Lehrstuhl für Biochemische Pflanzenpathologie

# "*Arabidopsis thaliana* legume lectin-like proteins at the interface between systemic acquired resistance and abiotic stress responses"

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## II ABBREVIATIONS

| | |
|---|---|
| bp | Base pairs |
| cfu | Colony forming units |
| DNA | Deoxyribonucleic acid |
| dpi | days after infection |
| dsDNA | double stranded DNA |
| dNTPs | Deoxynucleoside triphosphates |
| DC3000 | *Pseudomonas syringae* pv. *tomato* DC3000 – virulent |
| h | Hour(s) |
| hpi | hours after infection |
| JA | Jasmonic acid |
| Kb | Kilobases |
| kDa | Kilodalton |
| LB | Luria-Bertani |
| ml | Milliliter(s) |
| min | Minute(s) |
| mM | Millimolar |
| M | Molar |
| nm | Nanometers |
| nt(s) | Nucleotide(s) |
| OD | Optical density |
| O/N | Overnight |
| PCR | Polymerase chain reaction |
| *Pst* | *Pseudomonas syringae* pv. tomato (DC3000) |
| qRT-PCR | Quantitative real time polymerase chain reaction |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| RT | Room temperature |
| SA | Salicylic acid |

| | |
|---|---|
| WT | Wild-type |
| Y2H | Yeast-Two-Hybrid |
| µl | Microliter(s) |
| µM | Micromolar |
| $\lambda_{ex}$ | Extinction wavelength |
| $\lambda_{em}$ | Emission wavelength |

Amino acids and nucleotides are expressed according to the IUPAC code (International Union of Pure and Applied Chemistry). All base units and derived units are used following the convention of the SI-system (Système International d'unitès).

**III Summary**

Systemic acquired resistance (SAR) is a salicylic acid (SA)-dependent, long-lasting defence response and occurs in the systemic healthy tissue of locally infected plants. *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) encodes one of the main regulators of SA signalling and is essential for SAR signal generation. New SAR regulatory proteins, such as the LEGUME LECTIN-LIKE PROTEIN1 (LLP1), a predicted carbohydrate-binding protein, were examined. Mutations in *LLP1* and RNAi-mediated silencing of its two closest homologues *LEC1* and *LEC2* compromised SAR but not local resistance to *Pseudomonas syringae*. These data suggest that *LEC1*, *LEC2* and *LLP1* specifically promote systemic resistance. SAR induction in transgenic plants undergoing RNAi-mediated silencing of *LEC1*, *LEC2* and *LLP1* resulted in the induction of systemic susceptibility to *P. syringae*, suggesting that *LEC1*, *LEC2* and *LLP1* act additively during SAR. Strikingly, the same transgenic plants also displayed phenotypes that are associated with jasmonate (JA) and abscisic acid (ABA) signalling, which are thought to antagonize SAR. Considering that the lectins were positively associated with the defence response to a necrotrophic pathogen, these results suggest that *LEC1*, *LEC2* and *LLP1* promote synergism between SA and JA. ABA treatment reduced the transcript accumulation of *LEC1, LEC2 and LLP1* in Col-0, and *LEC1* and *LEC2* in *eds1-2*, but induced *LLP1* in *eds1-2.* Hence, *LLP1* might be negatively influenced by EDS1 under ABA treatment. Furthermore, it seems that the plant response to high salinity was negatively influenced by the lectins and this occurred in an EDS1-independent manner. Together, the data suggest an inverse correlation between *LEC1*, *LEC2* and *LLP1* and the defence response to salt stress. In turn, reduced accumulation of all three lectins might lead to an exaggerated ABA response during SAR, resulting in the observed enhanced systemic susceptibility. In conclusion, the legume lectin-like *Arabidopsis thaliana* lectins LEC1, LEC2, and LLP1 might directly act upon the pathways of the phytohormones SA, JA, and ABA and could also affect cross talk between these pathways, thereby fine tuning systemic immunity.

Die systemisch erworbene Resistenz (SAR) ist ein natürlicher und langanhaltender Schutzmechanismus von Pflanzen welcher durch die Salizylsäure vermittelt wird und in den systemisch gesunden Blättern von lokal infizierten Pflanzen stattfindet. Das *EDS1*-Gen kodiert dabei einen der Hauptregulatoren des Salizylsäure-Signalweges und ist essentiell für die Signalerzeugung während der SAR. Es wurden SAR-regulierende Proteine untersucht, wie das LEGUME LECTIN-LIKE PROTEIN1 (LLP1), welches wahrscheinlich Kohlenhydrate binden kann. Mutationen in *LLP1* und posttranskriptionelles Gen-Silencing der zwei homologen Gene *LEC1* und *LEC2* führten dazu, dass die SAR unterdrückt wurde, jedoch nicht die lokale Resistenz gegen *Pseudomonas syringae*. Dies lässt darauf schließen, dass *LEC1*, *LEC2* und *LLP1* gezielt die systemische Resistenz

