acid pipecolic acid (Pip) and its bioactive derivative N-hydroxypipecolic acid (NHP) (Figure 3, Vlot *et al.*, 2020).

1.2.1 SA-dependent SAR

SA is a critical signal in SAR induction, with levels increasing in both local and systemic tissues (Vlot *et al.*, 2009). In mutants where SA accumulation is compromised (*SA induction-deficient2 (sid2)* and *enhanced disease susceptibility 5 (eds5)*) and where SA degradation is enhanced (*nahG*), SAR is not induced (Wildermuth *et al.*, 2001; Nawrath *et al.*, 2002; Van Wees and Glazebrook, 2003).

Because SA can move systemically in plants via the apoplast, it was initially considered as a potential SAR signal. However, as plants in which cuticle formation and thus apoplastic trafficking was disrupted were still able to produce SAR signals, the mobility of SA was deemed to be unnecessary for SAR establishment (Vernooij *et al.*, 1994; Lim *et al.*, 2020). Another possibility was for MeSA, the volatile SA derivative, to act as a mobile SAR signal (Park *et al.*, 2007). The transcript

accumulation of *SA-Methyltransferase* (*SAMT*) in the local tissue and activity of the methyl esterase SA-Binding Protein 2 (SABP2) in the systemic tissue are both needed for SAR (Kumar and Klessig, 2003; Park *et al.*, 2007). This leads to the theory that the balance between SA and MeSA regulates SAR establishment, and has been seen in tobacco and potato as well as *Arabidopsis* (Vlot *et al.*, 2020). However, the importance of MeSA depends on the time of day at which infection occurs, possibly due to phytochrome-mediated sensing of the red:far-red light ratio, and in *phyAphyB* mutant plants SAR is abolished (Griebel and Zeier, 2008; Liu *et al.*, 2011). It has been also been hypothesised that the conversion of SA to MeSA is actually a mechanism co-opted by pathogens in order to reduce the amount of SA available in the immune pathways, as the MeSA is volatile and may be lost by the leaves (Attaran *et al.*, 2009).

The establishment of both SAR and ISR is dependent on NPR1 functioning correctly and recognising increased SA levels (Pieterse *et al.*, 1998). EDS1 is another SA-associated regulator that is required for SAR. It is required in both the local and systemic tissues for a full SAR response (Breitenbach *et al.*, 2014), and is critical for systemic defence after a local ETI induction by the CC-NLRs RPM1 and RPS2, possibly due to a role in ROS homeostasis (Breitenbach *et al.*, 2014).

1.2.2 SA-independent SAR

Although SA is required for SAR induction, other signals are also involved that are required solely for systemic defence, and not involved in local immunity. As mentioned above, the SAR pathway is dependent on Pip. Pip biosynthesis occurs in the chloroplasts, and comprises a dual-step process in which L-lysine is converted to cyclic dehydropipecolic acid (DP) by AGD2-LIKE DEFENCE RESPONSE PROTEIN 1 (ALD1), and DP is then converted to Pip by SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 4 (SARD4) (Ding *et al.*, 2016*b*; Hartmann *et al.*, 2017). Once it is transported outside of the chloroplasts, Pip can be hydroxylated by FMO1 to NHP, which is assumed to be the bioactive variant of the molecule (Hartmann *et al.*, 2018; Chen *et al.*, 2018). The expression of *ALD1* and *FMO1* and the accumulation of Pip and NHP are both reliant on EDS1/PAD4 signalling independently of SA (Bartsch *et al.*, 2006). If any stage of this process is compromised, then the SAR response is lost

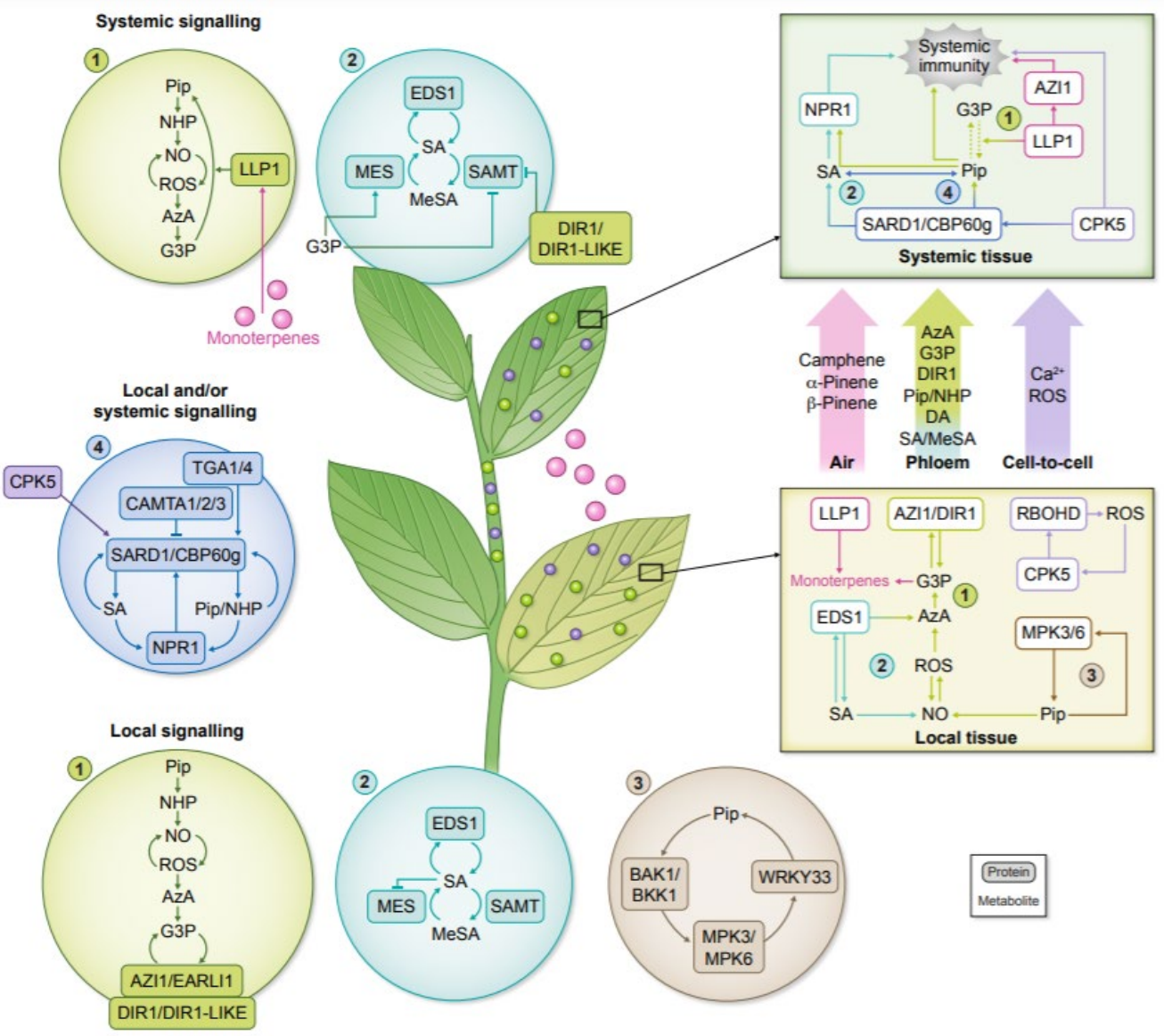

Figure 3: An overview of signalling pathways and feedback loops involved in SAR. Different signalling pathways are required in systemic tissue compared to local tissues during SAR. (1). An SA-independent pathway requires Pip, NHP, NO and ROS, AzA and G3P. In local tissues, AZI1/EARLI1 and DIR1 are also required to bolster the G3P signal. In the systemic tissue, propagation of this loop requires the presence of LLP1. A contribution of G3P is also required for SA level regulation in the systemic tissues (2). Another Pip feedback loop involving MPK3/MPK6 is required in the local tissues (3). Both SA and Pip pathways are regulated by feedback between NPR1 and SARD1/CBP60g locally as well as systemically (4). SAR signals move from local to systemic tissues via the phloem, cell-to-cell messengers, and via airborne compounds. Adapted from Vlot et al. 2020.

(Mishina and Zeier, 2006; Návarová *et al.*, 2012; Ding *et al.*, 2016*b*), with NHP especially being a key molecule (Bernsdorff *et al.*, 2016; Hartmann *et al.*, 2017, 2018).

Pip signalling in SAR involves three different feedback loops (Wang *et al.*, 2018*a*,*b*; Wenig *et al.*, 2019). One of these directly feedbacks into Pip biosynthesis, and is dependent on MPK3/MPK6 (discussed in relation to PTI and ETI above) to enhance *ALD1* expression via the transcription factor WRKY33 (Wang *et al.*, 2018*b*). However, it appears that the biosynthesis of Pip is only required in systemic tissues in order to recognise the SAR signal, and not in the local tissues (Wang *et al.*, 2018*a*).

The other Pip feedback loops are specific to SAR signalling. Pip induces signalling via ROS and NO, which then simultaneously bolsters the Pip signal (Wang *et al.*, 2018*a*). It is thought that the signal from ROS promotes the degradation of C18 lipids to produce the C9 di-carboxylic acid azelaic acid (AzA) (Wang *et al.*, 2014; Wittek *et al.*, 2014). AzA was initially associated with SAR after being found at heightened level in the petiole exudates of plants undergoing a systemic defence response (Jung *et al.*, 2009). If AzA is applied exogenously to local leaves it is able to induce resistance in systemic tissues in a process that is dependent upon both Pip and SA signalling. This exogenous AzA also becomes systemically mobile, suggesting AzA as a possible candidate for the mobile SAR signal. The activity of another key protein in the Pip cascade, AZELAIC ACID INDUCED 1 (AZI1) is, as the name would suggest, induced by AzA, and is critical for SAR establishment. It sometimes acts with its functionally redundant homologue, EARLY ARABIDOPSIS ALUMINIUM INDUCED 1 (*EARLI1*). In fact, just the overexpression of AZI1 in local tissues is sufficient to induce systemic defence (Cecchini *et al.*, 2015). Additionally, AZI1 has been shown to interact with PLASMODESMATA LOCALISING PROTEIN 1 (PDLP1). This means that AZI1 could promote a SAR signal that is relayed via the plasmodesmata, rather than the apoplast or phloem (Cecchini *et al.*, 2015).

Whether AzA is necessary in the local or systemic tissues during SAR is still under debate (Vlot *et al.*, 2020). The AzA-mediated signalling pathway is dependent on the membrane metabolism intermediate glycerol-3-phosphate (G3P) (Chanda *et al.*, 2011; Yu *et al.*, 2013). It is thought that G3P alone is insufficient to induce SAR, but

may need to act in concert with other signals such as Pip or DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1) (Chanda *et al.*, 2011; Wenig *et al.*, 2019). DIR1 is a predicted lipid transfer protein that is only required in local tissues (therefore in either signal generation or transmission), and is associated with a similar plasmodesmata transfer mechanism as AZI1 (Yu *et al.*, 2013; Carella *et al.*, 2015). As G3P is also only required in the local tissue (Gao *et al.*, 2014), and AzA may also function as a systemic signal, DIR1 may be playing a role in the mobilisation of these molecules (Vlot *et al.*, 2020). G3P is also able to stabilise AZI1 and DIR1 transcripts, which feedback to promote G3P accumulation (Yu *et al.*, 2013). Pip signalling after SAR signal recognition in the systemic tissues depends on a pathway involving AzA and G3P, so these along with AZI1 and DIR1 may fortify and stabilise the Pip induced upregulation of systemic defence responses (Lim *et al.*, 2016; Wang *et al.*, 2018*a*).

1.2.3 Interactions between SA and Pip pathways

Although they may have some totally independent functions, it has recently come to light that there may be shared mechanisms of regulation between the SA and Pip pathways. A set of Calmodulin-binding Transcription Activator (CAMTA) transcription factors negatively influences the expression of both *ICS1* and *ALD1*, via the transcription factors *SARD1* and *CALMODULIN BINDING PROTEIN 60g* (*CBP60g*), and so are involved in both Pip and SA biosynthesis (Fig …, Kim *et al.*, 2020; Sun *et al.*, 2020). When the CAMTA proteins are mutated, then there is constitutive activation of defence responses (Sun *et al.*, 2020). SARD1-related defence is also activated by CALCIUM-DEPENDENT PROTEIN KINASE 5 (CPK5), which in turn is regulated by ROS signalling (Guerra *et al.*, 2020). Increased CPK5 results in accumulation of *ALD1, FMO1* and *SARD1* transcripts in the distal leaves and an overall enhanced SAR phenotype. However, the presence of both Pip and SA is required for this to take place, indicating another regulatory loop (Guerra *et al.*, 2020). The activity of CPK5 is calcium-dependent, so this may provide a link between calcium signalling and the systemic immune system, and present a cell-to-cell method for SAR signal transmission as opposed to long-distance transport via the vasculature or the apoplast (Vlot *et al.*, 2020).

As well as shared regulation, SA and Pip are able to positively feedback into not only their own, but also each other's biosynthesis and accumulation. SA-induced SAR is fully dependent on the presence of Pip, while Pip is able to partially induce SAR in SA-biosynthesis mutants (Bernsdorff *et al.*, 2016; Hartmann *et al.*, 2018). It has also been associated with the stabilisation of NPR1 (see above), which in turn regulates expression of both SA and Pip biosynthesis genes (Kim *et al.*, 2020). The reciprocal fortification between these cascades could thus be a critical factor in establishing a complete SAR response (Vlot *et al.*, 2020).

1.2.4 What is the mobile SAR signal?

In order for a successful SAR response to take place, there must be some method of relaying the signal from a local infection site to distal parts of the plant. As alluded to above, there are many possible candidate signal molecules which could play this role (Vlot *et al.*, 2008). With so many potential signalling molecules in the different SAR pathways, there is an ongoing discussion about which compound(s) is/are the long-distance signal(s) for SAR.

Although both Pip and SA have been shown to be systemically mobile, this mobility is not necessary for a normal SAR response. However, their derivative molecules give further possibilities. MeSA is required only in local tissues, and accumulation in the systemic tissues is not required for SAR (Park *et al.*, 2007). A similar situation exists for DIR1 and G3P (Maldonado *et al.*, 2002). These molecules could therefore act as phloem mobile signals, being released from the local tissue and moving systemically within the plant until they are recognised systemically by a yet unknown receptor (see Legume Lectin-like Proteins). DIR1 and G3P were also previously shown to support each other's translocation (Chanda *et al.*, 2011). Therefore, it may be that multiple signals are released which are also able to support the transmission of others.

Another possibility is the Pip derivative NHP or its *O*-glycosylated form, *N*-OGlc-Pip, which is also formed by FMO1. Whether NHP is phloem mobile has been debated. A local treatment with NHP can induce SAR, but free NHP was not detected in these samples (Hartmann *et al.*, 2018), while in another case NHP was found in systemic leaves of *fmo1* after a local treatment (Chen *et al.*, 2018). During SAR NHP

accumulation is detected in systemic leaves at higher levels than would be expected from concentrations in petiole exudates, indicating that it is likely that NHP is biosynthesised anew after recognition of the SAR signal, rather than being the sole mobile signal itself (Hartmann and Zeier, 2019). As glycosylation may stabilise NHP, then it is possible that *N*-OGlc-Pip is actually the phloem mobile signal (Chen *et al.*, 2018).

Volatile signals are emitted from the plant and can act as airborne signals relaying the SAR signal both between plants and potentially between leaves on the same plant (Figure 4, Riedlmeier *et al.*, 2017; Wenig *et al.*, 2019). It has been known for a long time that volatile signals can induce defences against herbivory and abiotic stressors in neighbouring plants, and this has now also been demonstrated for defence against biotrophs (Scala *et al.*, 2013; Riedlmeier *et al.*, 2017). The key signal molecules in these processes are called Volatile Organic Compounds (VOCs). The composition of the VOC profile detected by the plant determines which type of defence response will be induced (Brilli *et al.*, 2019). The main VOCs that induce SAR in particular are the monoterpenes α-pinene, β-pinene, and camphene; the emission of all three compounds is induced during ETI, in a pathway dependent on EDS1. These VOCs may act alongside MeSA, the volatile SA derivative. The chemical properties of monoterpenes mean they could potentially be absorbed through the cuticle as well as through the stomata of neighbouring leaves (Schmid *et al.*, 1992). This may also explain why in mutants with disrupted cuticles there is no SAR signalling (Xia *et al.*, 2009; Lim *et al.*, 2020). Plants that detect these signals will respond with increased defence through initiation of ROS signalling and defence gene upregulation, including *AZI1* and *CBP60g* (Riedlmeier *et al.*, 2017), as well as initiating *de novo* VOC synthesis in a pathway requiring Pip and G3P (Wenig *et al.*, 2019). Biosynthesis of these VOCs, and thus the entire plant-to-plant (PTP) SAR response, is dependent upon proteins including GERANYLGERANYL DIPHOSPHATE SYNTHASE 12 (GGPPS12) and TERPENE SYNTHASE 24 (TPS24) (Chen *et al.*, 2003, 2015; Wenig *et al.*, 2019).

1.3 Lectins in Plant Defence

Lectins were identified in plants as early as 1888 and represent a broad class of proteins found widely across phylogenetic kingdoms. Even when considered only

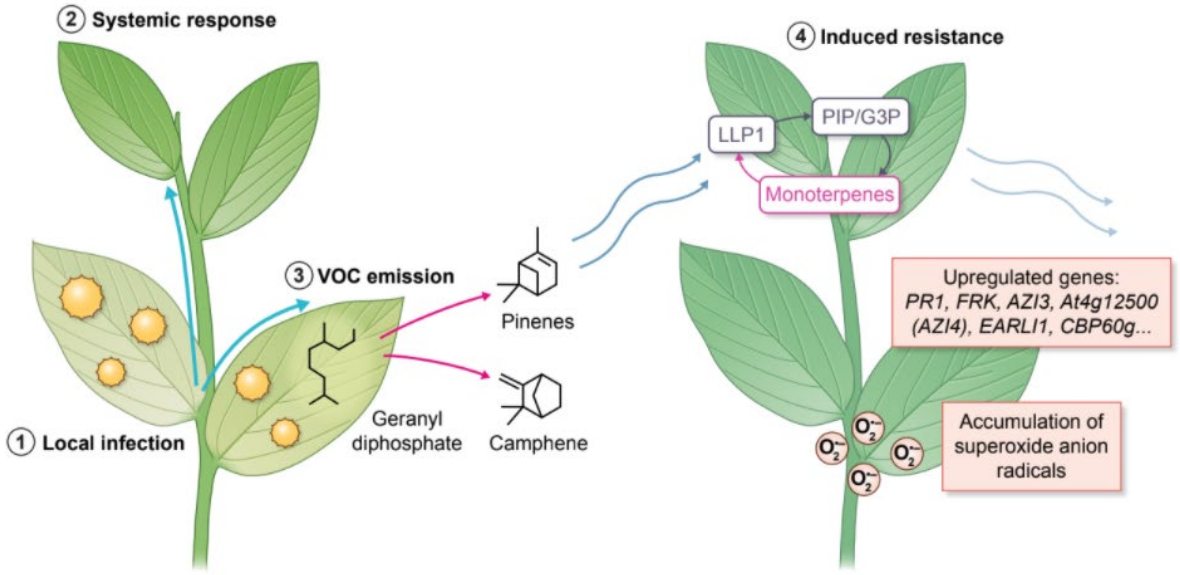

Figure 4: Airborne transmission of a defence signal between plants. After a local infection, plants can synthesise and emit VOCs such as monoterpenes from their leaves. These compounds are then recognised by neighbouring plants in a feedback loop dependent on LLP1 and Pip/G3P, which trigger downstream cascades leading to upregulation of defence genes in non-infected tissues. This renders the plant more resistant to later pathogen attack. Adapted from Vlot et al. 2020.

within plants, lectins still constitute a superfamily of proteins. Their structure can be either single-domain or multi-domain, with the latter often containing other protein domains such as kinase (Lectin receptor like kinases, or LecRLKs), F-box, or glycosyl hydrolase domains (Van Holle *et al.*, 2017). They are principally characterised by the presence of a carbohydrate recognition domain with no enzymatic activity that can reversibly bind to specific mono- or oligosaccharide residues (glycoconjugates) present in other molecules (Van Damme, 2007; Van Holle and Van Damme, 2018). These residues can either be from pathogens or from the host, which gives lectins a potentially important role in plant defence. This can occur by different routes. Some lectins bind directly to structures on the surface of bacterial cells and prevent direct access into the host cell. Others can function directly as PRRs, detecting both bacterial structures (PAMPs) or plant-derived components released into the apoplast after changes to cell wall integrity or cell death (DAMPs), and initiate the defence signalling cascades towards PTI (Figure 5, Wirthmueller *et al.*, 2013). LecRLKs and LecRLPs

(lectin receptor-like proteins) encompass some of the best known PRRs, such as CERK1, LORE and DORN1 (Miya *et al.*, 2007; Choi *et al.*, 2014; Ranf *et al.*, 2015). Additionally, effector proteins often require *N*-glycosylation in order to function, which can then be detected by intracellular lectins, giving them a role specifically in ETI (Chen *et al.*, 2014, 2017).

Although there are multiple methods of categorising lectins, one way is based on their evolutionary and structural relatedness (Mishra *et al.*, 2019). One of the largest groups that results from this classification is the Legume-Lectin family. Over 100 different proteins have been isolated from 70 species (Eggermont *et al.*, 2017). This breadth also leads to legume-lectins showing the widest range of saccharide

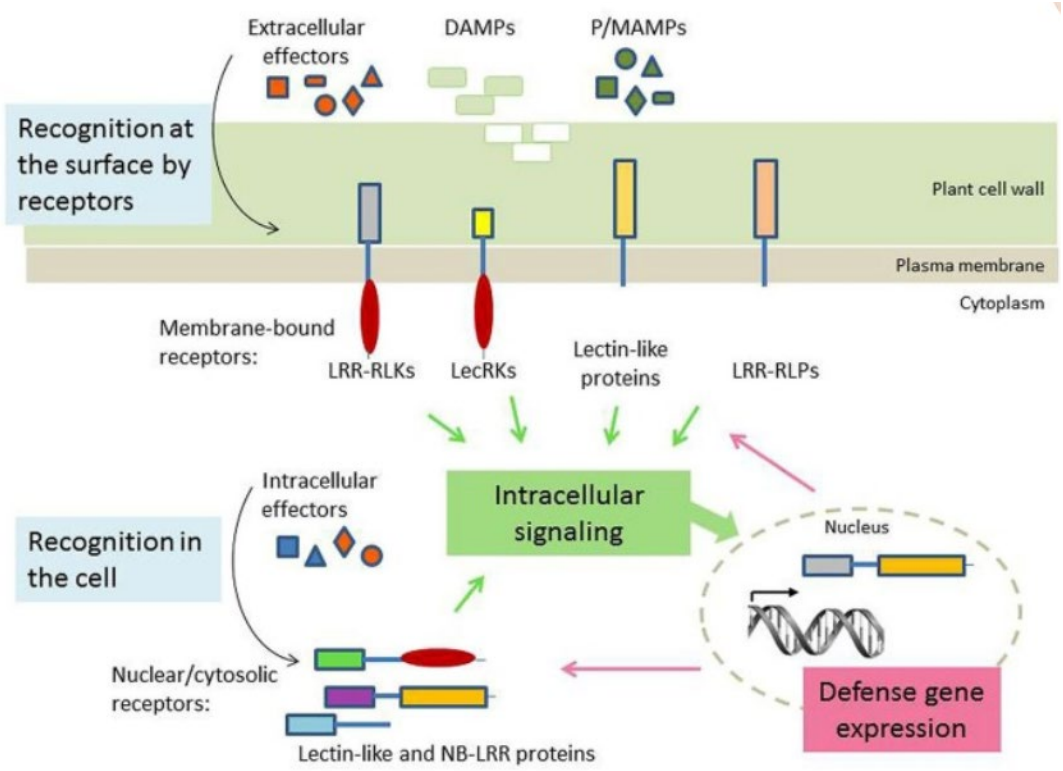

Figure 5: The role of lectins in plant immune signalling. In the plasma membrane, different forms of lectin receptors, including lectin-like proteins, can recognise DAMPS or P/MAMPs that are present in the extracellular space either directly from pathogens or from cell damage and death induced by them, and trigger PTI. Another set of lectins within the cell can recognise effector proteins, and then induce ETI. Both these processes result in intracellular signalling and upregulation of defence genes. (Adapted from Lannoo and Van Damme, 2014).

specificities when compared to other lectins studied so far (Buts *et al.*, 2007). Consequently, not every legume-lectin is involved in SA-mediated immunity, but can also initiate pathways including salt-stress tolerance and JA signalling, and be associated with both biotrophic and necrotrophic pathogen defence (Singh *et al.*, 2013; Zhang *et al.*, 2017*b*; Balagué *et al.*, 2017).

1.3.1 Legume Lectin-Like Proteins

Legume Lectin-like Protein 1 (LLP1) was identified in a proteomic analysis of apoplast-enriched extracts during ETI, and is induced both locally and systemically during SAR signalling (Breitenbach *et al.*, 2014). It is a glycoprotein with a predicted carbohydrate binding function, but no predicted enzymatic activity, and belongs to the lectin-legB family of legume lectin-like proteins (Vlot *et al.*, 2020). It is localised to the apoplastic side of the plasma membrane (Armijo *et al.*, 2013). Much of the research so far conducted on this protein has utilised the T-DNA insertion mutant, *llp1* (Breitenbach *et al.*, 2014). It was also shown that the presence of LLP1 is not required for PTI, and either unnecessary or having a very mild role in ETI, so its role in defence appears to be specific to systemic defence (Armijo *et al.*, 2013; Breitenbach *et al.*, 2014).

The accumulation of LLP1 is dependent on the key SA-signalling regulator, *EDS1* (Vlot *et al.*, 2009; Breitenbach, 2012; Breitenbach *et al.*, 2014). Thus it would initially appear that LLP1 is required for an SA-dependent pathway within SAR. However, local treatment with SA could still induce a systemic defence response in *llp1-1* (Breitenbach *et al.*, 2014), indicating that the presence of this protein is not necessary for SA-induced systemic defence. However, as described above, for biological SAR to occur two parallel signalling pathways are required (Vlot *et al.*, 2020). Pip is unable to induce SAR in *llp1-1* mutants, indicating that LLP1 is involved in the Pip rather than the SA branch of SAR (Wenig *et al.*, 2019).