unterstützen. Die Induzierung der SAR in transgenen Pflanzen bei denen durch RNA-Interferenz die Transkripte der Gene *LEC1*, *LEC2* und *LLP1* vermindert werden, zeigt eine systemische Empfindlichkeit gegenüber *P. syringae*. Daher wird vermutet, dass *LEC1*, *LEC2* und *LLP1* während der SAR additiv fungieren. Bemerkenswerterweise zeigen die gleichen transgenen Pflanzen, dass sie auch in den Signalwegen der Jasmonsäure (JA) und der Abscisinsäure (ABA) eingebunden sind, welche der SAR eigentlich entgegenwirken. Berücksichtigt man, dass während der Immunantwort gegen nekrotrophe Pathogene, die Lektine positiv assoziiert sind, kann man davon ausgehen, dass *LEC1*, *LEC2* und *LLP1* das Zusammenwirken von SA und JA fördern. Die Behandlung von ABA setzt die Gen-Induzierung von *LEC1*, *LEC2* und *LLP1* in Col-0 und von *LEC1*, und *LEC2* in *eds1-2* herab. Allerdings wird *LLP1* in der *eds1-2* Mutante induziert, was darauf schließen lässt, dass *LLP1* bei einer Behandlung mit ABA von EDS1 negativ beeinflusst wird. Des Weiteren scheint es, dass die Immunantwort der Pflanze bei Salzstress von den Lektinen negativ beeinflusst wird, wobei dies nicht von EDS1 abhängt. Somit deuten die Daten auf eine entgegengesetzte Korrelation zwischen *LEC1*, *LEC2* und *LLP1* und der Immunantwort gegen Salzstress hin. Im Gegensatz dazu führt der Mangel aller drei Lektine wahrscheinlich zu einer gesteigerten ABA-Immunantwort während der SAR. Dies macht sich verstärkt in der systemischen Anfälligkeit bemerkbar. Zusammenfassend lässt sich sagen, dass die Lektine LEC1, LEC2 und LLP1 vermutlich direkt auf die Biosynthesewege der Phytohormone SA, JA und ABA einwirken, sowie auf deren gegenseitigen Wechselbeziehungen zueinander und somit die systemische Immunität feinsteuern.

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# 1. Introduction

## 1.1 Plant defence

Terrestrial plants must be able to sense and respond to environmental changes. To this end, they developed an immune system with substantial defence strategies (Jones and Dangl, 2006; Tsuda and Somssich, 2015; Reimer-Michalski and Conrath, 2016). Plants are able to directly react to pathogen attack and they have adopted several strategies against invading microorganisms. The most common defence response exhibited by plants against the majority of pathogenic microorganisms is non-host resistance (NHR) (Cheng et al., 2012; Senthil-Kumar and Mysore, 2013). Multiple factors like plant structure and chemistry form the first barriers and impede non-host pathogens from penetrating the plant tissue or even to localise the plant. On-site pathogens need an appropriate source of nutrients to develop and proliferate. Hence, the metabolic status of the plant can selectively define its host/nonhost status (Stam et al., 2014). Natural openings like stomata, hydathodes or wounds in the epidermal layer are putative entries for some non-host pathogens. When these pathogens reach the apoplastic space, which is the major battleground in plant-bacteria interactions, plant defence mechanisms are activated to restrict non-host pathogen multiplication (Stam et al., 2014; Gill et al., 2015).

Reciprocally, pathogens developed many ways to infect plants by suppressing the defence response of the plant (Jones and Dangl, 2006; Asai and Shirasu, 2015). This co-evolution of plants and their associated microbes leads to a diverse array of exchanged signals and responses (Bent, 1996; Huot et al., 2013; Tsuda and Somssich, 2015) (Figure 1).