Although *LLP1* transcript accumulation is elevated both locally and distally to an infection, LLP1 is required only in the systemic tissue, but not locally, for a functional SAR response to occur (Wenig *et al.*, 2019). Along with its apoplastic membrane localisation (Armijo *et al.*, 2013), LLP1 could be acting as the receptor for the mobile SAR signal in the systemic tissues. The predicted lectin domain introduces the

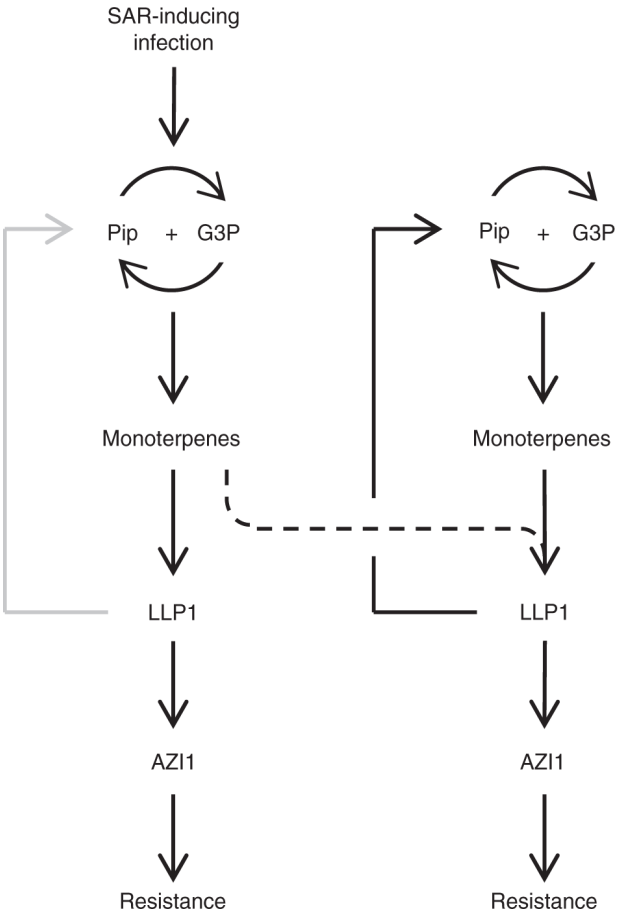

Figure 6: LLP1 is involved in a signalling feedback loop. A SAR-inducing local infection will initiate a signalling cascade involving Pip, G3P and monoterpenes. Downstream defence genes are regulated via AZI1. Monoterpenes can travel to neighbouring uninfected plants and upregulate defence in an LLP1-dependent manner. Adapted from Wenig et al. (2019).

possibility that LLP1 can recognise carbohydrate or free sugar molecules. However, as it lacks some of the features typically found in lectin binding domains, it is possible that the ligand of LLP1 is a different type of molecule, such as an amino acid or small lipid or peptide (Komath *et al.*, 2006; Vlot *et al.*, 2020). As many of the touted SAR signals, such as NHP, fall under this category, there are many different potential LLP1 binding partners.

Aside from its role in intra-plant SAR, LLP1 is also required for the emission and recognition of monoterpenes (see above) (Figure 6, Wenig *et al.*, 2019). After recognition of these molecules, LLP1 is crucial for initiating the pathway involving Pip and G3P needed to propagate further PTP signalling. This puts LLP1 as a key regulatory node between both phloem and airborne defence signals.

LLP1 has two homologues in the model plant *Arabidopsis*, *LLP2* and *LLP3*. LLP2 (At3g16530), which shares 66% similarity at the amino acid (AA) level with LLP1,

was identified as a possible SAR-associated protein along with LLP1 (Breitenbach et al., 2014). LLP1 and LLP2 respectively share 61% and 87% AA similarity with LLP3 (At3g15356; LECTIN in Lyou et al., 2009). Although some functions of LLP1 and LLP3 have been previously addressed (Lyou *et al.*, 2009*b*; Armijo *et al.*, 2013*a*), the combined effect of the proteins in different stresses is as yet unknown. The combined role began to be uncovered by previous work using a triple RNAi silencing line (*RNAi:LLP1-3*), in which the transcript levels of *LLP1*, *LLP2*, and *LLP3* were significantly reduced (Breitenbach, 2012; Wenig *et al.*, 2019). It has been shown that the *llp1-1* single mutant is unable to recognise mobile SAR signals. In *RNAi:LLP1-3*, it was shown that there is also a lack of signal generation as well as signal recognition (Wenig *et al.*, 2019). This gives us the first insight that in SAR the different LLPs are not functionally redundant, and at least LLP2 or LLP3 has a role differing from that of LLP1. Pabst (2017) also showed that there is an increased susceptibility in *RNAi:LLP1-3* to the necrotrophic pathogen *Alternaria brassicicola* compared to both wildtype and to the *llp1* single mutant. We therefore potentially see roles for these proteins in both biotrophic and necrotrophic defence. However, as discussed in Robert-Seilaniantz *et al.*, (2011), it is generally thought that a decrease in SA- or biotrophic defence should result in an increased tolerance to necrotrophic pathogens. Therefore, we are presented with a potential irregularity, as it appears that the transgenic lines in which the *LLPs* are compromised is susceptible to both systemic biotrophic and necrotrophic infection (Pabst, 2018). With this in mind, is it also possible that these mutants do not conform to the accepted dogma that when biotic stress tolerance is decreased, then abiotic stress tolerance will generally be improved.

1.4 Phytohormone crosstalk

When considering defence responses in a natural setting, it is rare that plants are only exposed to a single stressor. It is therefore of great importance to understand the crosstalk between SAR and other signalling cascades activated by abiotic and necrotrophic stress. The three key phytohormones that are associated with environmental stress responses are SA, ABA, and JA (Figure 7). It is generally thought that the overall balance between biotrophic, necrotrophic and abiotic stress

tolerance is dependent on these interactions, with an increase in one leading to susceptibility in the others (Robert-Seilaniantz *et al.*, 2011). This crosstalk would theoretically allow the plants to maximise their resource allocation, and ensure that fitness is compromised as little as possible while still activating sufficient defence (Mur *et al.*, 2005; Koornneef and Pieterse, 2008).

1.4.1 ABA defence signalling

ABA is a sesquiterpenoid hormone biosynthesised from zeaxanthin via a five-step process that takes place in plastids and then the cytoplasm (Xiong and Zhu, 2003; Hauser *et al.*, 2017). It is integral to signalling responses against a range of abiotic stresses including cold, drought, and high salinity (Christmann *et al.*, 2006), as well as in plant growth (Fernando and Schroeder, 2016). In a similar way to JA signalling, the transcription of ABA-responsive genes is repressed under standard conditions via the 2C-type protein phosphatase (PP2C)-mediated suppression of Sucrose non-fermenting Kinase-1-Related protein kinase 2s (SnRK2s). When ABA binds to one of its receptors (RCAR/PYR/PYL receptors), then as a complex they can bind to and inhibit PP2Cs, allowing SnRK2s to activate ABA-responsive element Binding Factors (ABFs) which in turn initiate transcription of ABA-responsive genes (Busk and Pagès, 1998; Ma *et al.*, 2009; Nishimura *et al.*, 2009). Depending on the type of stress response, different combinations of ABFs can initiate the appropriate downstream pathways (Choi *et al.*, 2000). As drought and salinity impart osmotic stress upon plant cells, ABA controls water balance via stomatal closure and cellular dehydration tolerance to enhance cell survival (Fernando and Schroeder, 2016). Downstream components that indicate an ABA-mediated abiotic stress response include the dehydrin family proteins RAB18 and RD29b (Pandey *et al.*, 2005).

1.4.2 ABA-SA crosstalk

Due to its role in abiotic stress tolerance, and the frequency with which plants experience this concurrently with disease, the crosstalk between ABA and SAR can be expected to have significant impact on the effectiveness of systemic defence after experiencing simultaneous environmental pressures.

The closure of stomata and callose deposition to reduce pathogen spread during biotic stress are mediated by ABA signalling. Stomatal closure, especially, is a very early

response to pathogen interaction, and in this context ABA supports biotic defence (Melotto *et al.*, 2006; Hewage *et al.*, 2020). In fact, a key virulence mechanism of *Pst* is to overcome ABA by producing coronatine to target guard cell ROS production and so to reopen stomata (Toum *et al.*, 2016). However, ABA can also have a general antagonistic effect on biotic defence by reducing SA accumulation and the expression of SA-mediated defence genes, and by disrupting JA-mediated defence against necrotrophs (Figure 7) (Spoel and Dong, 2008; de Torres Zabala *et al.*, 2009; Sánchez-Vallet *et al.*, 2012). This is also used as a virulence mechanism by *Pst* to suppress PTI (de Torres-Zabala *et al.*, 2007).

ABA and SAR are mutually antagonistic. In their 2008 paper, Yasuda et al. demonstrated that the induction of SA-mediated SAR in *Arabidopsi*s by the inducers BTH and BIT is directly inhibited both upstream and downstream of SA synthesis by the application of exogenous ABA, independently of JA or ethylene perception. Although the direct point of interaction is unknown, ABA has been shown to increase catalase activity in some cell types, and thereby increase the scavenging of free radicals (Jannat *et al.*, 2011). This could reduce the activation of SAR via ROS signalling (Wang *et al.*, 2014). However, the specific catalase enzymes affected, and in which way, also depends on whether ABA is applied exogenously or endogenously (Tan *et al.*, 2019), and so this mode of action when the plant is also being acted upon by pathogens is debatable. ABA also enhances the proteasome-mediated degradation of the SA-receptor NPR1, which is also required for a normal SAR response (Cao *et al.*, 1997; Mou *et al.*, 2003; Ding *et al.*, 2016*a*).

Pathways activated after salinity stress were also implicated in this ABA-SA/BTH antagonism. Plants with accelerated ABA degradation did not show repression of BTH and BIT-induced genes after NaCl treatment. Ergo the repression of SA/BTH signalling by NaCl is dependent on ABA. The converse effect was also observed, as a BIT pre-treatment reduced the expression of ABA-responsive and –biosynthetic genes after salt treatment, indicating that not only is ABA-mediated abiotic stress able to suppress SAR, but SAR is also able to suppress ABA-mediated stress responses (Yasuda *et al.*, 2008). It appears that this crosstalk is specific to BTH-induced systemic resistance rather than SA signalling in general, as BTH and BIT

showed different levels of ABA antagonism compared to a direct SA treatment in plants under high humidity (Moeder *et al.*, 2010). This crosstalk between SAR and ABA signalling is not limited to *Arabidopsis*. The SA-mediated SAR inducers BTH and BIT were able to reduce the susceptibility towards *Pst* and *Phytophthora capsici* incurred by ABA-mediated salt stress in tomato (Pye *et al.*, 2013, 2018), and ABA pre-treatment suppressed both SAR and SA accumulation induced by BIT treatment (Figure 7) (Kusajima *et al.*, 2017).

Components of the SA-independent SAR induction pathway have also been associated with ABA-mediated abiotic stress responses. The lipid transfer protein AZI1 is required for both SAR and priming (Jung *et al.*, 2009; Yu *et al.*, 2013), but when overexpressed confers drought susceptibility, and is upregulated in some ABA mutants (Atkinson *et al.*, 2013). Conversely, AZI1 appears to improve freezing tolerance and salt tolerance through an ABA-dependent mechanism (Xu *et al.*, 2011; Pitzschke *et al.*, 2014).

ROS signalling, which is critical in several SAR pathways, is also an important trigger in salt stress tolerance, as after extended periods of high salinity plant cells begin to undergo ionic stress as well as osmotic stress, and show disrupted ion fluxes and homeostasis (Miller *et al.*, 2010). The transcription factor WRKY33, which is linked to the Pip/ MPK3/6 feedback loop, has a positive role in salt tolerance, and its induction is dependent upon ABA signalling (Jiang and Deyholos, 2009), although this seems to be specific to *Arabidopsis* (Bao *et al.*, 2018). This could point to a difference of ABA interaction with each of the SAR pathways, showing antagonism towards SA-signalling while theoretically being able to support local Pip feedback loops.

1.4.3 SA-JA crosstalk

As a major phytohormone, JA is involved in a wide variety of stress responses in both positive and negative regulatory capacities (see section on JA in defence).

SA and JA generally show antagonism towards one another (Figure 7) (Glazebrook, 2005). For instance, if SA accumulation is compromised, then JA responsive genes will be expressed at higher levels, indicating that they are normally repressed by SA. SA-antagonism of JA occurs both through reduction in JA biosynthesis and directly at

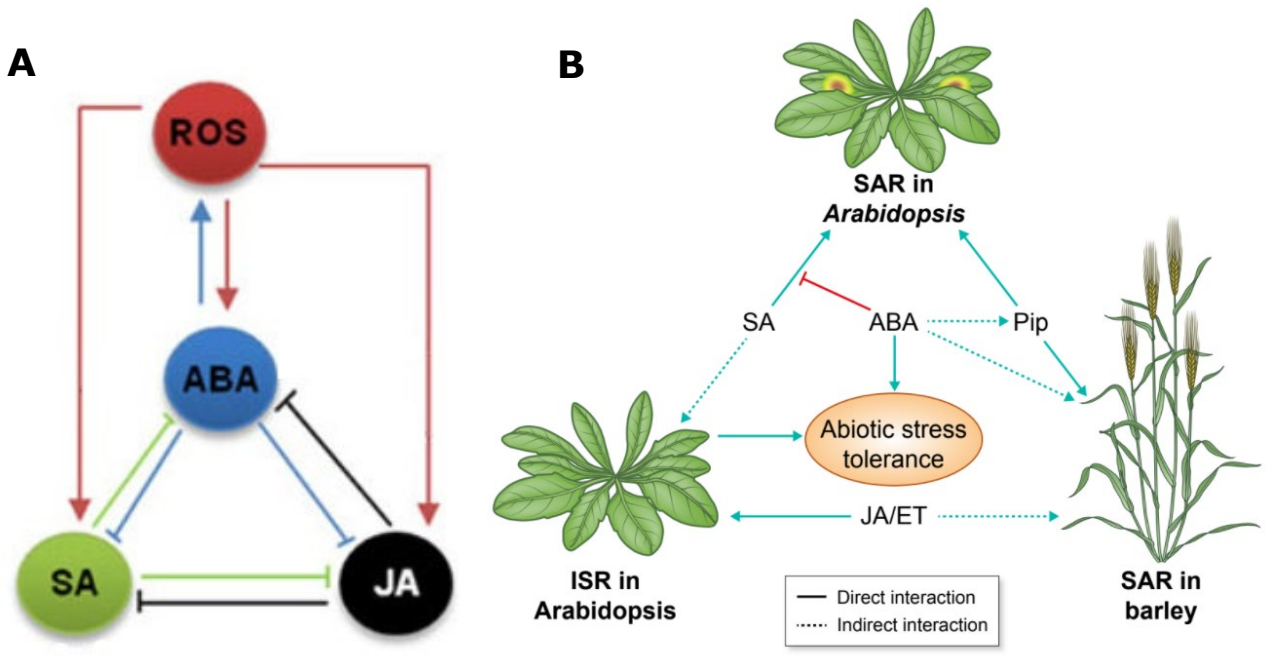

Figure 7: Phytohormone interactions are complex and control different stress responses. A) There is generally thought to be antagonism between the three major stress hormones, SA, JA and ABA, although ROS signalling plays a role in all of these pathways. B) Although some mechanisms are conserved, key signalling pathways have different levels of impact when considering SAR in *Arabidopsis* compared to monocotyledonous species. In a similar way, some, but not all signalling pathways are required for both SAR and ISR in *Arabidopsis*. ABA negatively regulates the SA-dependent pathway but may contribute to the Pip pathway in *Arabidopsis* and SAR in barley. Adapted from Carvalho *et al*. 2015 and Vlot *et al.* 2020.

the downstream gene transcriptional level. This repression requires the SA receptor NPR1 and TGA transcription factors. As the same TGAs can target the promoter of *ORA59*, itself a key transcription factor in JA signalling, this may be the mechanism through which NPR1-dependent SA signalling can antagonise the ERF1-mediated JA pathway (Zander *et al.*, 2014). Alternatively, the presence of a GCC-box in the promoter of JA-responsive genes is sufficient for SA repression of JA signalling. As this motif is present in the promoters of several JA-regulated genes, and would otherwise be bound to by JA-mediated transcription factors, SA may repress JA through the repression of transcriptional activation (Zarei *et al.*, 2011; Van Der Does

et al., 2013). Interestingly, NPR1 is normally required in the nucleus to propagate the SA signal, but for JA-repression it is only required in the cytoplasm, suggesting that NPR1 can perform different roles depending on its subcellular localisation (Spoel *et al.*, 2003). The cytoplasmic function of NPR1 may be important in sensing the JA signal necessary for ISR, and could act as the integration point between JA and ET signals in that pathway (Leon-Reyes *et al.*, 2009). The ERF1 branch of JA-singalling may be affected by the positive regulation of EDS1 on ICS1 and thus SA levels. SA is involved in promoting the downstream degradation of ORA59, and thus reducing the transcription of ORA59/JA-responsive genes (Van Der Does *et al.*, 2013; Cui *et al.*, 2018). The inhibition of MYC2 by EDS1 blocks COR/JA signalling, and thus protects the SA defence sector.

Another point of crosstalk between SA and JA is the transcription factor WRKY33. This gene is required for necrotrophic defence against *B. cinerea,* and loss of function results in overactivation of the SA pathway (Birkenbihl *et al.*, 2012). WRKY33 also directly targets redox homeostasis, salt tolerance, and Pip signalling in local tissues during SAR. In Pip feedback regulation WRKY33 is directly targeted by MPK3/MPK6, which also have positive regulatory functions on MYC2 signalling (Wang *et al.*, 2018*b*). Thus, the functions of MPK3/MPK6 and WRKY33 could present a key node for integration of signals from different hormone pathways.

1.4.4 SA-JA crosstalk in SAR

It has generally been acknowledged that the basal SA signals that are needed for SAR initiation are mutually antagonistic with JA (Glazebrook, 2005), but this is not so clear cut regarding systemic defence. Potatoes show an accumulation of the JA precursor OPDA after a SAR inducing infection with *P. syringae* pv *maculicola* (Landgraf *et al.*, 2002), and both SA and JA are required for defence against Tobacco Mosaic Virus in *Nicotiana benthamiana* (Zhu *et al.*, 2014). In *Arabidopsis* there are contradicting reports on the necessity of JA for SAR (Truman *et al.*, 2007; Attaran *et al.*, 2009). It also seems to be the case that while a SAR-inducing *Pst* infection confers susceptibility to necrotrophic pathogens via JA signal suppression at the local level, systemically there is neither an increase nor a decrease in susceptibility. This is possibly because the systemic SA accumulation associated with SAR is not high

enough to overcome the threshold needed for SA-JA antagonism to occur (Spoel *et al.*, 2007).

Some pathogens, such as the necrotrophic fungus *Botrytis cinerea*, have the capacity to co-opt SAR pathways as a pathogenesis mechanism to repress and therefore evade JA defence in tomato and tobacco (El-Oirdi *et al.*, 2011; Frías *et al.*, 2013). On the other hand, biotrophic pathogens are able to induce JA signalling as a virulence mechanism for suppressing PTI. A key example of this is seen in *Pst,* which is able to produce the JA-Ile mimic, COR (Zhao *et al.*, 2003; Uppalapati *et al.*, 2007). This binds to the COI1 receptor, and activates JA-mediated responses (Katsir *et al.*, 2008), in turn mitigating SA-mediated defence and allowing *Pst* to propagate more freely. *Pst* can also directly induce JA signalling independently of COI1 through effectors such as HOPX1 and HOPZ1 (Gimenez-Ibanez *et al.*, 2014).

It has long been a consideration in plant breeding that lines with higher defence capacities show a corresponding deficit in growth promotion, and vice versa (Huot *et al.*, 2014). Crosstalk between SAR and other defence pathways, and the differences between these pathways in dicotyledonous and monocotyledonous species adds further complexity to these considerations (Figure 7). These are factors that must therefore be taken into account when researching the potential applications of SAR elicitors in agriculture.

1.5 Aims of this work

The main aim of this work is to further investigate the roles of Legume Lectin-like Proteins 1, 2 and 3 in plant defence responses. Because data in a prior thesis (Pabst, 2017) showed that reduced transcript accumulation of *LLP1, LLP2,* and *LLP3* influences seedling responses to salt stress, I particularly aim to characterise the role of these genes in abiotic stress responses. This work also aims to put the *LLPs* in the context of phytohormone signalling crosstalk. The interactions of local biotrophic defence with other defence pathways has been widely studied. However, the interaction of SAR specific pathways with JA- and ABA-mediated defence is less clearly defined. The known role of *LLP1* in SAR signalling in systemic tissues and not

local defence may therefore help to situate systemic defence signalling in the wider landscape of phytohormones.

llp1-1 and *llp3* will be used to investigate the roles of the *LLPs* in biotrophic defence, both locally against avirulent *Pst* and during SAR. In order to study the joint roles of the *LLPs,* as well as potentially shed light on the function of *LLP2*, the triple silencing line *RNAi:LLP1-3,* in which the transcript accumulation of all three *LLPs* is significantly reduced, will also be used for all assays.

Specifically, I will look at how the *llp1-1, llp3* and *RNAi:LLP1-3* lines respond to salt stress at different development stages, and how they are affected at both a phenotypic and transcriptional level. I will also investigate the association between the *LLP* accumulation and JA and ABA, as well as the requirements for JA in systemic defence signalling.

I will mainly investigate these questions by using local defence, SAR and root growth assays. A variety of different mutant lines will also be used to identify the importance of the *LLPs* to known phytohormone cascades. To delve further into the molecular aspect of these questions, I will use quantitative PCR, and LC-MS to see the effect of the *LLPs* on phytohormone accumulation during the defence responses. Because no secondary line existed for *llp3*, this project aimed to generate a complementation line in order to explore the individual contribution of *LLP3* to defence in depth.

By using these approaches, I aim to unravel the role of *LLP1, 2* and *3* in phytohormone signalling and crosstalk. This will allow a better understanding of how plants respond to simultaneous stress, and will inform future possible application of systemic defence elicitors in plant protection.

2. Materials and Methods

2.1 Materials

2.1.1 Plant materials

A. thaliana ecotype Columbia-0 (Col-0) was used as the wildtype control throughout all experiments. Transgenic lines *llp1-1, eds1-2, ald1, ggpps12, tps24-1, tps24-2,* and *RNAi:LLP1-3* have been described previously (Mishina and Zeier, 2006; Bartsch *et al.*, 2006; Breitenbach *et al.*, 2014; Wenig *et al.*, 2019). The TDNA insertion line llp3 (SALK_030762, previously *lec1*) was obtained from the Nottingham *Arabidopsis* Stock Centre (NASC) and bred to homozygosity in a previous project (thesis of Elizabeth Pabst). All seeds were stored in dark, dry conditions at ambient room temperature.

2.1.2 Microorganisms and culture conditions

Table 2: Bacteria used in experiments

Species	**Strain**
Pseudomonas syringae pathovar Tomato (Pst)	DC 3000
	AvrRpt2
	AvrRpm1
Agrobacterium tumefaciens	GV3101::pMP90

Table 3: Antibiotics used in this work

Antibiotic	**Working Solution**	**Manufacturer**	**Use**
Carbenicillin	250 µg/ml	Sigma Aldrich, Germany	Sterile plant media
Cefotaxim	100 µg/ml	Applichem, Germany	Sterile plant media

Gentamycin	25 µg/ml	Roche, Germany	Selection of Agrobacterium tumefaciens GV3101::pMP90
Kanamycin	50 µg/ml	Roth, Germany	Selection of *Pseudomonas syringae pv tomato* (virulent and avirulent strains)
Rifampicin	100 µg/ml	Duchefa Bichemie, Germany	Selection of *Pseudomonas syringae pv tomato* (virulent and avirulent strains) and *Agrobacterium tumefaciens* GV3101::pMP90

All antibiotics were stored in powdered form at +4°C. To produce working concentration, all antibiotics (except Rifampicin) were dissolved in double distilled H_2O. Rifampicin was dissolved in 100% DMSO. Solutions were sterile filtered using a 0.22 µM filter (Millipore, Billerica, United States) and either stored at +4°C and used within 24 hours or aliquoted and stored at -20°C.

2.1.3 Chemicals, Solutions and Media

Table 4: Chemicals used in this work

Chemical	**Manufacturer**
Abscisic Acid	Sigma-Aldrich (St. Louis, USA)
Azelaic Acid	Sigma-Aldrich (St. Louis, USA)
BASTA® (Glufosinate)	Hoechst (Frankfurt, Germany)
Indole-3-Acetic Acid	Roth (Karlsruhe, Germany)
Lithium Chloride	Sigma-Aldrich (St. Louis, USA)
Mannitol	Roth (Karlsruhe, Germany)
MES (2-(N-morpholino)ethanesulphonic-acid)	Roth (Karlsruhe, Germany)
Methyl Jasmonate	Sigma-Aldrich (St. Louis, USA)
Murashige-Skoog + vitamins	Duchefa (Haarlem, Netherlands)
Phytoagar	Duchefa (Haarlem, Netherlands)
Salicylic Acid	Roth (Karlsruhe, Germany)

Silwet	Lehle Seeds (Texas, USA)
Sodium Chloride	Roth (Karlsruhe, Germany)
Tween-20	Calbiochem (San Diego, USA)

Table 5: Solutions and their compositions used in this work

Buffer/solution	**Composition**	**Use**
Floral dip solution	5% sucrose 0.03% silwet Sterile H_20	Suspending *Agrobacterium tumefaciens* for *Arabidopsis* floral dip
Bacterial isolation solution	10 mM $MgCl_2$ 0.01% Silwet	Isolating bacteria from leaves post-harvest
Mock buffer	10 mM $MgCl_2$	Mock control treatment for infiltration experiments
RNA-extraction buffer	3.05 g ammonium thiocyanate, 9.44 g guanidinium thiocyanate, 5 ml glycerol, 3.33 ml 3 M sodium acetate pH 5.2, 40 ml H2O, adjust pH to 5.0, 38 ml Roti-Aqua-Phenol	RNA extraction
RNA-extraction further solvents	Phenol Chloroform 2-Propanol Ethanol	RNA extraction

Table 6: Media composition used in this work

Media	**Composition**	**Use**
LB	10 g tryptone 5 g yeast extract 10 g NaCl add 1 L H_2O, adjust pH to 7 15 g agar-agar	*Agrobacterium tumefaciens* culture
Murashige-Skoog (MS)	4.302g Murashige & Skoog + vitamins 10g Sucrose 0.5g MES add 1 L H_2O, adjust pH to 5.8	Sterile plant culture

	12 g Phytoagar	
NYGA	5 g proteose peptone 3 g yeast extract 20 ml glycerol add 1 L H_2O, adjust pH to 7 18 g agar-agar	*Pseudomonas syringae pv tomato* culture

2.1.4 Molecular Biology materials

Table 7: Kits used for transcript accumulation quantification

Kit	**Manufacturer**	**Use**
SensiMix SYBR Low-Rox Kit	Bioline Reagents, London, UK	qPCR
SuperScript II Reverse Transcriptase	Invitrogen/ Thermo Fisher Scientific, Waltham, USA	cDNA synthesis

Table 8:Primers for genotyping

Name	**Sequence 5' → 3'**	**Description**	**T_m(°C)**
LLP1F	TGAGTAAACAGCAGTTACGA	LLP1 expression	60
LLP1R	TGACGCCATCAGAAGCAGGA	LLP1 expression	60
LEC1F	TTTGGAGCTGGTCGTTTGAA	LLP3 expression	60
LEC1R	ATTCACTCTACAACAATTAC	LLP3 expression	60
LEC2F	TTTGGAGCTGGTCGTTTGAA	LLP2 expression	60
LEC2R	AGTTACCACTGAGTAGTATG	LLP2 expression	60
LLP3-check2F	ACCGAAGAGGCCTTTGATCC	LLP3 expression (without 3'UTR)	60
LLP3-check2R	ACCAGCAAAACCAGCGTACA	LLP3 expression (without 3'UTR)	60

Table 9: Primers for qPCR

Name	Sequence 5′ → 3′	Description	T_m(°C)
UbiqF	AGATCCAGGACAAGGTATTC		60
UbiqR	CGCAGGACCAAGTGAAGAGTAG		60
PR1F	CTACGCAGAACAACTAAGAGGCAAC		60
PR1R	TTGGCACATCCGAGTCTCACTG		60
RAB18F	TTCGGTCGTTGTATTGTGCTTT		60
RAB18R	CCAGATGCTCATTACACACTCATG		60
PDF1.2F	CCAAGTGGGACATGGTCAG		60
PDF1.2R	ACTTGTGTGCTGGGAAGACA		60
VSP2F	GTTAGGGACCGGAGCATCAA		60
VSP2R	AACGGTCACTGAGTATGATGGGT		62

2.1.5 Other materials

Table 10: Devices and Instruments

Instrument	Model	Manufacturer
Camera	Nikon DC 300	Minato, Japan
Centrifuges	Heraeus 17	Thermo Fisher Scientific, USA
Photometer	NanoPhotometer	Implen GmbH, Germany
RT-qPCR cycler	Applied Biosystems 7500 Fast Real-Time PCR system (ABI 750 Fast)	Applied Biosystems, Freiburg, Germany
Rotator	intelli-mixer rotator with vortexer	Neolab (Heidelberg, Germany)

Table 11: Software and web applications used in this work.

Software	Version	Use
7500 Fast System SDS Software	Version 1.3.1.21	qPCR programming and raw data analysis

Camera Control Pro 2	Version 2.0.0	Settings and image acquisition for Nikon DC 300
GraphPad	GraphPad Prism 8 for Windows	Statistical data analysis and graph design
ImageJ	Version 1.52k	Image analysis
Microsoft Office	Excel, Powerpoint, Word,	Data management and analysis, graph design
Primer Blast	https://www.ncbi.nlm.nih.gov/tools/primer-blast/ (Ye et al. 2012)	qPCR primer design

2.2 Methods

2.2.1 Plant growth conditions

Synchronised seeds and repeats from different batches were used where possible, to reduce batch effect. Plants were grown on potting soil (without fertilizer) (Floradur Anzuchtsubstrat B Seed, Floragard) mixed with silica sand in 5:1 ratio. Seeds were placed on pre-dampened soil, covered with clingfilm, and stratified for 2 days at 4°C to enhance the synchronicity of germination. For experimental procedures, plants were kept under short day conditions (10 hours (h) light with an intensity of 100 µE m^{-2} s^{-1} at 22°C and 14 h dark at 18°C, 70% relative humidity). For seed collection, plants were kept under long day conditions (18°C/22°C (night/day), 70% relative humidity, and 80-90 µE m-2 s-1 of light for 14 h days).

2.2.2 Seed sterilisation

To sterilise *Arabidopsis* seeds, old spin columns (e.g. from plasmid purification) were sterilised using 75% EtOH. After columns were dried and labelled using pencil or alcohol-resistant markers, seeds were added to the top part of the column. 700 µl 75% EtOH was added on top of the seeds, and columns were closed and rotated for 4 minutes. Columns were subsequently placed in a centrifuge and spun at 11,000 rpm for 1 minute. The excess EtOH was decanted, and 700 µl 100% EtOH was pipetted to the top of the column. Columns were again rotated for 4 minutes, then centrifuged for 1 minute. After the excess EtOH was again decanted, seeds were finally dried by centrifuging for 5 minutes. Seeds were then sown onto sterile plant media, and stratified for 2 days at 4°C before being transferred to growth chambers.

2.2.3 Microbial culture conditions

Pseudomonas syringae pv tomato (henceforth referred to as *Pst* or DC3000) and the avirulent strains *Pst AvrRpm1* and *Pst AvrRpt2* were grown on NYGA medium containing rifampicin and kanamycin (Table 3, Table 6). For experimental purposes, plates were inoculated and grown overnight at 28°C.

Agrobacterium tumefaciens strain GV3101::pMP90 was cultured on solid LB medium plates containing rifampicin and gentamycin (*Table 3*, *Table 6*) and grown at 28°C.

2.2.4 Local bacterial growth assay

Pst AvrRpt2 was maintained on NYGA media supplemented with antibiotics (Table 3, Table 6, Aarts *et al.*, 1998). Fresh plates were inoculated the previous day, and the bacteria were allowed to grow over night before infection at 28°C. For local immunity assays, the first two true leaves per plant were syringe-infiltrated with $1x10^5$ colony forming units (CFU)/mL of *Pst AvrRpt2* suspended in 10 mM $MgCl_2$. To measure the bacterial concentration, leaf tissue were either harvested at 2 days post infection to analyse transcript accumulation or at 2, 4 and 7 days post infection to analyse bacterial titres as described (Section 2.2.6, Breitenbach *et al.*, 2014).

2.2.5 SAR assay

In order to assess the role of different proteins in SAR, the following assay was performed with wildtype (Columbia-0) and mutant or transgenic plants. *Pst* and *Pst AvrRpm1* were maintained as described above (2.2.4). The evening before the initial infection, NYGA plates with antibiotics were freshly inoculated with the desired bacteria and incubated at 28°C overnight. On the day of the first infection, bacteria were removed from the plates and suspended in a 10 µM $MgCl_2$ solution using a vortex until there were no clumps of bacteria remaining. The OD_{600} of the solution was then measured using a spectrophotometer, and the stock solution was diluted to a concentration of $1x10^6$ CFU/ml. 4.5 week old *Arabidopsis* plants were syringe infiltrated in their first two true leaves (leaves 1 and 2 in Figure 8) with the *Pst AvrRpm1* solution, or with a 10 µM $MgCl_2$ mock control. The plants were then returned to an infection chamber under the same conditions as the growth chamber. Three days later, bacteria were prepared in a similar manner and two systemic leaves (leaves 3 and 4 in Figure 8) were syringe infiltrated with $1x10^5$ CFU/mL of *Pst,* again suspended in $MgCl_2$ solution, in all the plants that had previously been treated, regardless of whether the initial treatment was with *Pst AvrRpm1* or $MgCl_2$. The plants were again returned to the infection chambers in order for the bacteria to grow. 4

days after the secondary infection, leaves 3 and 4 were harvested, and the resulting *in planta* bacterial titres were determined as described (Breitenbach *et al.*, 2014).

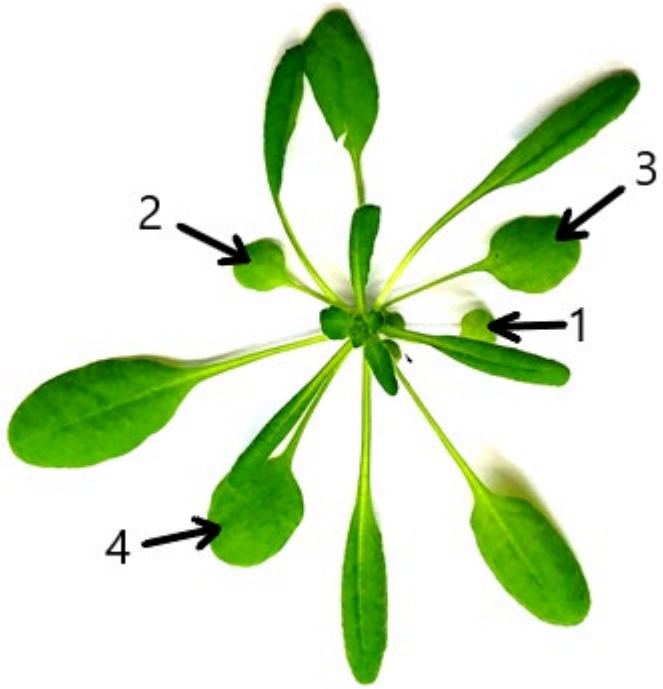

Figure 8: An *Arabidopsis* plant with indication towards the leaves infiltrated in a SAR assay.

2.2.6 Bacterial quantification from leaf tissue

To estimate the bacterial growth after various experimental treatments, leaf tissue was harvested at the time points described above. Leaf discs were cut using a leaf punch, and three punches from different plants were included per sample, to reduce the effect of external factors acting on a single individual plant. Leaf discs were then submerged in 500 µl of bacterial isolation solution in a 2 ml Eppendorf tube (Table 5) and shaken at 600 rpm at 26°C or ambient room temperature for 1 hour. The eppendorfs were then vortexed for 5 seconds to resuspend any clumps of bacteria that may have formed, and the solution was serially diluted by a factor of 10 five times in a $MgCl_2$ solution. 20 µl of each dilution was plated onto half a fresh NYGA plate (Figure 9), and the plates were allowed to completely dry. Bacteria were allowed to grow at ambient room temperature for 48 hours, before colonies were counted under a light microscope. Using the number of colonies and the dilution factor, the bacterial titre in the original leaf tissue was calculated.

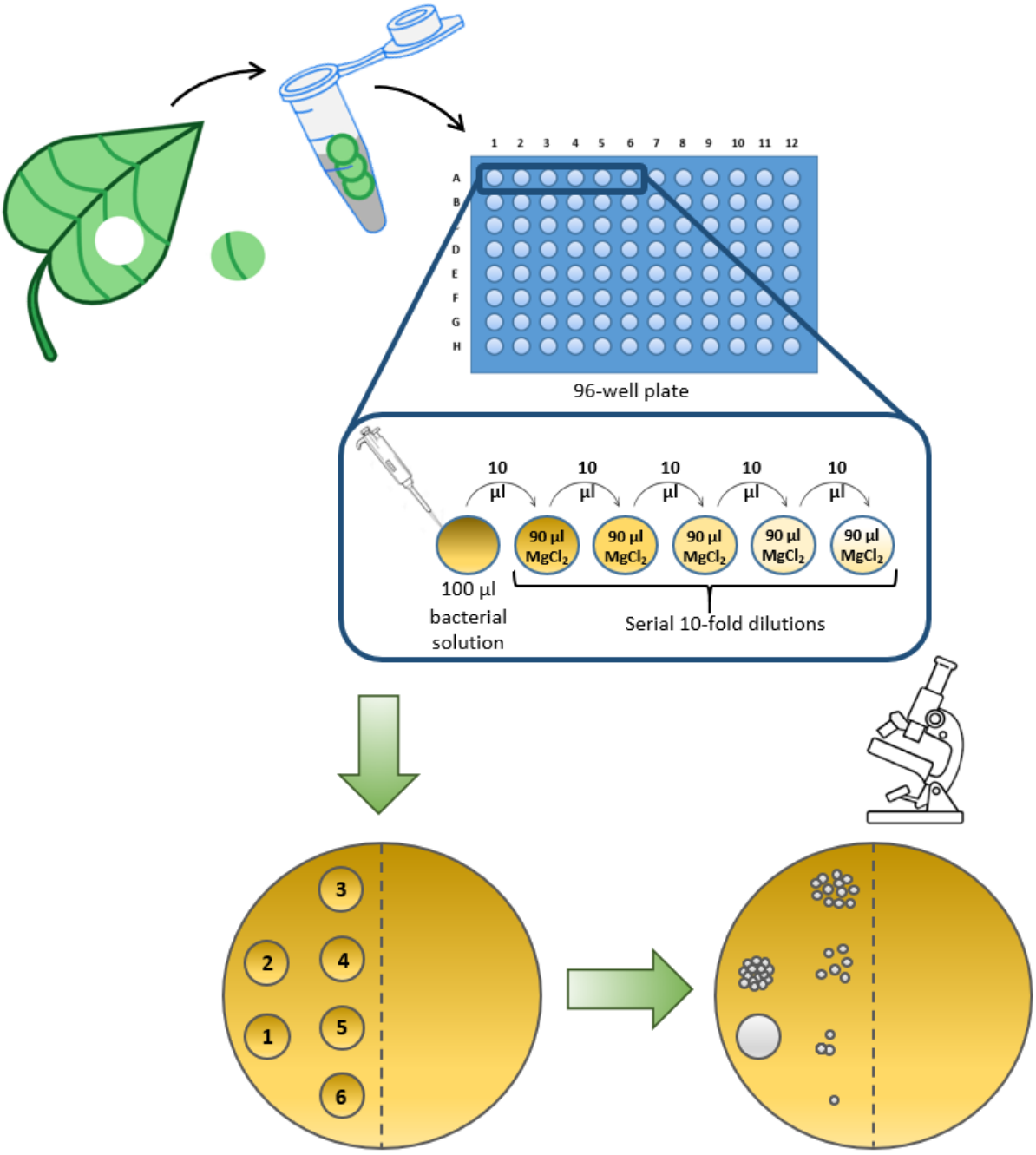

Figure 9: Quantification of bacterial titres in leaves. Leaf discs from three plants were shaken in bacterial isolation solution, before undergoing a serial dilution. 20 µl of each dilution was plated on NYGA and the colonies were counted after 2 days incubation at room temperature. The number of colonies and the dilution factor could then be used to calculate the bacterial titres in the original leaf tissue per cm^2.

2.2.9 Phytohormone treatments

To analyse *LLP1-3* transcript accumulation in response to phytohormone treatment, green tissues of 2- to 3-week old plants were sprayed until drop-off with 1 mM SA (Sigma Aldrich), 100 µM MeJA (Sigma Aldrich), or 100 µM ABA (Sigma Aldrich) dissolved in 0.1% $MgCl_2$, 0.01% Tween® 20, and 0.025% MeOH. Plants of the same age were sprayed with 0.1% $MgCl_2$, 0.01% Tween® 20, 0.025% MeOH as the mock control treatment. Leaf samples were taken at 8 and 24 h after treatment and flash frozen in liquid N_2 for qPCR analysis.

2.2.10 Phytohormone content measurement

ABA in seedlings was measured as described in Avramova *et al.*, (2018).

ABA, SA and JA in mature plants was measured by means of UHPLC-MS/MS. Thereby, the plant material (50-250 mg) was placed in 2 mL bead beater tubes (CKMix-2 mL, Bertin Technologies, Montigny-le-Bretonneux, France), filled with ceramic balls (zirconium oxide; mix beads of 1.4 mm and 2.8 mm). An aliquot of the internal standard (20 µL), containing (+)cis,trans-abscisic acid-d_6 (2.5 µg/mL), salicylic acid-d_4 (2.5 µg/mL) and (-)trans-jasmonic acid-d_5 (25 µg/mL), in acetonitrile was added to the plants and incubated for 30 min at room temperature. After the addition of ethyl acetate (1 mL) and extractive grinding (3 × 20 s with 40 s breaks; 6000 rpm) while using a bead beater (Precellys Homogenizer, Bertin Technologies, Montigny-le-Bretonneux, France) the supernatant was membrane filtered (0.45 µm), evaporated to dryness, resolved in acetonitrile (70 µL) and injected into the LC−MS/MS-system (2 µL).

For LC−MS/MS analysis a QTRAP 6500$^+$ mass spectrometer (Sciex, Darmstadt, Germany) was used to acquire electrospray ionization (ESI) mass spectra and product ion spectra. Negative ions were detected in the scheduled multiple reaction monitoring (MRM) mode at an ion spray voltage at −4500 V (ESI$^-$) and the following ion source parameters: curtain gas (35 psi), temperature (550°C), gas 1 (55 psi), gas 2 (65 psi), collision activated dissociation (−3 V), and entrance potential (−10 V). Positive ions were detected in the scheduled multiple reaction monitoring (MRM) mode at an ion spray voltage at 4500 V (ESI$^+$) and the following ion source

parameters: curtain gas (35 psi), temperature (550°C), gas 1 (55 psi), gas 2 (65 psi), collision activated dissociation (3 V) and entrance potential (10 V). The column oven temperature was adjusted to 40°C.

For analysis of ABA, SA and JA, the MS/MS parameters were tuned to achieve fragmentation of the $[M-H]^-$ and $[M+H]^+$ molecular ions into specific product ions: $[M-H]^-$: (+)cis,trans-abscisic acid-d_0 263→153 (quantifier) and 263→219 (qualifier), (+)cis,trans-abscisic acid-d_6 269→159 (quantifier) and 269→225 (qualifier), salicylic acid-d_0 137→93 (quantifier) and 137→65 (qualifier), salicylic acid-d_4 141→97 (quantifier) and 141→69 (qualifier); $[M+H]^+$: (-)trans-jasmonic acid-d_0 211→133 (quantifier) and 211→151 (qualifier), (-)trans-jasmonic acid-d_5 216→155 (quantifier) and 216→173 (qualifier). For tuning experiments, acetonitrile/water (1/1; v/v) solutions of each analyte and internal standard were introduced by means of flow injection using a syringe pump.

Chromatography was performed by means of an ExionLC UHPLC system (Shimadzu Europa GmbH, Duisburg, Germany) consisting of two LC pump systems ExionLC AD, an ExionLC degasser, an ExionLC AD autosampler, an ExionLC AC column oven – 240 V, equipped with a Kinetex F5 column (100 × 2.1 mm, 100 Å, 1.7 *µ*m, Phenomenex, Aschaffenburg, Germany) and an ExionLC controller. Operated with a flow rate of 0.4 mL/min using 0.1% formic acid in water (v/v) as solvent A and 0.1% formic acid in acetonitrile (v/v) as solvent B, chromatography was performed with the following gradient: 0% B held for 2 min, increased in 1 min to 30% B, in 12 min to 30% B, increased in 0.5 min to 100% B, held 2 min isocratically at 100% B, decreased in 0.5 min to 0% B, held 3 min at 0% B. Data acquisition and instrumental control were performed using Analyst 1.6.3 software (Sciex, Darmstadt, Germany).

2.2.11 Root growth assay

For root growth inhibition measurements, seedlings were sterilised as described in 2.2.2, dried, and sown on 1x Murashige Skoog medium including vitamins (Duchefa) with 0.1% Cefotaxim (Acros Organics) and 0.25% Carbenicillin (Roth) (see *Table 3*, *Table 6*). Seedlings were transferred after 6 days to treatment plates containing either 10 µM ABA, 100 mM NaCl (Sigma-Aldrich), 40 µM MeJA, 20 or 40 µM IAA, 25 µM LiCl, or 180 mM mannitol (Sigma-Aldrich), or to control MS plates. All plates were placed

upright in the growth chamber under long day conditions, and the seedlings were photographed 6 and 12 days post-transfer. Root length was measured using ImageJ, and the length on the treatment plates was normalised to the mock control for each genotype. The seedlings were harvested, pooled per genotype and treatment, and flash frozen in N_2 for RNA extraction.

2.2.11 Salt pouring assay

Plants were watered with distilled water or 300 mM NaCl three times with four day intervals starting from 4 weeks after germination until they had been treated for a period of 12 days. Leaf tissue was harvested 4 days after the final salt treatment, weighed to assess the impact on biomass, and flash frozen in liquid N_2.

2.2.12 Gene expression analysis

RNA extraction

Either leaf tissue or whole seedlings were collected and flash frozen in liquid N_2 following experimental procedures. Frozen samples were then ground to a fine powder to allow for more efficient RNA extraction. After grinding, samples were resuspended in 1 ml chilled extraction buffer (*Table 5*), and vortexed to ensure thorough dispersion. Samples were shaken at 8°C for 10 minutes at 1300 rpm. 400 µl ice cold chloroform was added to the samples and incubated on ice for 3 minutes before being centrifuged for 20 minutes at 13000 rpm at 4°C. The upper phase was then pipetted off, and added to 500 µl chloroform, and again incubated on ice for 3 minutes before being centrifuged for 20 minutes at 13000 rpm at 4°C. The upper phase was again collected and added to 250 µl 2-Propanol, and incubated on ice for 15 minutes, before centrifugation for 20 minutes at 13000 rpm at 4°C. The 2-Propanol was decanted, and the RNA pellet washed twice with 70% EtOH before being resuspended in Licrosolv water, and the concentration determined by measuring the absorption at 260 nm and 280 nm using a nanodrop spectrophotometer (*Table 10*). RNA was stored at -80°C before analysis.

cDNA synthesis

cDNA was synthesised from RNA using SuperScript II reverse transcriptase following the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, USA). The amount of RNA used for cDNA preparation was dependent on the number of qPCR samples required.

qPCR

To analyse RNA transcript abundance, reverse transcription quantitative PCR (RT-qPCR) was utilized. A fluorescent dye (in this case SYBR green from the SensiMix Low-Rox kit) binds to double stranded DNA as it is formed during the PCR reaction. The resulting complex emits light at 520 nm. As the PCR product increases, so does the resulting fluorescent signal, allowing the amount of DNA to be quantified in real time. cDNA was used as the target for the qPCR, to analyse only sequences that had been transcribed into RNA. This allowed quantification of accumulation of the transcripts of genes of interest (see *Table 9*). Ubiquitin was used as a standardised housekeeping gene against which other genes were quantified. qPCRs were performed using 7500 Real Time PCR System from Applied Biosystems (Darmstadt, Germany) and initial analysis was performed using 7500 Fast System SDS software (see *Table 10*, *Table 11*).

2.2.13 Complementation line generation

Agrobacterium tumefaciens containing transgenic constructs in which *LLP3* was expressed under its native promoter were generated during the doctoral thesis of Elisabeth Pabst. These were then used as the starting point for transgenic line generation.

Floral Dip

Arabidopsis plants to be used for floral dip were sown as described above (see section 2.2.1) and were maintained under long day conditions. The initial flowering stem was picked out, to produce multiple stems per plant. Plants were used for floral dip when

several buds were visible, and any buds with already visible petals were removed prior to dipping. At day 0, the required *Agrobacterium* glycerol stock was removed from the -80°C storage and a sample spread on an LB plate containing the appropriate antibiotics (see Table 3, Table 6). Bacteria were grown for 2 days at 28°C before being passaged to a new LB plate and grown for another 2 days. If bacterial growth was at a satisfactory level, the day prior to the floral dip 5 LB plates were inoculated per construct and allowed to grow overnight. The following day, bacteria were collected from the plates and suspended in 30 ml LB media until an OD_{600} of between 1.0 and 2.0 was achieved. The *Arabidopsis* plants were then suspended in the *Agrobacterium floral dip* solution (*Table 5*), and gently swirled for 20 seconds to ensure a thorough coating. Plants were then immediately lain on their sides in a covered tray to ensure humidity, and allowed to dry for 14 to 24 hours. Once dry, plants were placed upright and grown under long day conditions in the greenhouse until they set seed. Seeds were collected and stored as described in 2.1.1 until needed.

Transgenic seed selection

To select seeds containing the transgenic construct, seeds were sown on soil as described in section 2.2.1. At 8, 15 and 22 days post germination, seedlings were sprayed with diluted Basta (200g/L Glufosinate ammonium) until drop off. After treatment, surviving seedlings were repotted and grown under long day conditions. At approximately 4-6 weeks post germination, leaf tissue was harvested and used for qPCR to assess the transcript abundance of the *LLP3* transgene. New primers were designed using Primer blast (*Table 11*), to specifically target the transgene (genotyping primers used previously for the *llp3* line were ineffective, as they targeted the 3'-UTR which was not present in the transgene). Plants with the highest transcript abundance were selected for continued breeding. To allow breeding to homozygosity, segregation analysis was utilised in the T2 plants. A set number of seeds were sown, and after BASTA treatment lines were selected that showed a survival ratio of 3:1. This indicated a single insertion point, and these lines were again assessed for transcript abundance and used for T3. Due to time constraints, some initial experiments were conducted using the T3 seeds.

The new complementation lines are henceforth referred to as *llp3-LLP3:LLP3.* The three main complementation lines selected for this work were *llp3-LLP3:LLP3-3.02, 4.01* and *8.01.*

2.2.14 Statistics

Data was analysed in GraphPad Prism 8 for Windows. If necessary, outliers were removed using a Grubbs' test (α=0.05). Normal distribution of the data was checked using D'Agostino Pearson (α=0.01). Finally, data was tested using one-way ANOVA corrected for multiple comparisons. Data that showed normal distribution was tested for significance using an unpaired one-way ANOVA with Tukey's multiple comparison test, and data that was not normally distributed was tested using a Kruskal-Wallis test with a Dunn's multiple comparison test. Standard deviation is shown for results in which an example experiment is shown, standard error of the mean is shown for graphs with merged replicates.

3. Results

3.1 The role of EDS1 and LLP1-3 in SA-mediated immunity and local defence

SA is a key component for plant defence against biotrophic pathogens, in both local and systemic tissues, and is associated with the central signalling regulator *Enhanced Disease Susceptibility1* (*EDS1)* (Vlot *et al.*, 2009; Bhandari *et al.*, 2019). To investigate the responsiveness of *LLP1-3* to treatment with exogenous SA, and whether this response was *EDS1*-dependent, both Col-0 and *eds1-2* knockout plants were spray-treated with 1 mM SA. Treated tissues were collected after 24 h, and the levels of *LLP1*, *LLP2*, and *LLP3* transcripts were analysed by RT-qPCR. In concurrence with Armijo et al. (2013), SA induced significant upregulation of *LLP1* transcripts (Figure 10). As previously shown (Breitenbach et al., 2014), this effect was not dependent on *EDS1*, with a similar fold increase of *LLP1* transcripts after SA treatment of the *eds1-2* knockout mutant. In contrast to Lyou et al (2009), who detected a slight reduction in *LLP3* transcript abundance after treatment of plants with 50 µM SA, we did not observe a reproducible down regulation of *LLP3*. Similarly, *LLP2* transcript levels did not significantly change in response to SA in either genotype at any time point. Thus, *LLP1* transcript accumulation, and not that of its homologues *LLP2* and *LLP3*, is regulated by SA, and this regulation is independent of EDS1.

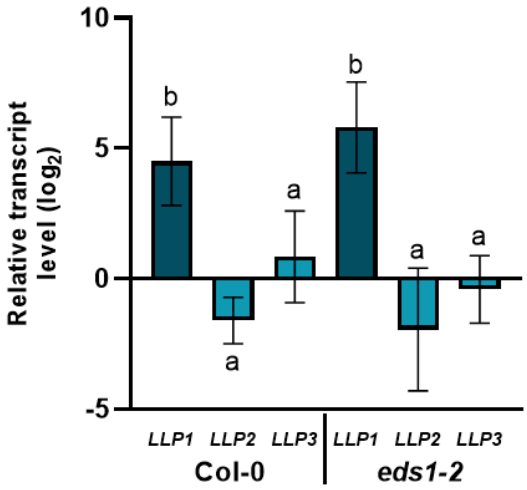

Figure 10: The transcriptional response of LLP1-3 to exogenous SA. 3-week-old plants of the genotypes Col-0 and *eds1-2* were spray-treated with 1 mM SA, and after 24 hours leaves were detached for RNA extraction and RT-qPCR. Data presented are the $\log_2$(mean) transcript levels of the *LLP* genes indicated below the panels relative to *UBI* and normalised to the appropriate mock controls ±SEM of three biologically independent replicates (one-way ANOVA).

LLP1-3 are not required for normal SA-induced local immunity and defence gene expression, as SA treatment can reduce pathogen growth in both *llp1-1* and *RNAi:LLP1-3* (Breitenbach *et al.*, 2014; Pabst, 2018). However, there are multiple pathways involved in local defence (such as the EDS1-mediated SA-independent pathway). It is therefore possible that although SA-signalling is functional, local responses to a pathogen may be altered. To test whether *LLP1-3* were still required for a complete response against hemibiotrophic pathogens, local growth patterns of the avirulent *Pst* strain *AvrRpt2* were tested to assess ETI induction. Mutation of *LLP1* was previously shown to impair SAR, but not local responses against virulent or avirulent *Pst* (Breitenbach et al., 2014). Here, the *RNAi:LLP1-3* line C3 13-1 showed a similar response. Plants were inoculated with *Pst AvrRpt2,* and bacterial titres were monitored at 2, 4, and 7 days post-inoculation (dpi). The bacteria grew to comparable titres in Col-0 and *RNAi:LLP1-3*, and to slightly higher titres in the *eds1-2* mutant, with levels increasing at day 2 and 4, and levelling off at around 10^7 CFUs/cm^2 by day 7 (Figure 11). These results were similar to those seen in C3 13-1 in response to *Pst* and *Pst AvrRpm1* (Pabst, 2018). Thus, the data suggest that reduced transcript accumulation of *LLP1, LLP2,* and *LLP3* does not compromise local immune responses against biotrophic pathogens.

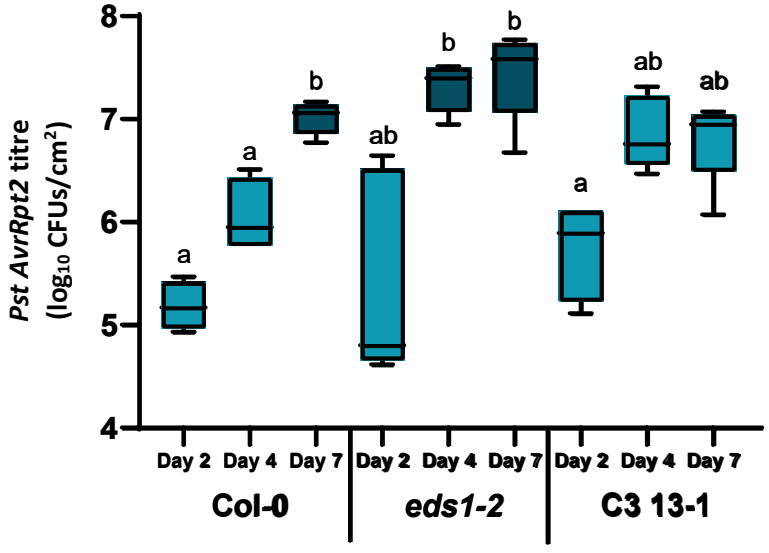

Figure 11: LLP1-3 do not affect bacterial growth titres in infected leaves. Col-0 (wildtype), *eds1-2*, and *RNAi:LLP1-3* (line C3 13-1) leaves were infiltrated with the avirulent

Pst strain *Pst AvrRpt2*, and bacterial titres were measured at 2, 4, and 7 days post-infection. A representative experiment is shown (n=3). Experiment was repeated three times. Box plots represent the average bacterial titres ±min and max values. Differences were analysed using a one-way ANOVA.

3.2 Generation of *LLP3:LLP3* complementation lines in the *llp3* mutant background

Extensive work has previously been completed on the role of LLP1 in SAR and its associated signalling pathways (Breitenbach *et al.*, 2014; Wenig *et al.*, 2019). LLP2 and LLP3 are thought to have similar or additive functions to LLP1 (Pabst, 2018). Because there are currently no T-DNA insertion lines available for *LLP2* and efforts to silence *LLP2* transcript accumulation have so far remained unsuccessful, this work focuses on *LLP3*.

In previous work a single T-DNA mutant, *llp3,* was identified (Breitenbach, 2012), but a second, independent mutant allele was not available. To ensure that results seen in the *llp3* plants were due to the disruption of the *LLP3* gene, and not to secondary, off-target effects, the *llp3* mutation was complemented by transformation of a full-length, functional copy of *LLP3* under the *LLP3* native promoter in the *llp3* mutant background. After floral dip, multiple independently transformed lines were selected. Further glufosinate treatment and segregation analysis was used to select plants across different generations to identify lines that were homozygous for the *LLP3:LLP3* transgene. To assess the levels of expression, RNA was extracted from individual plants and *LLP3* transcript levels quantified using RT-qPCR. Four independent lines with the highest expression of *LLP3* from three independent transformation events were selected for preliminary experiments. These lines were designated *LLP3-3.01*, *LLP3-3.02*, *LLP3-4.01*, and *LLP3-8.01*. *LLP3-3.01* had the highest level of *LLP3* expression in the T2 generation with approximately 45% transcript levels of wildtype (the *llp3* knockout mutant showed 0.9% and 1.1% as compared to wildtype levels in two repeats). *LLP3-3.02* showed approximately 30% of the level of *LLP3* gene transcription that was seen in wildtype, *LLP3-4.01* showed 29%, and *LLP3-8.01* showed ~10% (Figure 12 A). Other independent lines with lower levels of *LLP3 expression* were also tested and maintained in case of loss of the transgene expression in T3 in the four selected lines (Figure 12 B). Merged replicates

from different plants showed that while *LLP3* transcript accumulation levels in the complementation lines were lower than in wildtype plants, they were substantially higher than in the *llp3* mutant (Figure 12 C). Therefore, in the following these lines were used for experiments to elucidate the function of *LLP3* and support findings in *llp3* mutant plants.

A note: although the lines 3.01, 3.02, 4.01 and 8.01 consistently show higher expression of *LLP3* transcripts than the *llp3* knockout mutant, it appears that in later generations some of the lines, particularly 4.01, lost BASTA resistance. Nevertheless, the lines still show complementation of the *llp3* mutant phenotype. It may be the case that the qPCR primers are also able to amplify either *LLP1* or *LLP2* transcripts, and that these genes are upregulated to compensate for the loss of *LLP3.* However, it is not clear why this would occur in only the complementation lines and not the *llp3* mutant. Therefore, although the reason for the loss of herbicide resistance has not been fully resolved, I believe that these lines still provide us with valid experimental results due to the elevated *LLP3* expression, and thus enable conclusions on the function of LLP3.

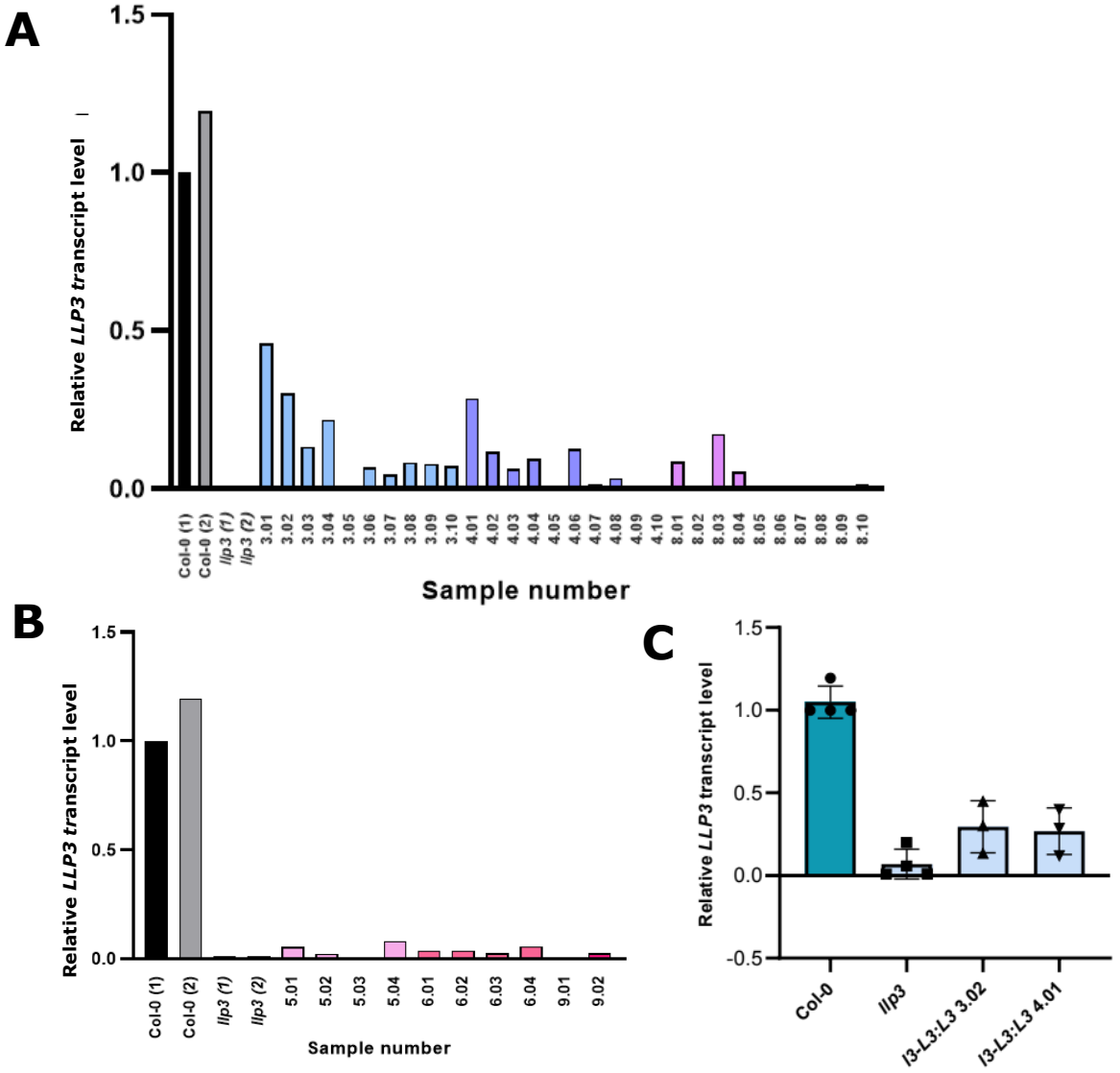

Figure 12: *LLP3* transcript accumulation in *LLP3:LLP3* complementation lines generated in the llp3 mutant background. (A and B) Shown is the relative quantification of *LLP3* transcripts relative to UBI compared to a positive control (Col-0) and a negative control (*llp3*). A value of 1=wildtype transcript levels. Each sample represents an RNA extraction from an individual plant from 3 independently transformed lines (3, 4, and 8). Ten T2 plants were selected for analysis from each line (n=1). (A) shows the transcript levels of *LLP3* in T2 lines. Lines 3.01, 3.02, 4.01 and 8.01 were selected for experimental use. (B) shows the *LLP3* transcript level in the lines reserved for backup (5, 6 and 9). (C) *LLP3* transcript levels in *llp3* and *llp3-LLP3:LLP3* complementation lines 3.02 and 4.01. *LLP3* transcript accumulation was determined in 5-to-6-week-old plants and normalised to that of *UBI*. *l3-L3:L3* 3.02 and *l3-L3:L3* 4.01 are two biologically independent transgenic lines carrying the *LLP3:LLP3* transgene

driving ectopic *LLP3* expression from its native promoter in the *llp3* mutant background. Bars represent the average of the indicated results ± SEM (one-way ANOVA, n=4 for Col-0 and *llp3*, n=3 for *l3-L3:L3* 3.02 and *l3-L3:L3* 4.01, F=51.41, DF=13).

3.3 The role of LLP1-3 in systemic immunity

A lack of change in defence in the local tissue does not preclude an altered defence response in systemic tissues, as there are several pathways involved only in SAR (Vlot *et al.*, 2020). It was previously shown that SAR is abolished in *llp1-1,* and there is enhanced systemic susceptibility in *RNAi:LLP1-3* (Pabst, 2018). This indicates that not only is either *LLP2* or *LLP3* also required for SAR, but there is an additional interaction that negatively effects systemic defence. As mentioned above, there is no single mutant to investigate the function of *LLP2.* However, using the *llp3* and *LLP3:LLP3* complementation lines, I was able to examine the role of LLP3 in SAR. To this end, the plants were initially infiltrated in two leaves with either *Pst AvrRpm1* or a 10 mM $MgCl_2$ mock control solution. Three days later, two leaves distal to the initial infection were infiltrated with virulent *Pst*. After another four days, the resulting *in planta Pst* titres were determined. In wildtype plants, a local *Pst AvrRpm1* infection reduced *Pst* growth in the systemic tissues compared to that in mock-treated plants, indicating the establishment of SAR (Figure 13). In the *llp3* plants there was no significant difference between plants pretreated with *Pst* compared to those pretreated with $MgCl_2$ (Figure 13). Therefore, *llp3* plants were unable to establish a SAR response. In *llp3* plants where *LLP3* was ectopically expressed under its own promoter (3.02 and 4.01), a reduction in bacterial titres was again observed at similar levels to those seen in wildtype (Figure 13). Thus, ectopic expression of *LLP3* complements the SAR-deficient phenotype of the *llp3* mutant. This supports my hypothesis that *LLP3* is required for a normal SAR response.

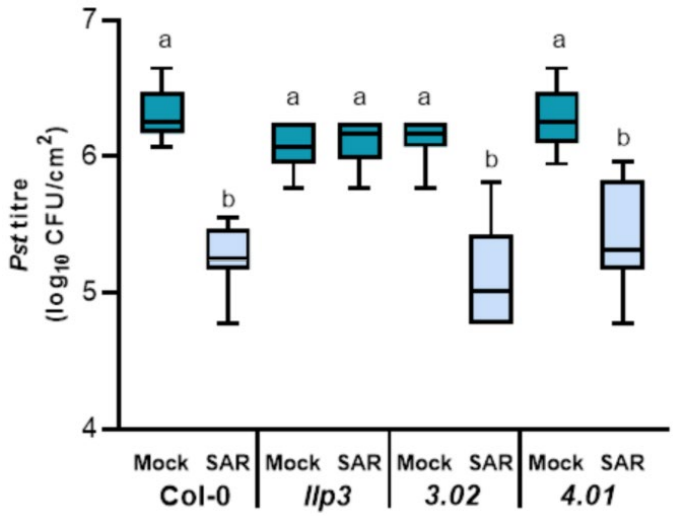

Figure 13: Systemic defence responses are compromised in the absence of *LLP3*. SAR assay. Plants from different Col-0 and various transgenic lines were infiltrated locally with either *Pst AvrRpm1* (SAR) or with 10mM MgCl2 mock control (Mock). Plant lines included *llp3* mutants and 2 independently transformed complementation lines carrying a transgene driving *LLP3* expression from its native promoter (3.02 and 4.01). Experiments were performed by Marion Wenig. Box plots represent average *Pst* titres in systemic leaves at 4 days post- inoculation (dpi) from 4 biologically independent experiments, including 3 replicates each ±min and max values. The letters above the box plots indicate statistically significant differences (Kruskal-Wallis test).

3.4 Involvement of LLP1-3 in the SA-independent SAR pathway

Multiple signalling pathways contribute towards systemic resistance pathways in *Arabidopsis.* As well as an SA-mediated component, a parallel pathway involving Pip is also required for the full distal response (Návarová *et al.*, 2012). Downstream in this pathway is the C_9 dicarboxylic acid AzA (Wittek *et al.*, 2014). In order to place the function of *LLP1-3* in the Pip pathway, local leaves were infiltrated with 1 mM AzA solution, and *Pst* growth was monitored after 4 days compared to a mock treated control. This showed that AzA is able to induce systemic resistance in both Col-0 and *llp1-1*. However, this resistance was lost in the *RNAi:LLP1-3* lines, indicating that while LLP1 is upstream, either or both of LLP2 and LLP3 are required downstream for a normal defence response (Figure 14). Further components of this pathway and their relative positions to LLP1 are discussed in (Wenig *et al.*, 2019).

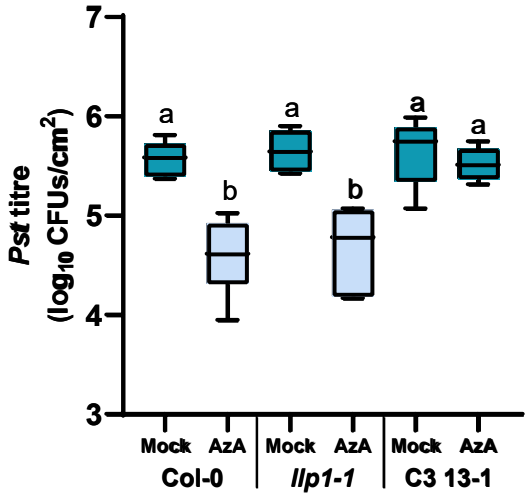

Figure 14: AzA-induced systemic resistance is not dependent on *LLP1*. Local leaves from wildtype (Col-0), *llp1* mutants and *RNAi:LLP1-3* (C3 13-1) plants were infiltrated with either AzA or a $MgCl_2$ solution. After 3 days systemic leaves were challenged with a virulent *Pst* strain, and bacterial titres were measured after four days. Box plots represent average *Pst* titres in systemic leaves at 4 days post-inoculation (dpi) from a representative biological replicate ±min and max values (n=3). The experiment was reproduced 3 times with similar results. Differences were analysed using a one-way ANOVA.

3.5 LLP1-3 influence responses to abiotic stress

3.5.1 Transcript response to ABA

Neither *LLP2* nor *LLP3* showed a significant response to SA treatment. However, a reduced level of the transcripts resulted in compromised systemic resistance against *Pst* as well as reduced *PR1* transcript accumulation in the systemic tissue during SAR. Importantly, the SA signalling pathway does not exist in isolation, which necessitates the consideration of interaction between biotic defence and other environmental stresses. Yasuda et al. (2008) showed that ABA, the key phytohormone controlling responses to abiotic stress, and ABA-dependent responses to salinity stress compromised SA signalling and potentially SAR. In order to investigate whether ABA also had an impact upon the transcript levels of *LLP1, LLP2,* and *LLP3*, plants were spray-treated with 100µM ABA and tissues were harvested 24 h later. In Col-0 plants, *LLP1* transcript levels were reduced in ABA- compared to mock-treated plants, while

the levels of *LLP3* transcripts were increased, albeit not significantly (Figure 15 A). This response was also seen after a longer treatment with ABA. Seedlings were germinated on MS plates, and then transferred after 6 days to MS plates supplemented with 10µM ABA. When seedlings were harvested after 12 days, they also showed a significant decrease in *LLP1* and *LLP2* transcripts on ABA compared to control plates, whereas *LLP3* transcripts were not significantly changed (Figure 15 B). In a similar way to the response of *LLP1* to SA, the ABA-induced changes in *LLP1* transcript accumulation was not dependent on *EDS1*, with transcripts also being significantly downregulated in *eds1-2* (Figure 15 A). *LLP3* transcript levels were not significantly upregulated or downregulated in either Col-0 or *eds1-2*. *LLP2* transcript levels were reduced, although not significantly, in response to ABA in both wildtype and *eds1-2* mutant plants (Figure 15 A).

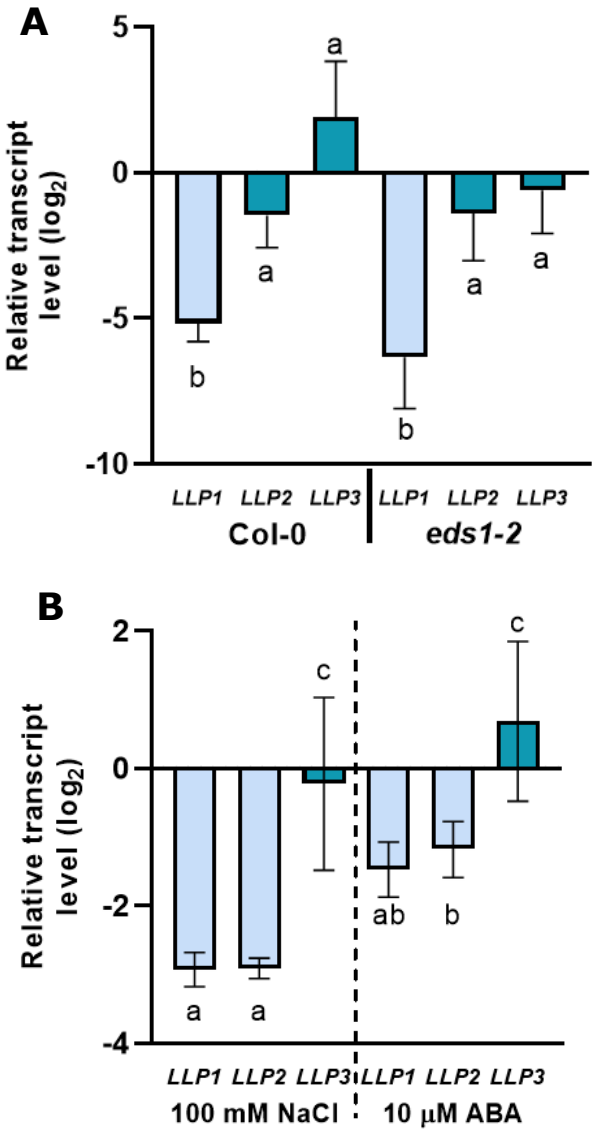

Figure 15: *LLP1-3* transcript accumulation after ABA treatment is *EDS1*-independent. (A) 3-week-old plants of the genotypes Col-0 and *eds1-2* were spray-treated with 100 µM ABA, and after 24 hours leaves were detached for RNA extraction and RT-qPCR. Data presented are the log_2(mean) transcript levels of the *LLP* genes indicated below the panel relative to *UBI* and normalised to the appropriate mock controls ±SEM of three biologically independent replicates (one-way ANOVA).

(B) *LLP1* and *LLP2* transcript levels decrease in response to salt and ABA. Col-0 seedlings were harvested 12 days after transfer to media supplemented with 100 mM NaCl or 10 µM ABA for RNA extraction and RT-qPCR. Data presented are the log2(mean) transcript level relative to *UBI* normalised to mock control ±SEM of three biologically independent experiments (one-way ANOVA).

3.5.2 LLP1-3 response to salt stress

ABA is an important phytohormone in abiotic stress signalling. Therefore, it seemed possible that if *LLP1* and *LLP2* are transcriptionally regulated by ABA, the *RNAi:LLP1-3* plants may show an altered phenotype under abiotic stress. To test for aberrant reactions to high salinity, seedlings were germinated and after 6 days transferred to treatment plates with 100mM NaCl. The length of the primary roots was measured at 6-and 12-days post transfer and normalised to those on control plates (to which

seedlings had also undergone transfer). While *llp1* plants had marginally longer roots than wildtype on control plates (Figure 16 C), the plants of all genotypes showed a significant reduction in root length when grown on salt compared to control conditions. Notably, both *llp1-1* and *RNAi:LLP1-3* plants showed more pronounced salt-induced root growth inhibition as compared to wildtype plants, and *RNAi:llp1-3* was significantly more affected than *llp1-1* (Figure 16 A,B).

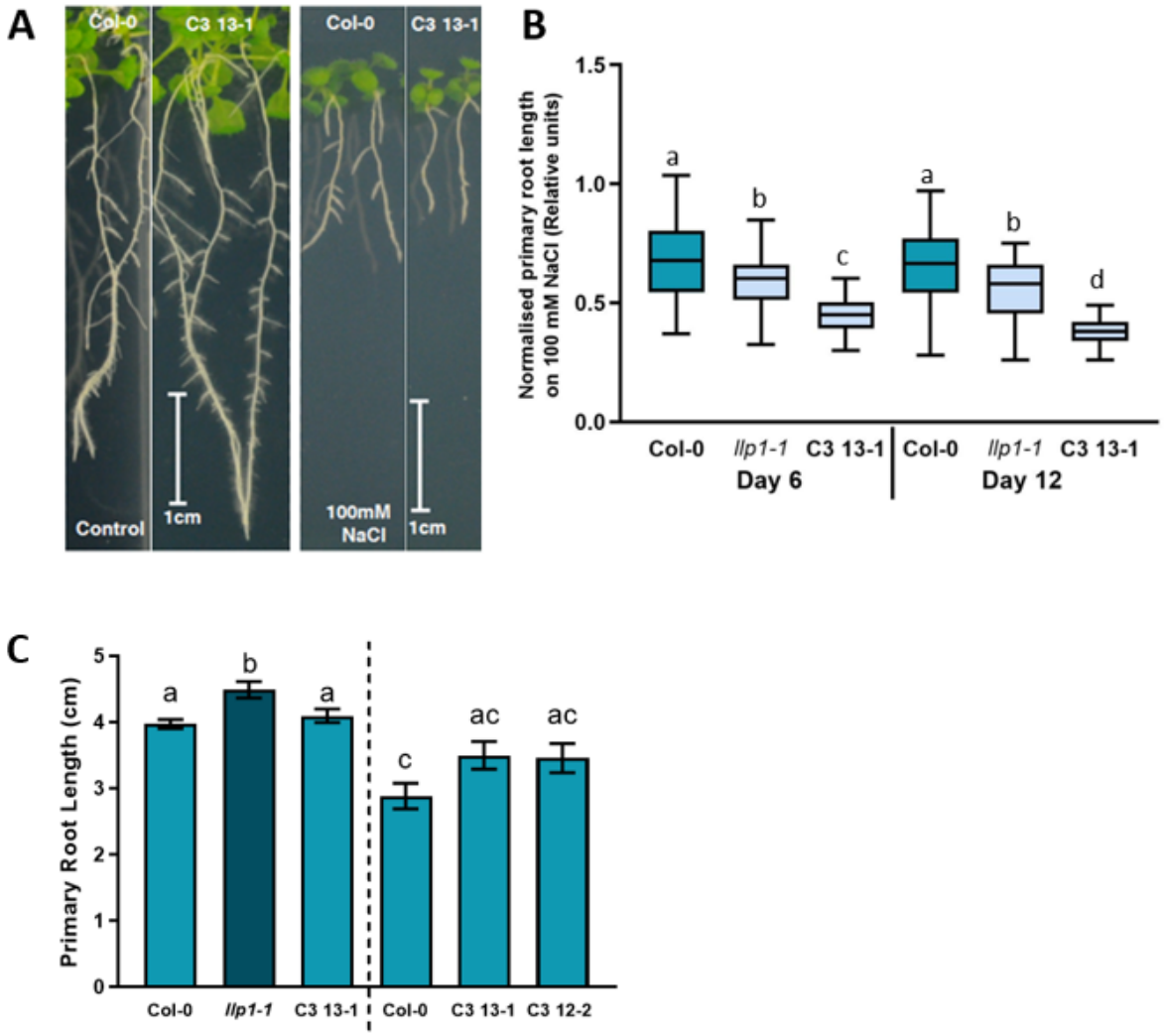

Figure 16: *LLP1-3* affect root growth in response to salt stress. (A) and (B) *RNAi:LLP1-3* lines show increased root growth inhibition on salt. Seedlings of Col-0 and transgenic lines were germinated on control MS plates, and after 6 days transferred to either further control plates, or MS plates supplemented with 100 mM NaCl. Primary root length was measured at 6- and 12-days post transfer. (A) Photos show representative root length for Col-0 compared to C3 13-1. (B) Root length of wildtype (Col-0), *llp1* mutants and *RNAi:LLP1-3* (C3 13-1) on

NaCl treatment plates was normalised to root length of the same genotype on control plates. Data presented is the normalised root length at day 6 and day 12 after transfer, ±min and max values. Differences were analysed with one-way ANOVA, (Day 6 Col-0 n=48, *llp1-1* n=38, C3 13-1 n=40, Day 12 Col-0 n=48*, llp1-1* n=29, C3 13-1 n=31.). This experiment was repeated 7 times with similar results. (C) The primary root length of *RNAi:LLP1-3* lines (C3 13-1 and C3 12-1) on control plates 12 days after transfer ±SEM (Kruskal-Wallis test (non-parametric, Col-0 n=447, *llp1-1* n=200, C3 13-1 n=239 from 9 biologically independent experiments, Col-0 n=56, C3 13-1 n=48, C3 12-2 n=60 from 3 biologically independent experiments).

3.5.3 LLP3 during abiotic stress

The loss of all three LLP proteins results in a more exaggerated response to abiotic stress compared to the mutation of the single *LLP1* gene. Therefore, it is plausible that either LLP2 or LLP3 have an additional role in abiotic stress tolerance. Because of this, the *llp3* mutant was tested to see whether it showed a similar inhibition of root growth to the *llp1-1* plants when placed under salt stress. *llp3* seedlings consistently showed a reduction in primary root growth when transferred to MS media containing 100 mM NaCl at both 6 and 12 days post transfer (Figure 17). To verify that this effect was caused by the lack of a functional *LLP3* gene, the complementation lines *LLP3-3.01, LLP3-3.02, LLP3-4.01* and *LLP3-8.01* were also tested in these experiments. At both 6 and 12 days post transfer, both *LLP3-3.01* and *LLP3-.801* showed root lengths that were not significantly different to the wild type control. *LLP3-3.02* and *LLP3-4.01* showed root growth that was significantly shorter than Col-0, but significantly longer than the *llp3* mutant (Figure 17). Thus, the *LLP3* transgene is, at least partially, successfully complementing the exaggerated root shortening phenotype caused by the loss of the native *LLP3* gene in *llp3* mutant plants. This suggests that the mutation in *llp3* is responsible for the observed primary root growth inhibition in *llp3* mutant seedlings, although we must further clarify the issue on lack of herbicide resistance in the complementation lines before a definitive result can be provided.

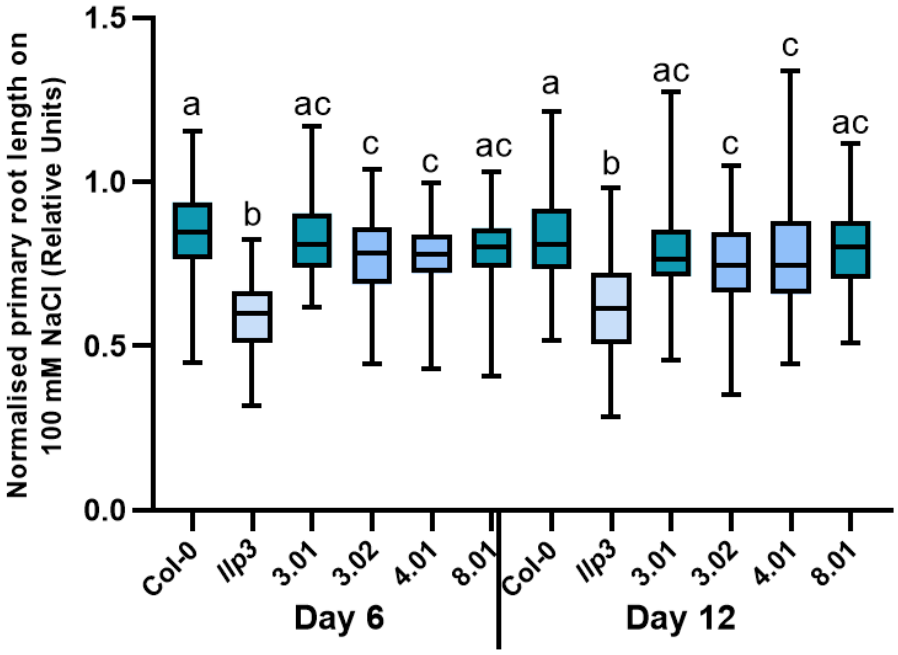

Figure 17: *LLP3* is required for normal root growth under salt stress. Seedlings from wildtype (Col-0), *llp3* mutants and *LLP3::LLP3* complementation lines (3.01, 3.02, 4.01, and 8.01) were grown on MS plates and transferred to plates with 100 mM NaCl after 6 days. Primary root length was measured 6 and 12 days after transfer. Data shown are the average primary root length ±min and max values of four biologically independent experiments. For Day 6, Col-0 n=83, *llp3* n=86, 3.01 n=95, 3.02 n=96, 4.01 n=89, 8.01 n=94. Day 12 Col-0 n=81, *llp3* n=84, 3.01 n=90, 3.02 n=91, 4.01 n=84, 8.01 n=92. Kruskall-Wallis was used for Day 6, one-way ANOVA for Day 12.

3.5.4 Salt-associated growth inhibition was not due to either osmotic stress, ion toxicity, or auxin signalling

NaCl can affect plant growth via a number of different effects. To ensure that the reduced root growth in the *RNAi:LLP1*-3 lines was indeed due to a hormonally-regulated response, seedlings were also transferred to plates containing either mannitol or Lithium Chloride (LiCl). The reduced root length phenotype was not observed under these conditions (Figure 18 A), excluding the premise that salt-associated phenotypes were due to either ion toxicity or osmotic stress, respectively. Changes in NaCl concentration have also been shown to affect meristematic structure

via redox regulation, and thereby change growth patterns in roots (Jiang *et al.*, 2016). This is partly due to a change in the distribution of auxin transporters. We therefore investigated whether the changes in root length after NaCl treatment may be due to misregulation of auxin signalling. 6 day old seedlings were transferred to plates containing either 20 µM or 40 µM indole-3 acetic acid (IAA), a naturally occurring class of auxin. Although for all genotypes there was a decrease in root length after treatment, there was no significant difference in length between wildtype and the transgenic lines (Figure 18 B).

This indicates that although exogenous auxin treatment does impact the remodelling of root growth, it does so via a mechanism that is separate from that induced by NaCl treatment, and is thus in a pathway not involving any of the LLP1, 2, or 3.

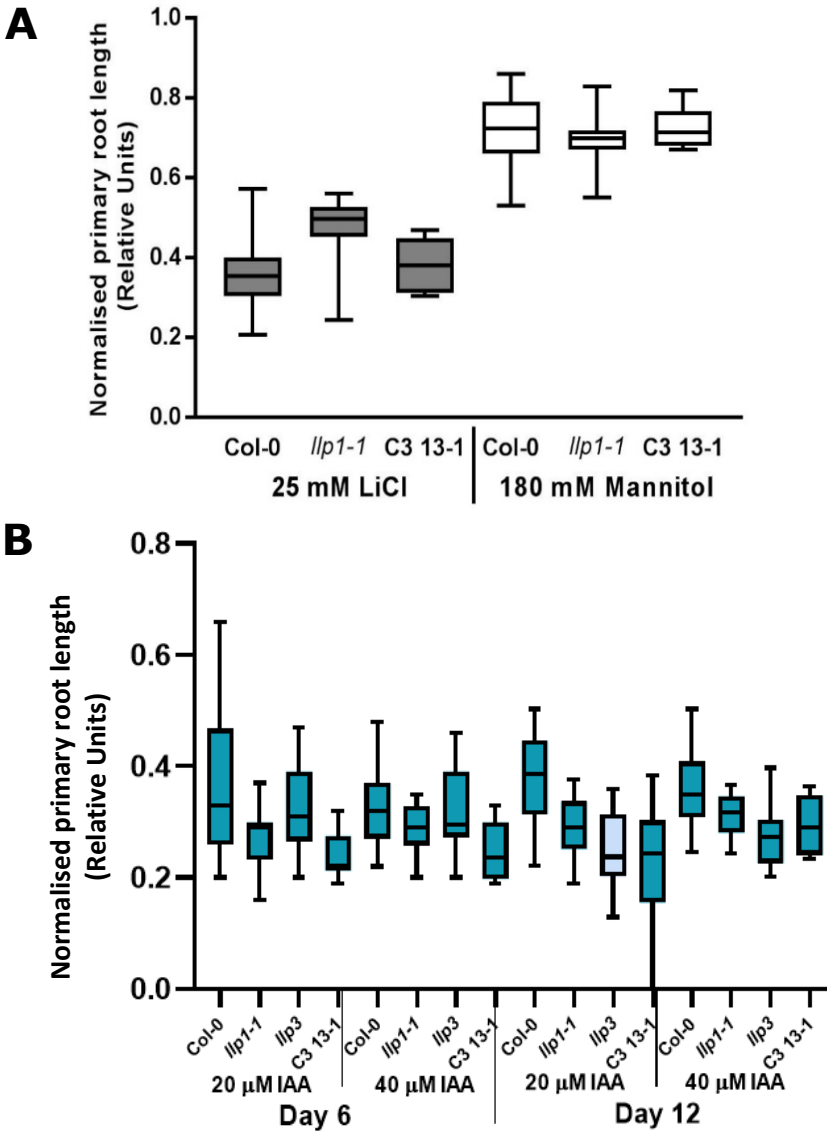

Figure 18: *LLP1-3* do not affect root growth in response to exogenous IAA, LiCl or mannitol. A) Seedlings from wildtype (Col-0), *llp1* mutants and *RNAi:LLP1-3* (C3 13-1) were grown on MS plates and transferred to plates with 25 mM LiCl or 180 mM mannitol after 6 days. Primary root length was measured at 12 days after transfer. Shown are the normalised primary root lengths at 12 days post transfer. Differences were analysed using a one-way ANOVA. (LiCl Col-0 n=43, *llp1-1* n=20, C3 13-1 n=8, Mannitol Col-0 n=44, *llp1-1* n=23, C3 13-1 n=10 from 1 biological replicate) B) Seedlings were grown as described in (A) (plus *llp3* mutants) and transferred to plates containing 20 or 40 µM IAA. Primary root length was measured at 6 and 12 days post transfer, and normalised to root length on control.(Day 6 20 µM IAA Col-0 n=18, *llp1-1* n= 12, *llp3* n= 14, C3 13-1 n=6, 40 µM IAA Col-0 n=19, *llp1-1* n=12, *llp3* n=14, C3 13-1 n=6, Day 12 20 µM IAA Col-0 n=18, *llp1-1* n=12, *llp3* n=15, C3 13-1 n=6, 40 µM IAA Col-0 n=16, *llp1-1* n=11, *llp3* n=15, C3 13-1 n=5 from one biological experiment).

3.5.5 LLP1-3 under salt stress in mature plants

The described sensitivity of *LLP1-3* transgenic plants to salt was primarily observed in seedlings grown on sterile media. As phytohormone signalling interactions can change significantly during different developmental stages, a salt pouring assay was also performed (with Gerardo Perez). 5-week-old plants were harvested after being poured with 300 mM NaCl solution every 4 days for a 12 day period. After harvesting, plants were weighed, and tissue samples taken for gene expression analysis. Although the salt treatment resulted in a significant reduction in biomass, there was no significant difference between Col-0 and either *llp1-1, llp3* or C3 13-1 (Figure 19).

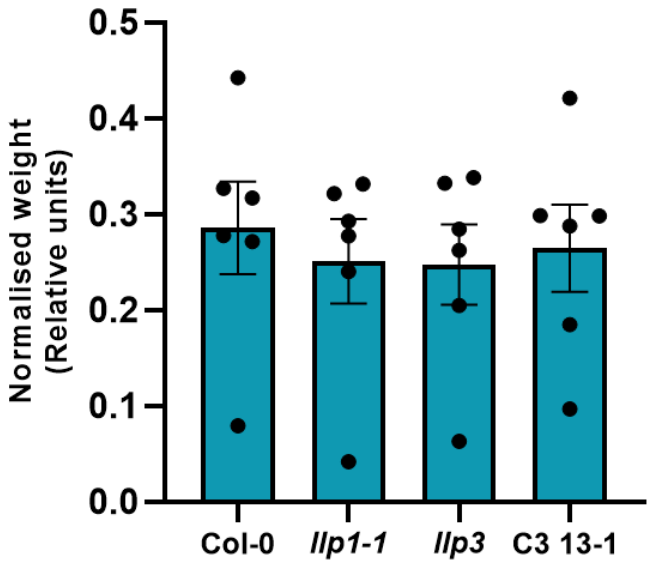

Figure 19: Mature *Arabidopsis* biomass is reduced after salt pouring, but is not affected by *LLP1-3*. Data shown are the average biomasses of *Arabidopsis* plants of wildtype (Col-0), *llp1* and *llp3* mutants, and *RNAi:LLP1-3* (C3 13-1) after pouring for 12 days with 300 mM NaCl. For Col-0, *llp3* and *RNAi:LLP1-3* n=5, for *llp1-1* n=4. Bars show the average fresh weight of biological replicates normalised to the untreated control ± SEM.

3.5.6 *LLP1-3* response to exogenous ABA treatment

We next tested if the exaggerated salt-induced root growth inhibition in *llp1-1* and *RNAi:LLP1-3* seedlings was associated with changes in ABA-mediated stress signalling, and whether the results seen on NaCl plates could be replicated by treatment of seedlings with exogenous ABA. First, sterile-grown seedlings were transferred to treatment plates supplemented with 10 µM ABA. Primary root length was measured at 6 and 12 days after transfer, and normalised to the root length on

control plates. At both day 6 and day 12, *RNAi:LLP1-3* showed no change in root growth compared to wildtype. At day 6 *llp1-1* showed no change, and was slightly reduced at day 12, but this result was not consistent across replicates (Figure 20 A). On the other hand, the *llp3* mutant showed consistently reduced root growth on ABA plates, which could be reversed by ectopic expression of *LLP3,* with 3.02, 4.01 and 8.01 all showing no difference in root growth compared to wildtype (Figure 20 B). This indicates that LLP3 may be sensitive to ABA signalling in a function distinct from LLP1. However, this sensitivity is affected by either *LLP1* or *LLP2*, as the exaggerated shortening is not seen in the *RNAi:LLP1-3* lines. Transcript levels of *RAB18*, an ABA marker gene, were induced by both salt and ABA in all genotypes, and the level of induction was not different in *llp1-1* and *RNAi:LLP1-3* compared to wildtype seedlings after 12 days of treatment, nor in *llp3* or its complementation lines (Figure 20 C and D).

Taken together, the data suggest that LLP1 and possibly LLP2 compromise plant responses to salinity stress in an ABA-independent manner. We therefore posit that the increased sensitivity of the *RNAi:LLP1-3* lines to high salinity is most likely mechanistically independent of ABA. LLP3 does appear to influence root shortening in response to ABA, but as there was no change in the ABA marker gene tested, it is unlikely that this is directly due to the ABA pathway.

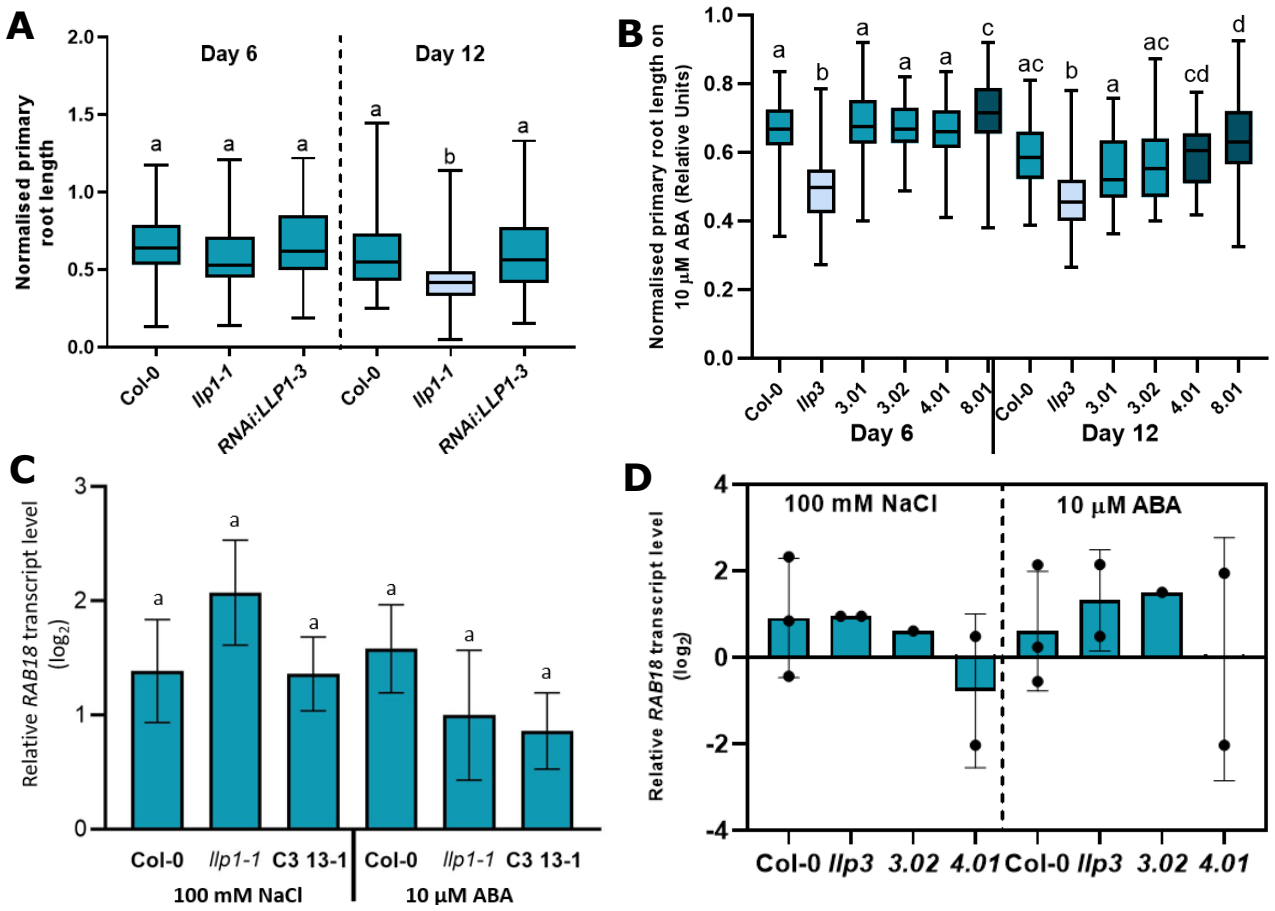

Figure 20: ABA-associated root shortening is only affected by *LLP3*, not *LLP1* or *2*, and does not affect ABA marker genes. (A) *LLP1* does not affect normal root growth in response to exogenous ABA. Seedlings were grown on MS plates and transferred to plates with 10 µM ABA after 6 days. Primary root length was measured 6 and 12 days after transfer. Data shown are the average primary root length ±min and max values of four biologically independent experiments. Day 6 Col-0 n=321, *llp1-1* n=136, C3 13-1 (*RNAi:LLP1-3*) n=125. Day 12 Col-0 n=290, *llp1-1* n=105, C3 13-1 n=138. One-way ANOVA. (B) *LLP3* is required for normal root growth in response to exogenous ABA. Assays were performed as described in (A) with seedlings from wildtype (Col-0), *llp3* mutants and *LLP3::LLP3* complementation lines (3.01, 3.02, 4.01, and 8.01). Data shown are the average primary root length ±min and max values of four biologically independent experiments. For Day 6, Col-0 n=77, *llp3*=86, 3.01 n=89, 3.02 n=98, 4.01 n=94, 8.01 n=97. For Day 12, Col-0 n=85, *llp3* n=90, 3.01 n=96, 3.02 n=100, 4.01 n=97, 8.01 n=97. One-way ANOVA was used for day 6, Kruskal Wallis test was used for day 12. (C) *LLP1-3* do not affect ABA downstream signalling components. Seedlings from 100 mM NaCl and 10 µM ABA treatment plates were used for RNA extraction and RT-qPCR after 12 days. Data presented is the $\log_2$(mean) transcript level of *RAB18* relative

to *UBI* and normalised to a mock treated control in four biological replicates, ±SEM. (D) *LLP3* does not affect ABA downstream signalling components. Data presented is the $\log_2$(mean) transcript level of *RAB18* relative to *UBI* and normalised to a mock treated control in four biological replicates, ±SEM. Data were analysed with one-way ANOVA, but no significant differences were found.

3.6 LLP3 responds to MeJA, and may be involved in JA-mediated abiotic defence responses

Another candidate pathway that has been shown to be involved in both biotic defence and salt stress tolerance is the JA pathway (Ismail *et al.*, 2012; Valenzuela *et al.*, 2016; Zhang *et al.*, 2017*c*), along with one of its key transcription factors, ERF1 (Cheng *et al.*, 2013). Lyou et al. (2009) showed that in *Arabidopsis* MeJA treatment is associated with an increase in *LLP3* transcript levels. Here, *Arabidopsis* plants were spray-treated with 100 µM MeJA, and after 24 h transcript accumulation of *LLP1*-3 was assessed via RT-qPCR. The accumulation of *LLP1* and *LLP2* transcripts was not significantly changed by MeJA. In contrast, there was a significantly increased accumulation of *LLP3* transcripts in both Col-0 and *eds1-2* plants, suggesting that MeJA induces *LLP3* in an *EDS1*-independent manner (Figure 21).

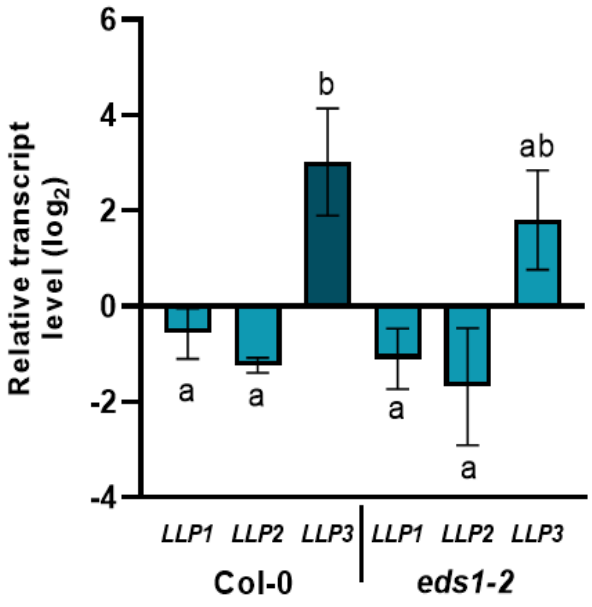

Figure 21: *LLP3* transcript accumulation is induced by MeJA. 3-week-old plants of the genotypes Col-0 and *eds1-2* were spray-treated with 100 µM MeJA, and after 24 hours leaves were detached for RNA extraction and RT-qPCR. Data presented are the $\log_2$(mean) transcript levels of the *LLP* genes indicated below the panel relative to *UBI* and normalised to the appropriate mock controls ±SEM of three biologically independent replicates (one-way ANOVA).

3.6.1 Root growth in response to exogenous JA

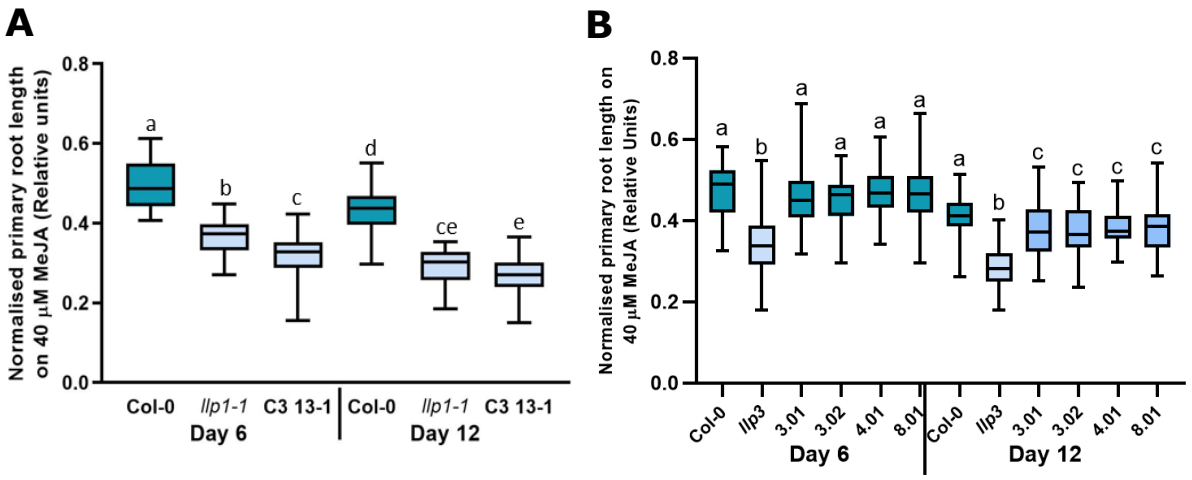

Figure 22: *LLP1-3* are involved in root shortening in response to MeJA. (A) Seedlings from wildtype (Col-0), *llp1* mutants and *RNAi:LLP1-3* (C3 13-1) were germinated on control MS media, and after 6 days transferred to either further control plates, or to MS plates supplemented with 40 µM MeJA. Plotted are the root lengths for each line normalised to their mock controls ±min and max values for a representative experiment, which was repeated 5 times with similar results. (one-way ANOVA, n=28-30). (B) *LLP3* is required for normal root growth in response to exogenous MeJA. Seedlings from wildtype (Col-0), *llp3* mutants and *LLP3::LLP3* complementation lines (3.01, 3.02, 4.01, and 8.01) were grown on MS plates and transferred to plates with 40 µM MeJA after 6 days. Data shown are the average primary root length after 6 and 12 days ±min and max values of four biologically independent experiments. For Day 6, Col-0 n=71, *llp3* n=85, 3.01 n=100, 3.02 n=94, 4.01 n=97, 8.01 n=95. For Day 12, Col-0 n=63, *llp3* n=73, 3.01 n=98, 3.02 n=94, 4.01 n=97, 8.01 n=95. One-way ANOVA was used for day 6, Kruskal Wallis was used for day 12.

I subsequently tested if compromised JA signalling could have been responsible for the root growth inhibition phenotype of the *RNAi:LLP1-3* seedlings on salt. To this end, I again used the root growth inhibition assay, but this time the treatment plates were supplemented with 40 µM MeJA. This treatment induced similar results as treatment with NaCl. There was a shortening of roots of all plant genotypes when grown on plates supplemented with MeJA as compared to control. Both the *llp1-1* mutant and *RNAi:LLP1-3* seedlings showed significantly enhanced root growth inhibition compared to wildtype 12 days after transfer (Figure 22 A). Therefore, we

can produce a phenocopy of the effect of high NaCl on root growth by treating the seedlings with exogenous MeJA. To see whether the presence of *LLP3* also contributed to this effect, we performed the root growth assay as described above with both the *llp3* knockout mutant and the *LLP3* complementation lines. At both 6 and 12 days post transfer to MS media containing 40 µM MeJA, the *llp3* plants showed significantly reduced primary root growth when compared to Col-0, as was also seen in the *llp1-1* plants (Figure 22 A). After 6 days, all of the *LLP3*-complementation lines showed no significant difference in root growth compared to the Col-0 wildtype. After 12 days, although all the *LLP3*-complementation lines were significantly shorter than Col-0, they were significantly longer than the *llp3* mutant (Figure 22 B). This indicates that complementation of the *LLP3* gene can at least partially restore the normal root growth in response to exogenous MeJA treatment. I can therefore conclude that LLP3 as well as LLP1 has a role in MeJA signalling, which is associated with normal abiotic stress responses.

JA downstream signalling pathways in the seedlings after 12 days on MeJA-supplemented plates were also aberrant in the *RNAi:LLP1-3* seedlings. Transcript accumulation of the JA marker gene *PDF1.2* was increased 12 days after transfer of wildtype and *llp1-1* seedlings from control to MeJA plates. By contrast, the induction of *PDF1.2* transcript accumulation was compromised in both of the *RNAi:LLP1-3* lines C3 13-1 and C3 12-2 (Figure 23A), while the transcript accumulation of *VSP2* remained unchanged (Figure 23B).

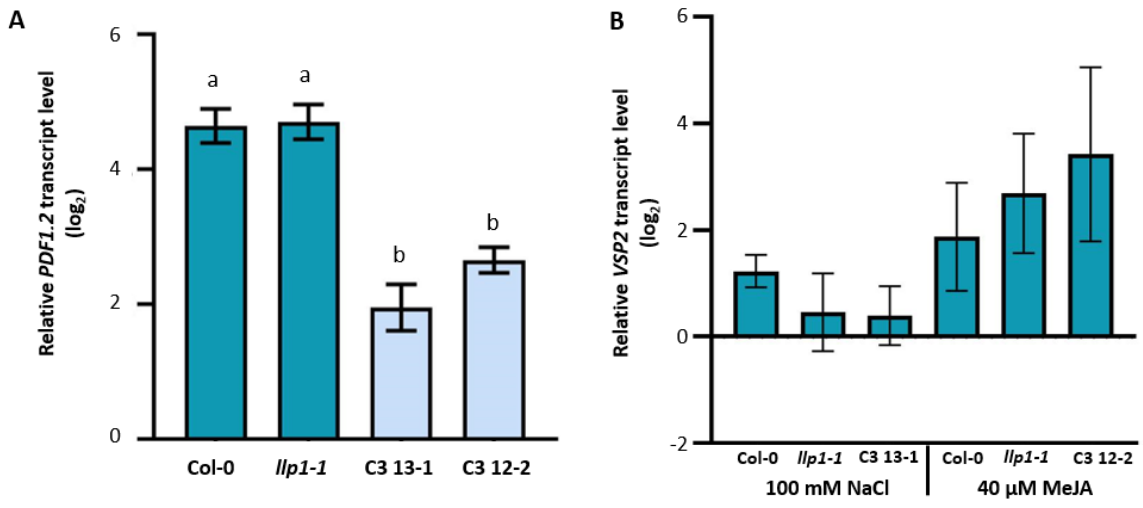

Figure 23: JA-marker genes show different responses to exogenous MeJA in the absence of *LLP1-3*. (A) *PDF1.2* transcript accumulation is reduced in *RNAi:LLP1-3* lines in response to MeJA. Seedlings from (Figure 13 A) were harvested after 12 days on treatment media, and used for RNA extraction and RT-qPCR. Data presented are the $\log_2$ mean transcript levels relative to *UBI* normalised to their own mock control ±SEM. (one-way ANOVA, n=3 for Col-0 and C3 13-1, n=2 for *llp1-1*, n=1 C3 12-2). (B) *VSP2* transcript accumulation is not significantly changed in *RNAi:LLP1-3* lines in response to NaCl or MeJA, as described for (A). Data presented are the $\log_2$ mean transcript levels relative to *UBI* normalised to their own mock control ±SEM. (One-way ANOVA, n=4-5 for Col-0 and C3 13-1, n=3 for *llp1-1* and C3 12-2).

3.6.2 Proteins associated with LLP1 in other pathways are not required for NaCl tolerance

As *LLP1* was recently shown to be involved in a positive feedback loop with pip and monoterpenes (Wenig *et al.*, 2019), mutant lines for other components of this pathway were also tested for their sensitivity to NaCl, in order to determine whether the observed susceptibility towards salt stress was associated with the role of *LLP1* in these pathways. Neither *ggpps12* (*geranyl geranyl diphosphate synthase12*), *tps24-1* or *tps24-2* (*terpene synthase24*) (monoterpene synthesis mutants), nor *ald1* (*agd2-like defence response protein 1,* a pipecolic acid synthesis mutant) showed a similar response to the *llp1-3* lines (Figure 24). These components in *LLP1*-associated signalling pathways also had no influence on root shortening mediated by exogenous

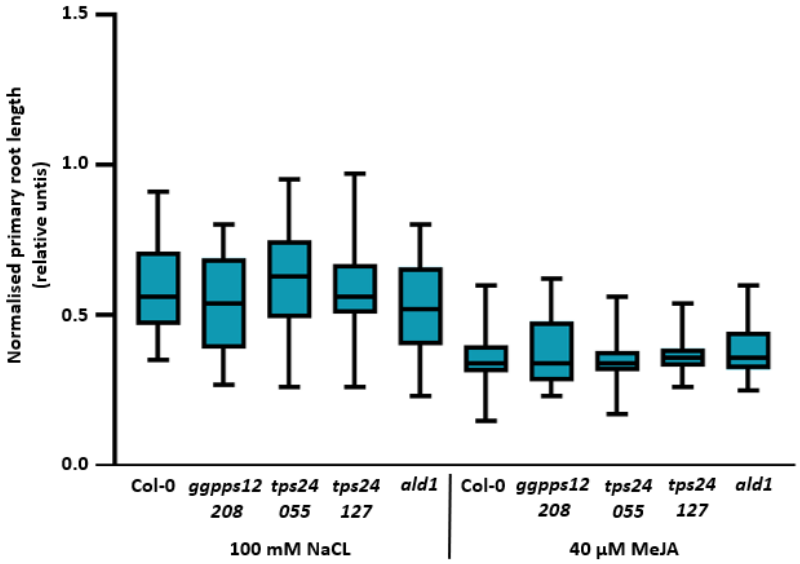

Figure 24: The interaction of the *LLP*s and root growth under salt stress or MeJA treatment is not associated with any other components of the *LLP1*-dependent monoterpene feedback loop. Seedlings carrying mutations in genes that are associated with monoterpene synthesis and signalling (*ggpps12, tps24 055, tps24 127*) and Pip biosynthesis (*ald1*) were transferred to treatment plates containing either 100 mM NaCl or 40 µM MeJA and the primary root length measured after 12 days. Data shown are the average primary root length normalised to a mock-treated control ±min and max values. Data was analysed with Kruskal-Wallis test (non-parametric data), Col-0 n=87, n=103. *ggpps12 208* n=117, n=117. *tps12 055* n=80, n=117. *tps12 127* n=85, n=118. *ald1* n=110, n=116 from 2 experiments.

MeJA treatment (Figure 24). Together, the data suggest that LLP1 is involved in the tolerance of salt stress of sterile-grown seedlings, in a manner that is independent of its previously identified functions in monoterpene recognition and feedback signalling.

3.6.3 Phytohormone content of transgenic lines under abiotic stress

To determine whether the loss of *LLP1-3* was affecting gene expression through changes in hormone biosynthesis, or through downstream interactions, the net content of SA, JA and ABA was measure in the transgenic lines after pouring with 100 mM NaCl (see section 3.4.5). Although there was an increase in net JA and ABA content after salt pouring, there was not a significant difference between wildtype and the transgenic lines (Figure 25 A, B and C). The net content of ABA in seedlings grown on sterile media containing 100 mM NaCl was also quantified. Again, although there was an increase in the net content of ABA after the seedlings had been subjected to salt stress, there was no significant difference between Col-0 and *RNAi:LLP1-3* (Figure 25 D). This indicates that the signalling aberration in the JA network does not occur due to changes in the biosynthesis of either JA, SA, or ABA, and that any crosstalk occurs in the pathways downstream of phytohormone biosynthesis.

Thus, the three LLP protein appear to promote JA-associated salt tolerance in a process that is occurring downstream of JA accumulation.

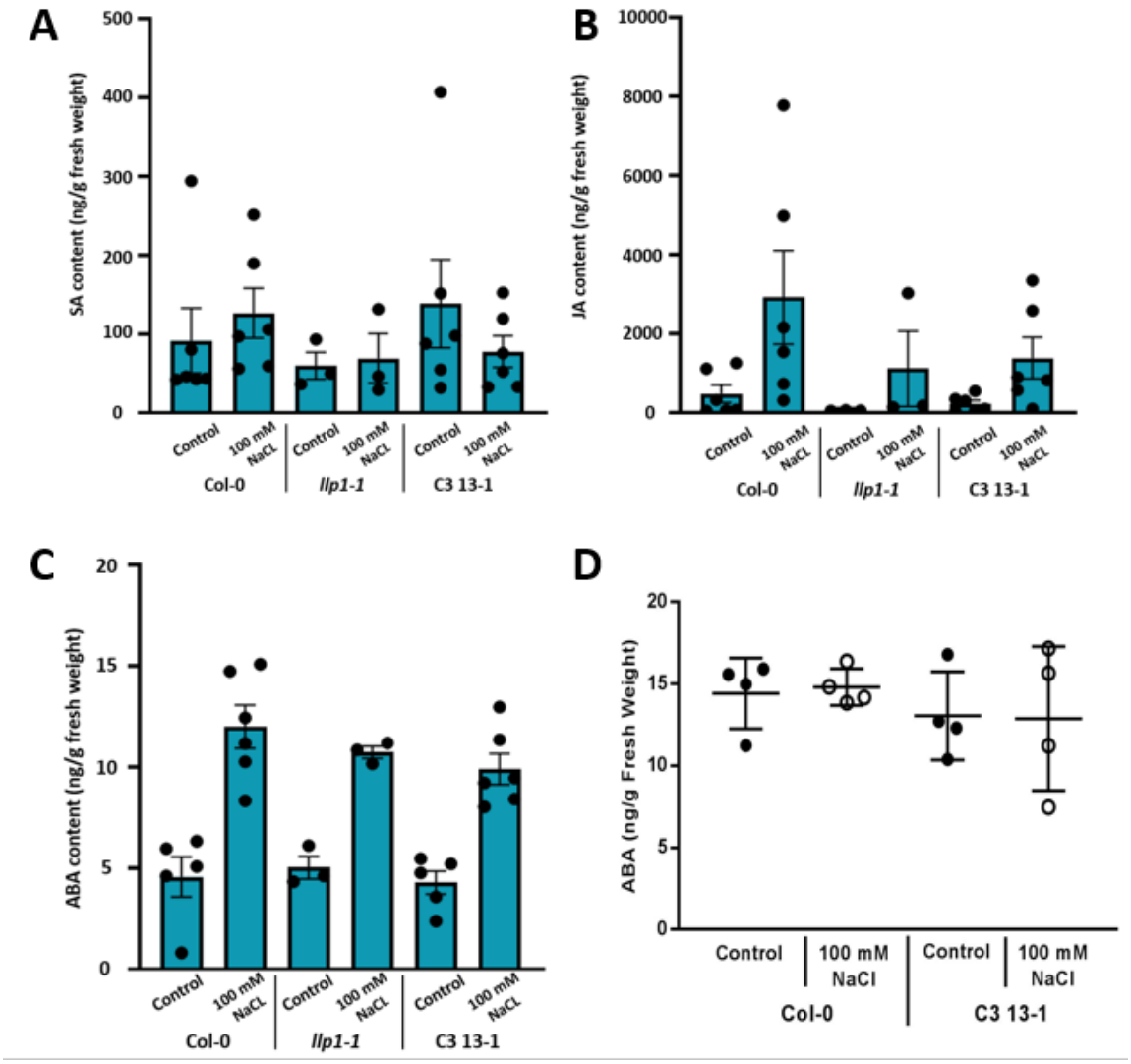

Figure 25: Transgenic lines show no significant difference in key phytohormone content after treatment by salt pouring. (A-C) 3 week old plants form either wildtype(Col-0), *llp1* mutants or *RNAi:LLP1-3* (C3 13-1) were treated with 100 mM NaCl pouring for 12 days, and then tissues were harvested and flash frozen for LC-MS analysis. (A) SA content does not significantly change after salt treatment. (B) JA content increases in all lines after salt treatment. The transgenic lines show a trend that is not significant, towards lower JA levels. (C) Both wildtype and transgenic lines show a comparable increase in ABA content after salt treatment. For (A-C) LC-MS was performed by Richard Hammerl and Corinna Dawid. (D) ABA content does not change in seedlings grown on MS with 100 mM NaCl for 12 days. LC-MS was performed by Wilfried Rozhon and Brigitte Poppenberger. Shown is average ±SEM, for Col-0 and C3 13-1 n=6, *llp1-1* n=3. Differences were analysed with Kruskal-Wallis test (for non-parametric data).

3.7 Crosstalk between JA and SA signalling pathways is misregulated in RNAi:LLP1-3 plants

From the above experiments, the *RNAi:LLP1-3* plants show a different level of JA marker gene transcript accumulation under abiotic stress, and different levels of SA marker transcripts under certain types of biotic stress. The SA and JA signalling pathways are known to have multiple points of interaction, normally resulting in antagonistic interactions (Glazebrook, 2005; Pieterse *et al.*, 2012). We therefore investigated whether *PR1* as a marker of SA signalling, shows a different pattern of behavior than normal under abiotic stress. To this end, sterile-grown seedlings were transferred to treatment plates containing 50 µM MeJA, and *PR1* transcript accumulation was monitored 12 days after transfer. In this assay, *RNAi:LLP1-3* C3 13-1 seedlings supported a two-fold higher level of *PR1* transcript accumulation than did Col-0 or *llp1-1* (Figure 26 A). Consequently, the data shows that absence of the LLP proteins can lead to outcomes in phytohormone pathways not originally targeted, suggesting a new point of crosstalk between cascades.

PR1 misregulation was also seen after watering of mature plants with salt. Four-week-old plants were subjected to salt stress by watering with 300 mM NaCl (experiment performed with Gerardo Perez). Four days after the final treatment, leaf tissue was harvested and analysed by RT-qPCR. *PR1* transcript levels were reduced in Col-0 after salt treatment when compared to a mock control, possibly due to the antagonistic relationship between SA and either ABA or MeJA. In the *llp1-1* plants, there was a slight, but insignificant increase in *PR1* transcripts when compared to Col-0, with very little difference between NaCl- and mock-treated *llp1-1* plants. Notably, *PR1* transcript levels were upregulated by ~80-fold in salt- compared to mock-treated *RNAi:LLP1-3* C3 13-1 plants (Figure 26 B). Hence, LLP1, LLP2, and/or LLP3 might co-operate in compromising JA-associated responses to salt or associated JA-SA crosstalk events resulting in enhanced SA-associated responses in *RNAi:LLP1-3* plants in response to salinity stress.

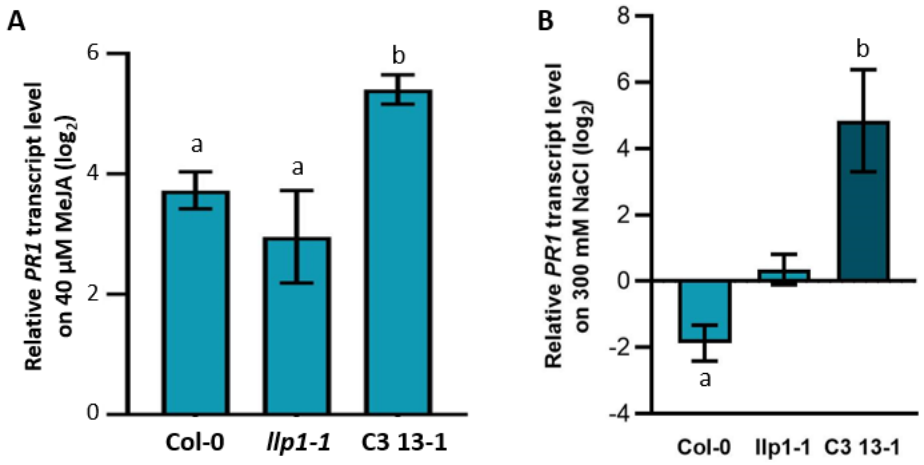

Figure 26: SA-signalling is misregulated in the absence of *LLP1-3*. A) *PR1* transcript levels are higher in *RNAi:LLP1-3* lines after MeJA treatment. Seedlings of wildtype (Col-0), *llp1* mutants or *RNAi:LLP1-3* (C3 13-1) were transferred to plates supplemented with 40 µM MeJA and harvested after 12 days for RNA extraction and RT-qPCR. Data presented are the $\log_2$ mean transcript levels relative to *UBI* normalised to mock control ±SEM (one-way ANOVA, n=4 for Col-0 and C3 13-1, n=3 for *llp1-1*). B) *PR1* transcript levels increase in plants after salt pouring. Tissues were sampled from 5-week-old plants that had been treated for 12 days with 300 mM NaCl poured directly to the soil. Data presented are the $\log_2$ mean transcript levels relative to *UBI* normalised to mock control ±SEM of 4 biologically independent experiments (one-way ANOVA).

3.7.1 MeJA can induce systemic resistance in the absence of LLP1

As MeJA associated stress was able to induce SA-dependent gene expression in *llp*-deficient plants, we investigated whether a local application of MeJA would be sufficient to reconstitute a systemic defence response in the same lines. Using a similar experimental setup to a classical SAR assay, two lower leaves were infiltrated with 100 µM MeJA, and systemic leaves infiltrated with virulent *Pst* three days later. The bacterial titres in the systemic leaves after 4 days indicated that the MeJA, while not affecting bacterial titres in wildtype, was able to reconstitute a SAR response in *llp1-1* but not the *RNAi:LLP1-3 lines* (Figure 27). This result indicates a key role of JA in LLP1-associated systemic immunity, but which is limited by the presence of either LLP2 or LLP3.

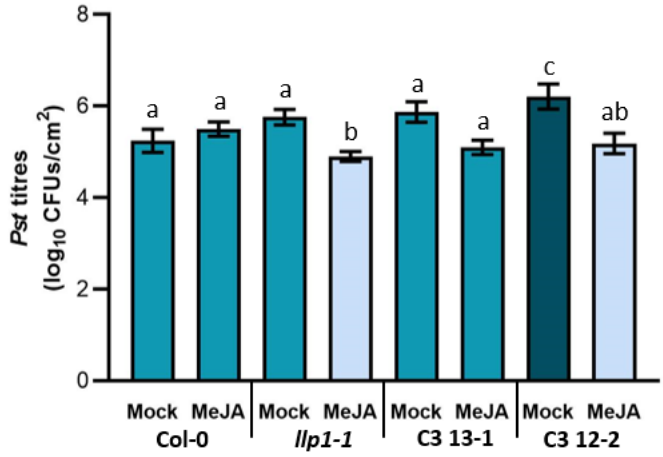

Figure 27: Local MeJA infiltration can induce systemic resistance in *llp1-1*, but not *RNAi:LLP1-3* (C3 13-1). Local leaves were infiltrated with 100 µM MeJA, and after 3 days systemic leaves on the same plant were infiltrated with *Pst*. Systemic leaves were harvested 4 days after infection, and bacterial titres quantified. Data presented are the *Pst* titres in systemic leaves ±SEM (n=11-19, four experiments for Col-0, *llp1-1*, and C3 13-1, three experiments for C3 12-2. Kruskal-Wallis test for non-parametric data).

In summary, *LLP 1, 2* and *3* appear to have non-redundant functions during both biotic and abiotic stress. Loss of *LLP3* abolishes SAR in a similar manner to the loss of *LLP1.* Both *LLP1* and *LLP3* are required for normal root growth inhibition in response to NaCl and MeJA, while *LLP3* is additionally required for normal root growth inhibition in response to exogenous ABA. This is reflected in the misregulation of downstream marker genes, including *PR1* and *PDF1.2,* but not *VSP2* or *RAB18.* There is also no change in the net content of either SA, MeJA or ABA, indicating that the interaction between the pathways occurs downstream of biosynthesis. However, MeJA appears to induce systemic resistance in the absence of *LLP1*, indicating a new possible role for JA signalling in SAR, which is mediated through the LLPs.

4. Discussion

One of the main issues facing modern society is crop security. With changes in climate comes growing demand for farmers to maximise food production under both the direct pressures of abiotic stresses such as drought and high soil salinity, as well as from previously unencountered pathogens that have expanded their ranges (Velásquez *et al.*, 2018; Simler *et al.*, 2019). This reduction in resources brings the threat of starvation, as well as civil unrest and outbreaks of violence in some of the most underprivileged countries in the world (Holleman *et al.*, 2017). To begin to mitigate these risks, better strategies must be employed to preserve crop yields in the face of multiple perils (Atkinson *et al.*, 2004; Juroszek and von Tiedemann, 2011; Storkey *et al.*, 2018). It is therefore of the utmost importance to be able to understand how plants respond to stress from multiple sources. The key to this understanding is in how the phytohormone pathways that control these stress responses interact (Wang and Irving, 2011; Altmann *et al.*, 2020).

Inducible systemic resistance responses such as SAR may present new possibilities for plant protection against both biotrophic and necrotrophic pathogens (Vlot *et al.*, 2020). However, the nature of the signalling pathways, such as the identity of the mobile SAR signal and its potential receptor, and the involvement of JA in both these pathways is still under discussion. It is also still unknown about how to maximise the strength of these defences when the plant also comes under abiotic stress. The aim of this work was to explore the involvement of three legume lectin-like proteins, LLP1, 2, and 3, in the context of systemic acquired resistance. Although LLP1 has been previously investigated to a greater extent than the other, the roles of LLP2 and LLP3, as well as the combined activity of these proteins, has yet to be fully elucidated.

In this work I show that the presence of two or more of the LLP1-3 proteins, while not needed for local immunity, is essential for the SAR response in *Arabidopsis*. These proteins are also necessary for the regulation of JA-mediated responses to salinity stress, on both a physiological and molecular level, as well as for the normal function of crosstalk between the SA and JA signalling cascades within these processes.

4.1 The role of *LLP1-3* in biotrophic defence

After the induction of ETI by avirulent *Pst*, LLP1 accumulates in an *EDS1*-dependent manner in both local and systemic tissues, although it is not required for SA-dependent defence (Breitenbach *et al.*, 2014). However, there is a marked transcript accumulation of *LLP1* induced by treatment with exogenous SA (Figure 10) or its functional analogue 1,2,3-benzothiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Breitenbach *et al.*, 2014; Pabst, 2018). EDS1 is a key regulatory protein in the SA pathway, and functions upstream of SA accumulation and signalling. However, it is also involved in SA-independent pathways, including for example *FMO1* and *NUDT7* (Bartsch *et al.*, 2006). It may therefore be the case that although *LLP1* transcript accumulation is induced by SA, it is functional in a different, SA-independent pathway. A probable candidate for this is the Pip pathway (see section 1.2.2).
The expression of *LLP2* and *LLP3* remains unaffected by exogenous SA treatment, in both a wildtype and *eds1-2* background (Figure 10). It would also seem that they are not involved in any SA-independent pathways in regard to local defence, in either an individual or compensatory capacity, as both *llp3* and *RNAi:LLP1-3* plants sustain normal growth of both avirulent (Figure 11) and virulent (Pabst, 2018) strains of *Pst.*

It was previously shown that the absence of *LLP1* is sufficient to abolish the SAR response (Breitenbach *et al.*, 2014). In this work I show that reduced transcript accumulation of *LLP3* also eradicates the SAR response, which can be reinstated by the expression of a transgene driving *LLP3* expression from its native promoter (Figure 12). Therefore, *LLP3* plays a critical role in SAR signalling, most likely via a role in the local tissue during SAR signal generation (Wenig and Vlot, personal communication). While the absence of functional *LLP1* or *LLP3* abolishes SAR, a SAR-inducing infection causes systemic susceptibility rather than resistance in *RNAi:LLP1-3* plants (Figure 13 A), suggesting additive effects of the three LLP proteins on SAR. This enhanced systemic susceptibility has not been observed in any other SAR pathway mutants, and therefore cannot be explained only by disruption of the SAR pathway. Instead, some combined function of LLP1-3 must actively inhibit defence responses in the systemic tissue while leaving local defence intact. Because only *LLP1* transcript accumulation is sensitive to SA-treatment, it is likely that *LLP2* and *LLP3*

contribute to the enhanced susceptibility because of their roles in other signalling cascades. The prime candidate for this is the jasmonate pathway, as *LLP3* displayed a transcriptional response to MeJA application in wildtype plants (Figure 21). Also, as *llp1, llp3* and *RNAi:LLP1-3* all demonstrated abnormal root phenotypes when exposed to MeJA, it may be that JA signalling interacts with SAR at multiple junctures. The *llp3* phenotype could be reverted at least partially back to wildtype-like root growth by expression of the *LLP3::LLP3* construct in the *llp3* mutant background, which confirms the role of *LLP3* in this interaction. *RNAi:LLP1*-3, but not *llp1,* also showed misregulation of gene expression in SA- and JA-responsive genes after treatment of seedlings with MeJA (Figure 23 A)(See section…). This indicates that the LLPs are involved in downstream signalling from more than one phytohormone pathway. It also indicates that at least *LLP1* and *LLP3*, as well as possibly *LLP2*, are required not only for SAR signalling, but also to prevent antagonism upon SAR signalling from other pathways.

The role of *LLP2* in this process remains undetermined. There was no increase in the transcript accumulation of this gene for any of the phytohormones tested, suggesting that if *LLP2* does have a role in SAR, or is responsible for the systemic susceptibility, it is not downstream in any of these cascades. Neither does it feedback into the regulation of their biosynthesis or activation. *LLP3* was previously shown by microarray analysis to be a strong, early-responsive gene after chitin treatment (Zhang *et al.*, 2002). In the same study it was shown that this chitin-mediated induction of *LLP2* was not dependent on either JA or SA signalling, with *jar1, npr1, pad4* and *eds5* mutants showing similar levels of expression as compared to wildtype plants. These results were verified with RT-qPCR by Ramonell *et al.* (2005). Chitin is in fact such a strong defence inducer that lobster shell extract, which contains high levels of both chitin and its deacetylated derivative chitosan, is used as a soil treatment to reduce incidence of plant disease (Ilangumaran *et al.*, 2017). This chitosan treatment had such a potent priming effect that it increased *PR1* expression by around 500-fold after *Pst* infection and *PDF1.2* by around 40-fold after *B. cinerea* infection. *MPK3* has also been highlighted as a chitin-responsive gene whose expression was not affected by the loss of JA or SA (Zhang *et al.*, 2002; Ramonell *et al.*, 2005). It may be the case that *LLP2* is required for initiating forms of resistance

that are triggered by fungal pathogens which are able to repress JA or SA signalling, and that this processes is aided by MPK3, which is also known to be involved as an upstream regulatory node in many other defence responses (Pitzschke *et al.*, 2009). As chitosan is able to behave as a PAMP and induce systemic resistance (Iriti and Faoro, 2009), the function of LLP2 may be in this specific pathway, having diverged from the role of LLP3 in mediating SA/JA/Pip-dependent responses.

SAR is mediated via two key signalling pathways: SA and Pip. For a time it was thought that the functions of these pathways were in parallel, and had minimal influence upon each other (Wendehenne *et al.*, 2014). Above, I show that *LLP1* transcript accumulation is increased in response to SA (Figure 10), despite having no role in local SA defence pathways (Breitenbach *et al.*, 2014; Pabst, 2018). However, the presence of LLP1 is crucial for SAR, and Wenig *et al.* (2019) showed that it is required specifically in systemic tissues for signal recognition, and not at the local site of infection. LLP1 is also involved in the Pip signalling pathway and in monoterpene production and sensing (Wenig *et al.*, 2019). Armijo *et al.* (2013) found that the LLP1 peptide has a glycosylation site at N129. Because glycosylation occurs on the luminal side of the ER, when the protein is translocated to the cell membrane it will be facing the apoplast. This apoplastic localisation, along with the protein only being required in systemic tissues, puts LLP1 in a prime location to act as a SAR signal receptor.

Ideally, we would further characterise the LLPs by finding their spatial and temporal expression and subcellular localisation patterns. Some work towards this has already been carried out, for example with Lyou *et al.* (2009) using GUS staining to find the tissues in which *LLP3* is expressed. They showed that while there were high expression levels of *LLP3* in the rosette leaves, primary inflorescences, and mature plant roots, but not in the cauline leaves, siliques, or secondary inflorescences. However, more detailed subcellular localisation has not been characterised so far. Although work towards this has been started (also in the frame of this thesis), it appears that overexpression of *LLPs* tagged with GFP proves lethal for the plants if the proteins are expressed from constitutive (35S) or even from estradiol-inducible (but leaky) promoters (Breitenbach, 2012). In Pabst (2018) and during this thesis

work, attempts were initiated to express *LLP1, LLP2,* and *LLP3* under their native promoters. Whereas successful in some instances (for example the *LLP3* complementation lines expressing untagged *LLP3* from its own promoter, *LLP3::LLP3,* including lines 3.01, 3.02, 4.01 and 8.01), expression levels under the native promoter appear very low (Figure 12). Transient expression of constructs containing, for example, *LLP1:LLP1-GFP* have been successfully expressed in *Nicotiana benthamiana* (Pabst, 2018), but a more detailed approach with *Arabidopsis* is still needed. Knowledge of the subcellular localisation of the LLPs, and how this changes during different stress responses, may shed further light on their function.

The potential role of LLP1 as a receptor is unlikely to involve the potential signalling molecule AzA, as treatment with exogenous AzA in local tissue can still induce systemic resistance in the *llp1-1* mutant (Figure 14). LLP1 and AzA both function downstream of Pip in SAR signalling, with Pip pouring being able to induce SAR in wildtype plants but not in the *llp1* mutant (Wenig *et al.*, 2019). Application of Pip is able to induce a SAR response in the *sid2* SA mutant, indicating that the Pip pathway is not completely dependent upon SA (Bernsdorff *et al.*, 2016). However, systemic defence induced in *sid2* is not as robust as wildtype. It is therefore conceivable that the SA pathway has an effect of bolstering the Pip pathway through the upregulation of *LLP1* in systemic tissue, leading to a stronger SAR response.

4.2 LLPs and abiotic stress

Understanding the crosstalk between multiple environmental stresses is key in moving towards the creation of crop lines that are able to show increased tolerance to abiotic stress as well as pathogen resistance. Because of the antagonism seen between ABA and SA or JA pathways, it has so far often been the case that if a plant shows increased resistance to a pathogen, then it is more susceptible to abiotic stressors such as high salinity (Anderson *et al.*, 2004; Yasuda *et al.*, 2008; Berens *et al.*, 2019). The LLPs therefore present somewhat of a rarity in that they are required for the normal growth of plants under salt stress (i.e. the phytohormone-mediated changes in metabolism and physiology that are incurred after osmotic and ionic stress from excess NaCl), as well as for necrotrophic and systemic biotrophic defence. When treated with 100 mM NaCl, *llp1-1, llp3* and *RNAi:LLP1-3* all showed a significantly

exaggerated root shortening phenotype compared to Col-0, indicating that at least two of the LLPs, probably *LLP1* and possibly to a greater extent *LLP3,* are required for normal salt tolerance (Figure 16). Salt stress also affected mature *Arabidopsis* plants, with changes in key regulatory genes being noted in both SA and JA signalling pathways, although there was no effect on biomass (Figure 26).

As mentioned previously, the key phytohormone associated with abiotic stress is ABA (Vishwakarma *et al.*, 2017). Exogenous treatment with ABA also induced a significant down regulation in the transcript levels of *LLP1,* although it did not affect transcript accumulation of *LLP2* or *LLP3* (Figure 15). It is therefore possible that the increased sensitivity of *RNAi:LLP1-3* seedlings to high NaCl is due to misregulation of the ABA signalling (Julkowska *et al.*, 2014; Waskiewicz *et al.*, 2014).
However, when seedlings were grown on plates containing ABA, only *llp3* plants and not *llp1-1* or *RNAi:LLP1-3* showed a significant reduction in primary root length compared to wildtype. This increased shortening was not observed in the *llp3-LLP3::LLP3* lines. This suggests that ABA may be able to target the signalling cascade LLP3 is involved in, but this effect is negated when LLP1 is also lost. In none of the lines was there a significant change in the accumulation of the ABA marker gene *RAB18* (Figure 20 C) (Jeannette *et al.*, 1999), suggesting that the effect of ABA on LLP3 may be routed via its interaction with a different phytohormone signalling pathway, rather than through the main ABA cascade (Valenzuela *et al.*, 2016).

The other key phytohormone that has been associated with salt stress in dicotyledonous plants is JA. JA accumulation was associated with increased salt tolerance in tomato and sweet potato (Pedranzani *et al.*, 2003; Zhang *et al.*, 2017*c*). However, it is also associated with exaggerated shortening of roots in response to salinity stress, both in *Arabidopsis* and other plant species (Staswick *et al.*, 1992; Brodersen *et al.*, 2006; Wu *et al.*, 2015; Valenzuela *et al.*, 2016). The role of JA regarding salt tolerance in monocotyledonous species is not so clear, with it apparently protecting wheat, while increasing the susceptibility of rice to exposure to increased salt levels (Qiu *et al.*, 2014; Peethambaran *et al.*, 2018). To explore whether the root shortening phenotype that was displayed in *llp1, llp3* and *RNAi:LLP1-3* when grown on high salt media was due to misregulation of JA signalling

rather than of ABA signalling, seedlings were also grown on media containing MeJA. On these plates the transgenic lines exhibited exactly the same phenotypic response as on salt, with *llp1, llp3* and *RNAi:LLP1-3* all exhibiting significantly enhanced primary root shortening as compared to that observed in wildtype plants. This effect was mitigated by *LLP3* expression in the *llp3* mutant (Figure 22). As it appears that a signal between LLP1 and LLP3 may be required for systemic defence (Wenig and Vlot, personal communication), the enhanced root shortening under high salinity may also be dependent on both the presence and the interaction of *LLP1* and *LLP3*, and possibly *LLP2* with JA-mediated signalling.

JA activates two separate signalling pathways, depending upon which other signals/factors are detected at the same time (Figure 28**:**). This allows the plant to use JA to fine-tune responses to multiple stresses (Kazan and Manners, 2012). One of the key transcription factors is MYC2 (Figure 28**:**), which orchestrates a wide variety of downstream responses, including the action of other transcription factors (Kazan and Manners, 2013). Aside from its role in the JA pathway, MYC2 acts as a functional activator for ABA signalling (Abe *et al.*, 2003), and is closely associated with abiotic stress responses (Zhu, 2002; Christmann *et al.*, 2006), and herbivore defence (Dombrecht *et al.*, 2007). It is therefore possible that the increased sensitivity of *RNAi:LLP1-3* seedlings to high NaCl was due to misregulation of the ABA signalling pathways by MYC2 in the *RNAi:LLP1-3* genetic background. However, this explanation can be excluded, as the MYC2 marker gene, *VSP2*, in a similar manner to *RAB18,* showed no significant difference in *RNAi:LLP1-3* after salt pouring (Figure 20 C). It therefore is unlikely that the jasmonate responses seen are due to MYC2 regulation.

The alternative JA pathway is regulated by ERF1. In salt signalling, the particular pathways involved in stress tolerance are dependent upon the action of MED25 in complex with PHYTOCHROME AND FLOWERING TIME 1 (PFT1) (Çevik *et al.*, 2012). This complex can bind to both ERF1 and MYC2, as well as transcription factors involved in ABA signalling. Downstream, ERF1 targets DRE motifs, found in the promoters of salt-tolerance genes and thereby activates them (Cheng *et al.*, 2013).

It can also feedback into the MED25/PFT1 complex, further enhancing salinity-tolerance mechanisms (Figure 28**:**) (Kazan, 2015). *PDF1.2* is a downstream marker gene for ERF1 signalling, and is thought to have a role in salinity tolerance as it is constitutively upregulated in salt-adapted species such as salt cress (Taji *et al.*, 2004). I therefore tested its transcript accumulation in seedlings after MeJA treatment. Although *llp1-1* showed a similar response to wildtype, *RNAi:LLP1-3* showed significant decreases in the accumulation of *PDF1.2* transcripts in response to MeJA treatment, indicating that either one or more of the LLPs is involved in the ERF1 signalling cascade.

There is also a possibility that this pathway could involve ethylene (ET) signalling, which was not investigated in this work. Ethylene is known to have an important role in salinity tolerance (Cao *et al.*, 2007). Loss of the EIN2 receptor, which functions

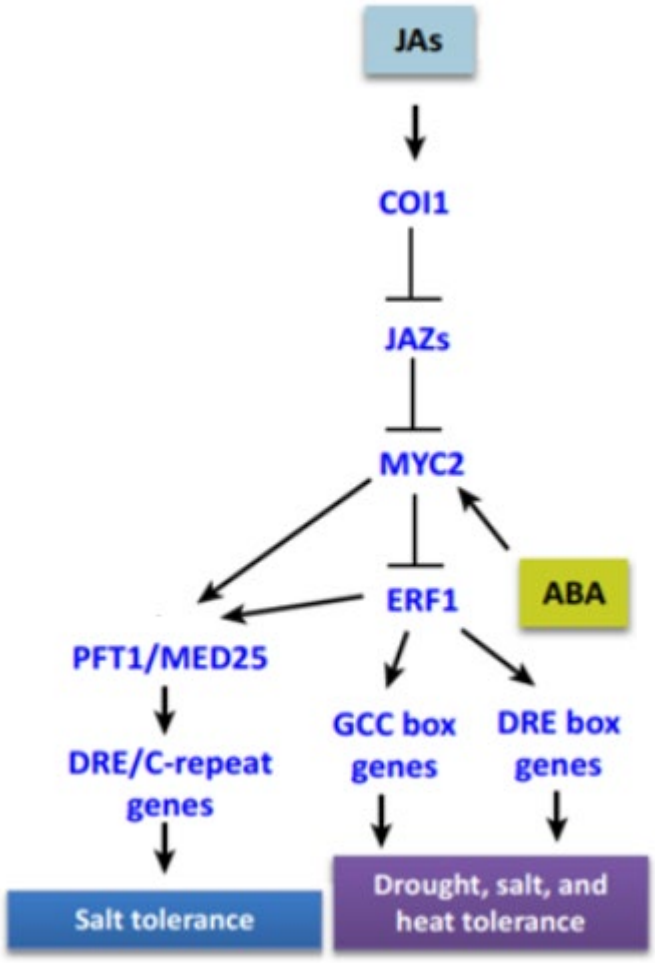

Figure 28: JA signalling is mediated through two key transcription factors, MYC2 and ERF1. These pathways are mutually antagonistic, but both can positively regulate tolerance to high salinity. After COI1 recognises a JA signal and lifts repression by JAZ proteins, MYC2 and ERF1 can both complex with the mediator protein MED25, and recognise specific motifs, such GCC or DRE boxes, in the promoters of genes associated with salt tolerance. ABA is also able to feed into these pathways via MYC2. Adapted from Kazan (2015).

upstream of EIN3 and ERF1 in ET signalling, induces severe salt sensitivity in *Arabidopsis* (Cao *et al.*, 2007). This could theoretically be responsible for salt-induced changes in *PDF1.2* transcript accumulation, as this is a marker gene for the ERF1 pathway. The JA pathway converges with that of ET in EIN3 and ERF1 (Figure 29, Lorenzo *et al.*, 2003; Takaoka *et al.*, 2018). Therefore, we cannot definitively say whether JA or ET is the causal signal for the change in *PDF1.2* transcript levels (Figure 23). ET-mediated signalling has a strong negative regulatory effect on the function of MYC2 (Song *et al.*, 2014). Ergo if ET was involved in the change in stress responses observed in *llp1-1, llp3* and *RNAi:LLP1-3,* then it should also have had an effect on the levels of MYC2-dependent *VSP2* transcript accumulation. The fact that we did not see this suggests that the impact of ET signalling on LLP function is minimal.

LLP1 is known to have an important role in the pathway leading to monoterpene synthesis and propagation of VOC emission during PTP SAR, as well as in the Pip-associated systemic SAR pathway. To identify whether one or both of these roles are associated with the role of LLP1 in salt tolerance, I performed root assays using media supplemented with both NaCl and MeJA for two different monoterpene synthesis mutants and a Pip biosynthesis mutant. These mutants have all been shown to be

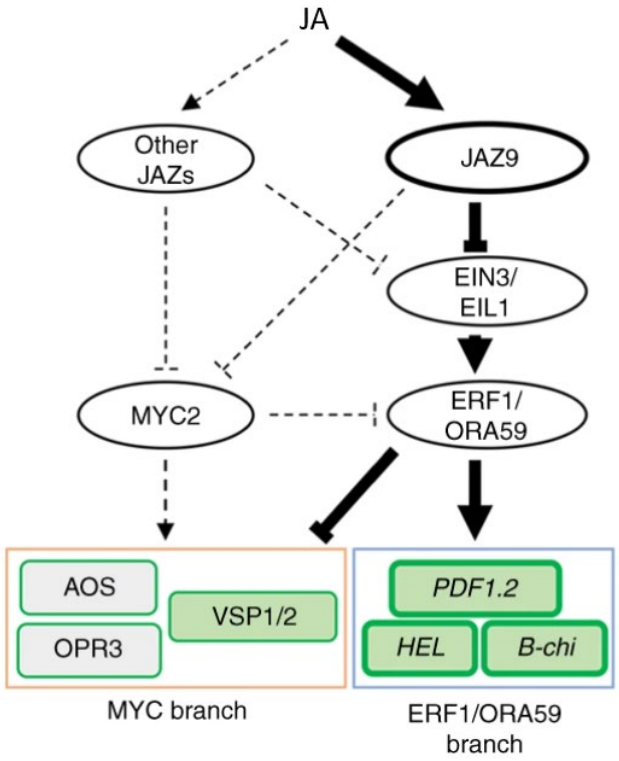

Figure 29: JA signalling can occur via two pathways mediated by different transcription factors. After repression by JAZ9 is lifted from EIN3, there is signal transduction via ERF1 that results in the upregulation of genes including PDF1.2. This pathway antagonises that mediated by MYC2, and thus inhibits the upregulation of marker genes such as *VSP2*. Adapted from Takaoka et al 2018.

unable to perform PTP signalling (Wenig *et al.*, 2019). The exaggerated root shortening phenotype seen in *llp1* was either lacking or reduced in the Pip-synthesis mutant *ald1* and in the monoterpene synthesis mutants *tps24-1* and *tps24-2*(Figure 24). This indicates that the pathways controlling Pip signalling and monoterpene transmission, and therefore *LLP1*'s role within them, is not involved in salt tolerance. However, it also suggests that *LLP1*, along with either *LLP2* or *LLP3,* acts in a separate pathway that can be induced by jasmonates and is involved in salt stress tolerance. *LLP1* therefore has distinct and separate roles in multiple stress response pathways (Sales *et al. in preparation*; Breitenbach *et al.*, 2014; Wenig *et al.*, 2019). A role of *LLP1* in multiple stress responses brings into question the current model that SAR is induced via two separate and distinct pathways, as well as the dogma that SA- and JA-pathways always are mutually antagonistic. An issue with past work into the elucidation of phytohormone function has been to consider them as separate pathways. More recently, their interaction has begun to be considered more as a signalling network, where multiple pathways are interconnected (Altmann *et al.*, 2020). This allows a more subtle view of interactions, albeit while requiring a more bioinformatics-based approach.

My experiments indicate that in the *RNAi:LLP1-3* plants, when under salt stress, root growth is inhibited to a greater extent than in wildtype, a result that can be phenocopied by treatment of the same plants with exogenous MeJA (Figure 22). In addition, *llp1, llp3* and *RNAi:LLP1-3* plants have an increased susceptibility to the necrotrophic pathogen *A. brassicicola* (Pabst, 2018). This would indicate that there is a misregulation in signalling at a point upstream of one of JA's two key pathway regulators, MYC2 and ERF1. A key function of ERF1 is defence against necrotrophic pathogens (Van Wees *et al.*, 2003). As *VSP2* transcript levels, which is a marker of the MYC2 pathway, remain unchanged, while *PDF1.2* levels, which is a marker of the ERF1 pathway, are reduced, it leads to the conclusion that *LLP1-3* have a role in ERF1-mediated JA signalling. *LLP3* is the only gene out of the *LLP*s studied here that shows increased transcript accumulation in response to MeJA treatment (Figure 21), while the *llp3* mutant displayed increased root shortening on media containing MeJA (Figure 22) and increased susceptibility to the necrotroph *A. brassicicola* (Pabst,

2018). Therefore, it would seem that *LLP3* is primarily responsible for interacting with JA signalling, thus presenting a previously unidentified role for LLP3 in integrating JA pathways with systemic defence and abiotic stress tolerance. However, although its transcript levels are unresponsive to exogenous MeJA, *llp1* mutants also displayed enhanced MeJA-induced root shortening and susceptibility to necrotrophs (Figure 22, Pabst, 2018). It is therefore possible that *LLP3* is involved in signalling with *LLP1* during salt stress, in a similar manner to the dynamic that is seen during systemic defence, and thus *LLP1* is affected by JA via *LLP3*. Additionally, *llp3* is the only line to show exaggerated ABA-induced root shortening (Figure 20). As function of ERF1 is likely to be ABA-independent (Cheng *et al.*, 2013), any effect of ABA on *LLP3* could be acting to fortify the original signal from jasmonate signalling to mediate salinity tolerance.

4.3 Jasmonates in SAR

If the LLPs are needed for JA-mediated signalling in salt stress, then could they also be responding to JA signals during systemic defence signalling? The role of JA in regards to SA-mediated defence and SAR is rather convoluted. There is much evidence to suggest that this interaction is mainly antagonistic in nature, most likely through the influence of JA on MPK4 (Figure 0) (Petersen *et al.*, 2000; Thatcher *et al.*, 2005; Glazebrook, 2005; Brodersen *et al.*, 2006; Spoel *et al.*, 2007; Van Der Does *et al.*, 2013).

However, it has more recently been shown that the response is highly dependent on concentration, spatial distribution, and circadian rhythms (Mur *et al.*, 2005; Betsuyaku *et al.*, 2018; Karapetyan and Dong, 2018). Additionally, during RPS2-mediated ETI the accumulation of SA and downstream signalling through the NPR3 and NPR4 receptors initiates *de novo* JA synthesis (Liu *et al.*, 2016), and the SA sector in *Arabidopsis* immune networks activated in PTI is dependent upon the JA sector (Hillmer *et al.*, 2017). Given that PTI and ETI have some convergent signalling pathways, including through SA accumulation (Sun *et al.*, 2015; Peng *et al.*, 2018), it is likely that JA may have a more important role in biotrophic immunity than has

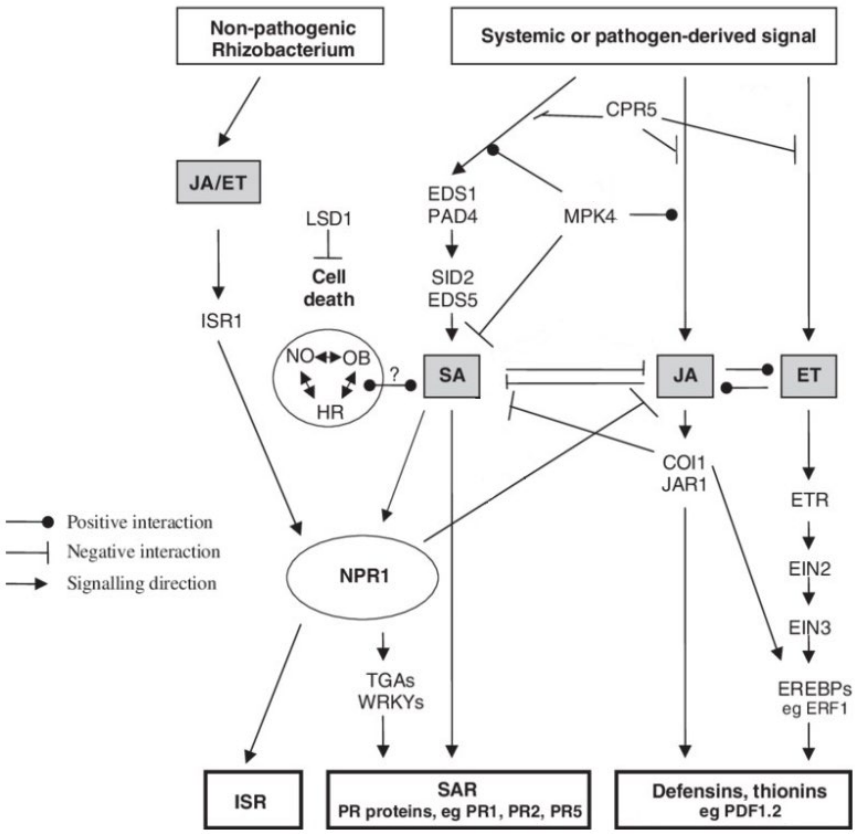

Figure 30: An overview of previously identified points of interaction between SA and JA pathways after induction of systemic defences by microbes. SA can repress JA signalling both directly and via NPR1, while JA can also supress SA directly and via COI1 signalling. (ETR = Ethylene Receptor, LSD1 = Lesion Simulating Disease1). Adapted from Thatcher et al. 2005.

been traditionally recognised. If we also take into account the possible and debated contribution of JA to SAR (Truman *et al.*, 2007; Attaran *et al.*, 2009), and the induction of SAR by SA application in *llp1-1* plants (Pabst, 2018), then it seems that the LLPs may be responding to a JA signal rather than a SA signal (Figure). The multiple branches of the JA pathway mean that it not only must be activated correctly, but if there is incorrect activation then it may further antagonise itself (i.e. by the action of MYC2 on ERF1). Therefore, an interruption in JA rather than SA signalling may be responsible for the observed inverted SAR phenotype in *RNAi:LLP1-3* plants (Fig. 2A, Figure 1). This is further supported by the induction of *LLP3* transcript accumulation after exogenous MeJA treatment (Figure 21). Additionally, when *RNAi:LLP1-3* seedlings were exposed to MeJA, there was an increase in the expression of *PR1*, a key SA marker gene. This increase was further compounded in mature plants that were treated by salt pouring for 12 days, and subsequently showed a pronounced upregulation of *PR1* compared to both wildtype and *llp1-1* (Figure 26). Therefore, JA and abiotic stress regulate these proteins in a way that can potentially directly affect biotrophic defence pathways.

In a recent paper, Betsuyaku *et al.* (2018) showed that during ETI, in the area directly affected by programmed cell death and HR there is active SA-signalling. However, a small circumference of cells surrounding the HR lesion shows active JA signalling, highlighting a strong spatial component to the phytohormones involved in local immunity. The role of LLP3 in SAR has been shown to be limited to the local tissues, with functional LLP3 being required for SAR signal generation but not recognition (Wenig and Vlot, personal communication; Sales *et al., in preparation*). As *LLP3* expression is also inducible by JA, it may be that LLP3 has some function in sensing the JA signal from the HR response, and then initiating signalling towards generation of a mobile SAR signal (Figure 1).

In this work, I show that while a local MeJA treatment did not induce changes, either negative or positive, in systemic responses to *Pst* infection in Col-0, *llp3* or *RNAi:LLP1-3*, it was able to reinstate a systemic defence response in *llp1-1* (Figure 27). The reason for this systemic induction is not immediately clear. It has been shown that MeJA is phloem-mobile (Thorpe *et al.*, 2007), so it is possible that the exogenous MeJA provided in the experiment is moving directly to the distal tissues. JA signalling has been associated with the biosynthesis of some monoterpene molecules (Filella *et al.*, 2006; Taniguchi *et al.*, 2014). Although it has never been directly associated with pinenes, perhaps the MeJA signal is able to induce VOC production downstream of LLP1, and thus kickstart the systemic response. However, if LLP3 is involved in the sensing or transport of a jasmonate signal in the local tissues, then locally applied MeJA cannot be mobilised in plants were the LLP3 protein is compromised (*llp3* or *RNAi:LLP1-3*). Additionally, LLP1 cannot act to recognise a potential signal resulting from LLP3 function in local tissues, and we do not see the induction of systemic resistance in the *llp3* or *RNAi:LLP1-3* lines where both LLP1 and LLP3 are compromised (Figure 27). This indicates that a potential role of LLP1 in the systemic tissues is able to recognise a JA-dependently produced signal from the local tissues, which would therefore confirm the necessity of JA in SAR responses.

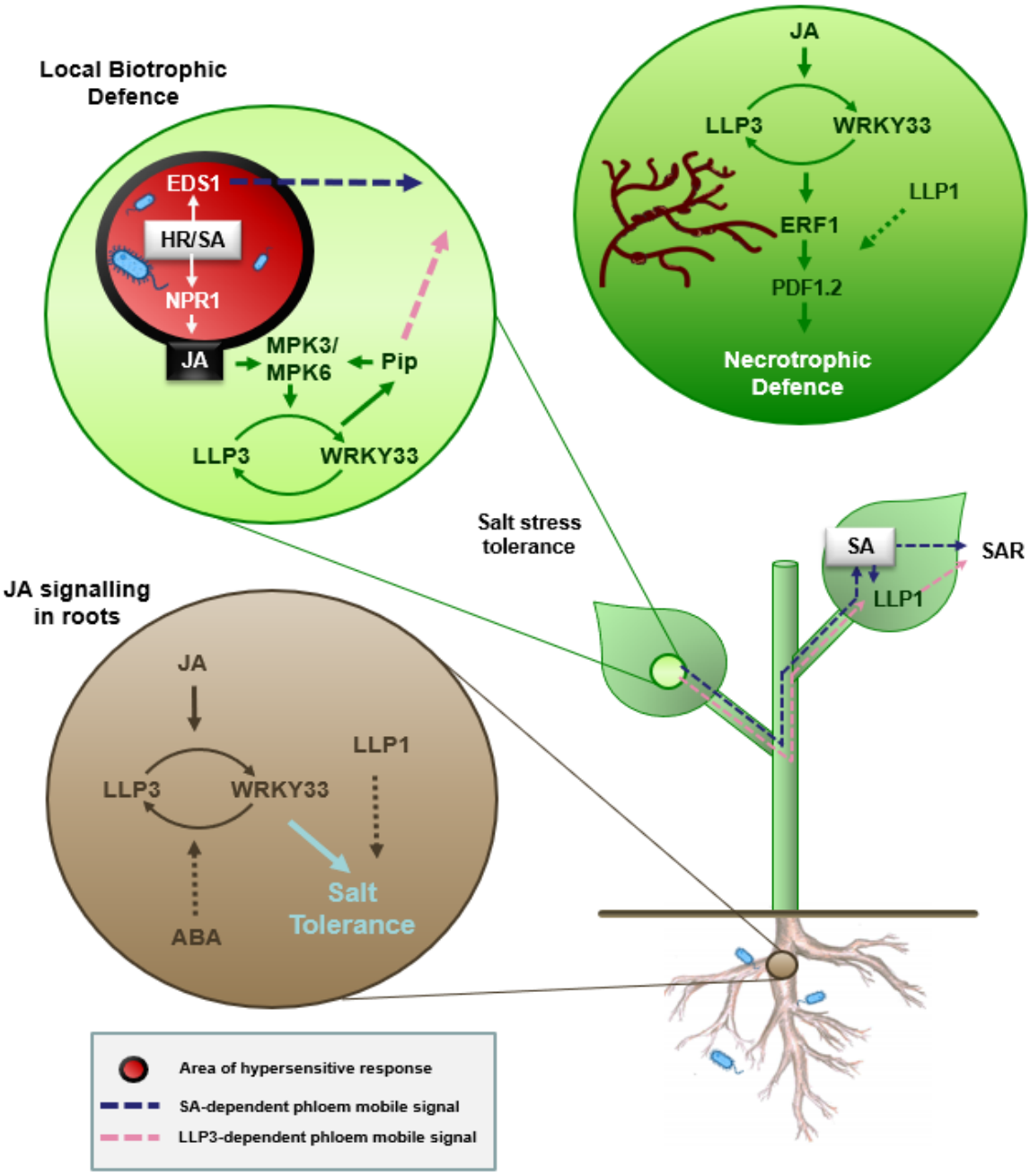

Figure 31: An overview of the function of LLPs within systemic signalling and salinity tolerance. LLP3 is required in local tissues to generate a SAR signal. It may function in a pathway with WRKY33 and Pip that is sensitive to JA produced in the perimeter of HR lesions. LLP1 is required for recognition of the SAR signal in systemic tissues. Both LLP1 and LLP3 are required for necrotrophic defence and salinity tolerance. It is possible that the role of LLP3 in these processes is again in combination with WRKY33, as both proteins are known to be required for all responses.

4.4 The possible involvement of LLP3 and WRKY33

LLP3 is only required for local signal generation and not for systemic signal recognition during SAR (Wenig and Vlot, personal communication; Sales *et al., in preparation*). In *llp3* plants SAR is lost, but we do not see the systemic susceptibility observed in the *RNAi:LLP1-3* lines (Figure 13). The *llp3* mutant also shows exaggerated root growth inhibition under both high salinity and JA treatment (Figure 17, Figure 22), and this phenotype is lost by ectopic expression of *LLP3* from its native promoter. Although some work has previously been conducted with the *llp3* mutant, until now only a single mutant line had been available, and thus it had not been possible to prove that any results were due to loss of LLP3 function and not because of a secondary T-DNA insertion at a different genomic site. The results shown in this work using *LLP3::LLP3* complementation lines in the *llp3* mutant background allow first conclusions about biological functions of LLP3. As discussed above, my data suggest that LLP3 significantly contributes to the regulation of cross talk between SAR and JA signalling, because of the *llp3* mutants' compromised systemic resistance and salt sensitivity, and the misregulation therein of both *PDF1.2* and *PR1*. Notably, *LLP3* is also the only gene studied here that affected root shortening in response to ABA. This indicates that not only does *LLP3* have a different function to *LLP1*, but it may also be a point of interaction between SAR and ABA signalling (Figure 1). It is therefore unlikely that it is functioning in SA-MeSA homeostasis, and not directly in the Pip-AzA-G3P cascade, as these are required in both local and systemic tissues (Vlot *et al.*, 2020).

One of only a few known SAR pathways that are required solely for SAR signal generation is the feedback loop between Pip, MPK3/6 and WRKY33 (Figure 3) (Wang *et al.*, 2018*b*). This is an interesting coincidence, because, similarly to LLP3, WRKY33 also plays key roles in necrotrophic defence and tolerance to high salinity.

WRKY33 is a transcription factor with a wide range of predicted targets (Petersen *et al.*, 2008). It therefore has the potential to be involved in the regulation of a range of biological processes. After a *B. cinerea* infection, around 2600 genes were differentially regulated in *wrky33* compared to Col-0 (Liu *et al.*, 2015). This only accounted for around 30% of predicted WRKY33 targets. Also, although WRKY33

binding motifs were enriched in the promoters of various *PRR* genes, the *wrky33* mutant did not show an altered local defence response against *Pst* (Zheng *et al.*, 2006). This may indicate that for transcriptional control of some WRKY33 targets cofactors are required. Concomitantly, necrotrophic defence is severely compromised in *wrky33,* with the mutant plants showing increased susceptibility to *B. cinerea* and *A. brassicicola* (Zheng *et al.*, 2006; Birkenbihl *et al.*, 2012). In a resting state, WRKY33 forms a complex with other proteins and MPK4. After activation of defence mechanisms, MPK4 releases WRKY33, and it can then be directly phosphorylated by MPK3 and MPK6 (Andreasson *et al.*, 2005; Qiu *et al.*, 2008; Mao *et al.*, 2011). WRKY33 has a negative regulatory effect on most of its downstream targets, with the exceptions of a set of genes required for camalexin biosynthesis. Although camalexin is not immediately accumulated in systemic leaves during SAR, there is a priming effect, and biosynthesis increases more rapidly upon a secondary infection (Návarová *et al.*, 2012; Gruner *et al.*, 2013).

In a similar manner to ERF1, LLP1, and LLP3, while WRKY33 positively regulates necrotrophic defence, it negatively regulates responses against herbivory and wounding (Dombrecht *et al.*, 2007). It thus has the opposite role to MYC2. In fact, MYC2 can have a repressive effect on WRKY33 in a similar way to ERF1. However, the role of WRKY33 in supporting necrotrophic defence does not rely on the antagonism between JA and SA-signalling, as is seen in some other stress responses, because the *wrky33* mutant does not show any changes in susceptibility to *B. cinerea* infection compared to *wrky33sid2* double mutant (Birkenbihl *et al.*, 2012). As *LLP3* also appears not to reply to SA-regulation, nor in turn affects SA-signalling, it also shares this commonality with WRKY33.

WRKY33-mediated pathways are also associated with enhanced salt-stress tolerance. For example, WRKY33 was shown to be induced shortly after salt treatment, and acts upstream of the Cytochrome P450 gene *AtCYP94B1*, which enhances suberin deposition which acts as an apoplastic diffusion barrier in seedlings to improve salinity tolerance (Krishnamurthy *et al.*, 2020). The *wrky33* mutant exhibits enhanced sensitivity to salt stress, suggesting a possible redundancy with other transcription factors (Jiang and Deyholos, 2009).

The different phenotypes associated with misregulation of WRKY33 signalling are very similar to those seen in the *llp3* mutant. *llp3* shows compromised systemic but not local defence against biotrophic pathogens, susceptibility to necrotrophic pathogens, sensitivity to ABA and salt stress, and misregulation of ERF1-mediated JA signalling (Figure 11, Figure 13, 17, 20) (Pabst, 2018). It is also sensitive to JA treatment, possibly to repress the antagonism by ABA that is needed for a successful necrotrophic response. However, the NaCl-induced transcription of WRKY33 is partially ABA-dependent (Jiang and Deyholos, 2009). I would therefore suggest that LLP3 is involved in a regulatory feedback loop with WRKY33, that also involves Pip and MPK3/6, and is responsible for the generation of the systemic SAR signal. Only downstream signalling targets of this loop are dependent on NPR1 and EDS1 (Kim *et al.*, 2020). This would explain why there is no difference in *LLP3* expression upon treatment with exogenous SA, JA and ABA in the *eds1-2* mutant as compared to wildtype plants, as the induction of LLP3 is likely acting upstream of EDS1 (Figure 10, Figure 15, Figure 21). However, the recognition of this signal in the systemic tissue requires LLP1. Although LLP1 is primarily dependent on Pip signalling, during local defence it also requires input from the SA pathway via EDS1 (Breitenbach *et al.*, 2014). Therefore, there is a distinct possibility that the systemic role of LLP1 is also dependent on *EDS1*, which is supported by the SA-induced upregulation of LLP1 transcripts in an *EDS1*-dependent manner (Figure 10). The increased susceptibility seen in systemic tissues when both LLP1 and LLP3 are lost might be due not only to the loss of the signal which induces enhanced defence, but also because of misregulation through WRKY33 which stops the repression of antagonism between the SA and JA phytohormone pathways. An interaction of LLP3 with WRKY33 would also explain why the *llp3*, but not the *llp1* mutant, showed increased sensitivity in root growth to exogenous ABA treatment, as well as to NaCl and MeJA. It is possible that in this context, the LLP3-WRKY33 interaction is regulated upstream by MPK4 rather than MPK3 and MPK6, allowing finetuning of defence responses against either abiotic or pathogen stress.

It has also been shown with other genes that WRKY33 is able to recruit duplicated genes into its regulon through recognition of transposable elements in the enhancers of the respective genes. This, for example, includes the retrotransposon *EPCOT3* in

the enhancer region of *CYP82C2* (Barco *et al.*, 2019). The high sequence homology between *LLP2* and *LLP3* suggests that they may have diverged as a result of a sequence duplication event, and thus WRKY33 may mediate signalling through both these proteins, although LLP2 needs further characterisation.

4.5 Conclusion

The complexity and extent of crosstalk between hormone cascades, different patterns of induction between biotic and abiotic stress, and the gaps in knowledge regarding the exact intricacies of these interactions provide an important area for further research. The involvement of LLPs in multiple pathways demonstrates a potential target for crop improvement against not only pathogen attack, but also increasing salt stress faced in agriculture. Their feature of being positively involved in biotrophic, necrotrophic and salt stress defences provides an opportunity to tackle multiple stresses, which is a unique feature amongst systemic immunity mutants.

Because of the differences in phytohormone-induced transcriptional responses and their additive role in systemic defence, it appears that LLP1, 2, and 3 may have different functions in SAR (Figure 1).

- I hypothesise that *LLP1-3* respond to a jasmonate signal produced by salt stress and in leaf tissue surrounding areas of infection after HR. This signal is then relayed through the *ERF1* pathway, and affects salt tolerance, systemic immunity and necrotrophic defence. *LLP3* plays an important part in this pathway, as I have now shown that it has a role separate from that of *LLP1* in systemic resistance and abiotic stress tolerance, which can be reverted back to wildtype by expression of an *LLP3:LLP3* transgene in the *llp3* mutant background.
- It is possible that this role is JA-dependent, SA-independent, and supported by ABA signals, and functions in concert with WRKY33 to control systemic signalling responses in different tissues.
- The role of *LLP1* in systemic resistance has been confirmed and is also shown to be necessary individually for salinity tolerance in an ABA-independent manner.

- The loss of *LLP1* and *LLP3* has a direct negative effect on SAR through the loss of signal generation and perception. However, the interaction with other phytohormone pathways, such as LLP3 with ABA, imparts an extra layer of negative regulation, and thus results in enhanced systemic susceptibility.

This work highlights the differences in the functions of *LLP1* and *LLP3*, despite their high sequence homology. Although *LLP2* may also be involved in these responses, the lack of a single mutant or transgenic line precludes us from making definite conclusions surrounding its function. The potential multiple roles of *LLP1* and *LLP3* in phytohormone signalling may also allow them to act as signalling nodes within SAR to integrate both the SA- and Pip-dependent systemic defence signals, thus creating an interwoven network necessary for SAR, as well as between biotic and abiotic stress response pathways.

5. Outlook

This work suggests that the Legume Lectin-like Proteins 1, 2, and 3 are key nodes in SAR and abiotic stress signalling. They could therefore be promising candidates for therapeutic targets against crop stresses. However, before this could be a possibility, more research is required into the function of these proteins.

For some of the assays I performed, data from the complementation lines is missing. For example, we do not yet know whether there is an effect individually of *LLP3* on the change in net phytohormone content. We saw no change in SA, JA or ABA content in the *RNAi:LLP1-3* plants after salt treatment, so the loss of *LLP3* in conjunction with the other *LLPs* does not have an effect, but this experiment should be repeated with the *llp3* single mutant and the complementation lines.

The exact position of the LLPs in the different signalling cascades remains to be defined. Many membrane-bound lectins, such as RLPs, can act as receptors, but do not individually have any enzymatic activity. For this they require the interaction with other accessory transmembrane proteins, which they are able to activate and thus propagate a signal to the interior of the cell (Wang *et al.*, 2008; Jamieson *et al.*, 2018). Given the apoplastic localisation of LLP1 (Armijo *et al.*, 2013), but the lack of predicted domains other than the predicted carbohydrate binding site, it is highly likely that LLP1 acts in concert with other, as yet unidentified proteins. Therefore, identifying these proteins would be the next logical step in understanding the role of LLP1, and potentially also LLP2 and 3, in phytohormone signalling. In this thesis work, an initial attempt was made to find protein-protein interaction partners of LLP1 by using a yeast-2-hybrid (Y2H) screen (together with Melina Altmann and Pascal Falter-Braun, Helmholtz Zentrum München). Unfortunately, this did not provide any reproduceable interaction partners. Ergo, it may be necessary to utilise another protein-protein interaction screen, such as by co-immunoprecipitation and then verify any potential interactions with a split-ubiquitin system (Müller and Johnsson, 2008; Avila *et al.*, 2015; Xing *et al.*, 2016). These pipelines would also utilise mass spectrometry approaches, and could be used to put any LLP interactions in the context of microdomains or the cell membrane interactome (Chen and Weckwerth, 2020).

It may also be interesting to use a yeast-1-hybrid screen to identify which transcription factors interact with the *LLP* promoters. Specifically, WRKY33 and the main transcription factors in JA signalling, such as ERF1 and EIN3 (Solano *et al.*, 1998), would be of interest for *LLP3,* and NPR1 with TGAs for *LLP1* (Fan and Dong, 2002). This may help to understand at which point in phytohormone cascades the *LLPs* act. As the LLPs are thought to have cell membrane localisation, it is unlikely they themselves directly control transcription, and thus it would not be necessary to perform a Y1H in the other direction. In sum, these approaches may help to understand at which point in phytohormone cascades the *LLPs* act.

Further characterisation of the LLPs could involve investigating whether any of the proteins undergo posttranslational modifications, such as phosphorylation or glycosylation (Arsova *et al.*, 2018). To look for phosphorylation sites, a targeted proteomics with chromatography and mass spectrometry could be utilised. Tandem mass spectrometry and fragmentation techniques could be used for identification of *N*-glycosylated residues (Ford *et al.*, 2015).

Although experiments using *RNAi:LLP1-3* plants start to give us some idea, the exact function of *LLP2* remains unknown. There are no T-DNA insertion lines available for this gene, and RNAi-mediated silencing of the gene without at the same time affecting *LLP3* has not proved possible so far. A different approach could be to use CRISPR/CAS_9 gene editing to create a *llp2* single mutant, and thus better elucidate the function of *LLP2* in both SAR and potentially other stress responses. However, as efforts to mutate or silence *LLP2* have been unsuccessful so far (Pabst and Vlot, personal communication), there is the possibility that LLP2 plays a core function in *Arabidopsis,* for example in development*,* and so any disruption of the gene may be lethal in plant embryos. The high sequence homology of *LLP2 and LLP3* could indicate similar functions/interactions. As ABA is critical in the regulation of seed germination (Schopfer *et al.*, 1979). It may be that LLP2 is playing a role in ABA interactions during development, while LLP3 is recognising a similar signal, albeit it during defence induction. This may explain why *LLP2* but not *LLP3* mutation appears to be embryo lethal.

Ideally, we would further characterise the LLPs by finding their spatial and temporal expression and subcellular localisation patterns. Transient expression of constructs containing, for example, *LLP1:LLP1-GFP* have been successful in *Nicotiana benthamiana* (Pabst, 2018), but a more detailed approach with *Arabidopsis* is still needed. Knowledge of the subcellular localisation of the LLPs, and how this changes during different stress responses, may shed further light on their function.

The final and most important stage in characterising the functions of the LLPs would be the discovery of the ligands for their binding sites. Identification of the LLP1 ligand would be greatly helpful in understanding whether LLP1 is directly acting as a receptor for a mobile SAR signal, or if it is sensing signals in a local capacity, possibly from cell-to-cell communication, or feedback into the Pip pathway. Once a potential ligand is identified, further parameters such as binding affinity, kinetics, and ligand specificity could be investigated. There are a range of techniques, such as MicroScale Thermophoresis, which could be used in this endeavour (Sandoval and Santiago, 2020). It would also be important to characterise the physiological function and long-distance mobility of the potential ligand, for example through petiole exudate experiments.

It would be of interest to investigate the involvement of *LLP1-3* with other phytohormone pathways, such as ET. As discussed above, ET can also interact with jasmonate signalling. One could specifically investigate a possible role of ET by treatment of seedlings with aminocyclopropane-1-carboxylic acid (ACC), an ethylene precursor, and by observing *LLP1-3* transcript accumulation after ACC treatment in mature plants.

The role of *LLP3* in abiotic stresses other than that imposed by high salinity may also be of interest. A protein that is involved in supporting not only biotic defence and salt stress tolerance, but also protection against drought, extreme temperatures or water logging, would be an important agrochemical target. *llp1* and *RNAi:LLP1-3* were already included in progressive drought assays testing parameters such as water consumption and water use efficiency, with neither line showing a difference from wildtype (Liu and Grill, personal communication). However, *llp3* was the only mutant line to show sensitivity to exogenous ABA in seedlings, and it may therefore be

worthwhile to see if this mutant responds differently in responses which are primarily influenced by ABA, such as drought stress tolerance (Takahashi *et al.*, 2020). This could clarify the extent of the importance of *LLP3* in ABA pathways.

While knowledge of signalling pathways in SAR in *Arabidopsis* can often have potential applications for other economically useful dicotyledonous species, such as members of the *Brassicaceae* and *Solanaceae*, many of today's important crops are monocotyledonous. Therefore, before potential therapeutic applications can be developed for crops such as wheat and barley, SAR must be better understood in monocots. The *LLPs* have no direct homologous in, for example barley. However, many of the components of the signalling pathways they are known to interact with do. Pip and NO, which regulate systemic signalling dependently upon LLP1, are involved in systemic resistance in barley (Lenk *et al.*, 2019). Additionally, SA and NPR1 signalling and homologues of WRKY33 have been found in monocots (Zhou *et al.*, 2015*b*), highlighting potential further involvement of *LLP*-associated pathways. If identification of LLP paralogues in monocots was possible after further characterisation of the proteins, then this may be able to better inform strategies on crop protection.

Overall, further characterisation of the subcellular interactions of the LLPs is required. Particular attention should be paid to LLP2, as very little is currently known about its function. Additionally, the identification of the LLP1 ligand could be a key step in fully elucidating SAR pathways. Nevertheless, the identification of the LLPs as key nodes in multiple phytohormones signalling pathways represents an important shift in our understanding of the interaction of SAR with abiotic stress. It may also provide a route by which both biotrophic and abiotic defences could be simultaneously activated, without compromising either, thereby solving one of the main problems in developing plants suitable for changing environments.

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