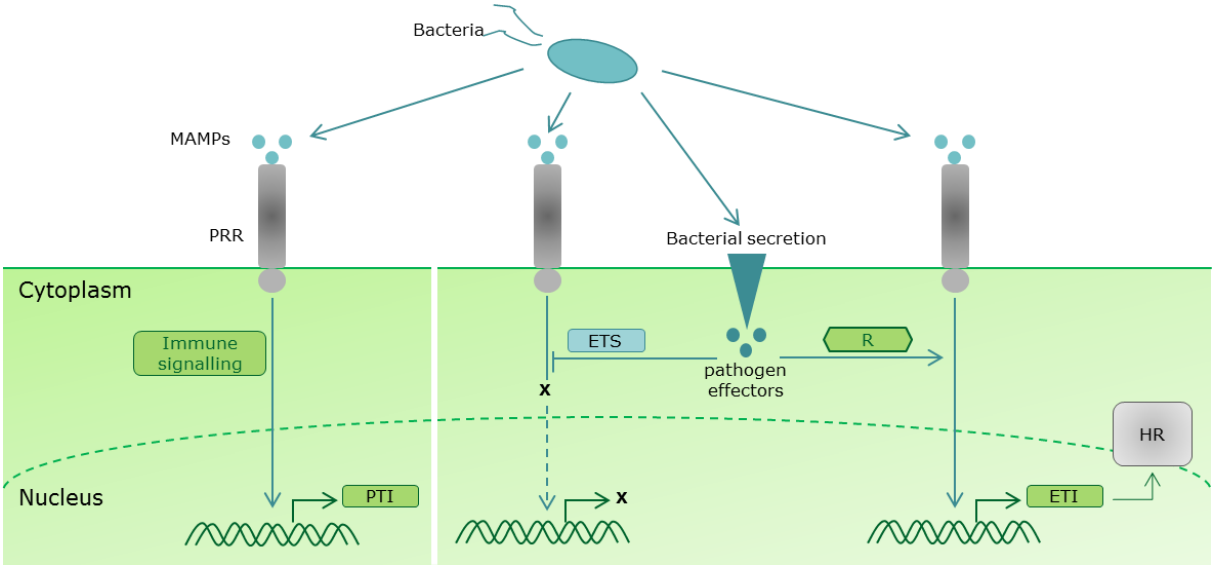


**Figure 1: Multi-layered immune system**

Model of plant disease resistance that illustrates the evolved interplay of plant and pathogens to supress and overcome each other's responses; left: immune response triggered by PAMPs; middle: Suppression of immune response by pathogen effectors; right: Immune response triggered by effectors (MAMPs: microbe-associated molecular patterns, PRR: pattern-recognition receptors, PTI: PAMP-triggered immunity, ETS: effector-triggered susceptibility, R: pathogenesis-related proteins, ETI: effector-triggered immunity, HR: hypersensitive response)

The recognition of common features of (host-adapted) microbial pathogens activates a primary immune response. These elicitors are called pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) (Jones and Dangl, 2006; Macho and Zipfel, 2014). These PAMPS or MAMPS activate pattern-recognition receptors (PRRs) to activate a basal resistance that is called PAMP-triggered immunity (PTI) (Figure 1, left) (Chisholm et al., 2006). PRRs are able to promote resistance to most unadapted pathogens and contribute to basal immunity during infection (Zipfel, 2014). PRR-triggered PTI can avert multiple microbes, due to the conserved nature of PAMPs (e.g., fungal chitin, bacterial flagellin) across species, families, or classes of pathogens (Hamdoun et al., 2013). As a result of natural selection, pathogens evolved effectors that suppress PTI and lead to effector-triggered susceptibility (ETS). These successful pathogens are repressing basal immunity and thus the expression of defence genes (Figure 1, middle). In turn, plants evolved immune responses to recognize specific pathogen effectors by RESISTANCE (R) proteins and activate effector-triggered immunity (ETI), an amplified version of PTI (Figure 1, right). ETI often results in hypersensitive cell death (HR), which is necessary to keep the pathogen isolated in the infected tissue (Jones and Dangl, 2006; Rajamuthiah and Mylonakis, 2014). Studies suggest that the kinetic differences between ETS and ETI are dependent on the doses of the pathogen (Hamdoun et al., 2013). PTI and ETI include the accumulation of PATHOGENESIS-

RELATED (PR) proteins and synthesis of secondary metabolites, including salicylic acid (SA). PTI and ETI further trigger the production of ROS (reactive oxygen species) and NO (nitric oxide) as well as cell wall strengthening (Silipo et al., 2010; Torres, 2010; Wu et al., 2014). The latter will be discussed in detail in section 1.5 The induction of systemic acquired resistance.

## 1.2 The phytohormone salicylic acid – one among others

Various plant hormones are involved in plant defence and play their role in plant immune signalling during stress responses. In addition to salicylic acid (SA) the phytohormones jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) primarily regulate plant defence to pathogens.

SA plays a substantial role in the regulation of various biological processes throughout the entire lifespan of the plant (Liu et al., 2015). The defence mechanisms PTI and ETI, for example, lead to an accumulation of SA, which in turn activates defence mechanisms against pathogen attack (Dempsey et al., 2011). Low concentrations of SA may enhance the antioxidant capacity in plants, but high concentrations of SA may cause cell death or susceptibility to abiotic stresses (Hara et al., 2012). SA is also important for proper plant growth, development, ripening and flowering (Rivas-San Vicente and Plasencia, 2011; Hara et al., 2012).

Enhanced SA levels induce NONEXPRESSOR OF *PR* GENES1 (NPR1)-mediated expression of *PR* genes and confer enhanced resistance (Shah and Zeier, 2013). NPR1 is present as an oligomer in the cytosol but is monomerized upon an increased SA level due to SA-triggered changes in cell redox potential and the direct binding of SA to NPR1 (Spoel and Dong, 2012; Janda et al., 2015). This facilitates the localization of NPR1 to the nucleus where it interacts with specific transcription factors to regulate gene expression including the induction of *PATHOGENESIS RELATED 1 (PR1)* (Veloso et al., 2014). In addition to mediating these signalling events downstream of SA, NPR1 is part of a homeostatic feedback loop regulating SA signalling by suppressing *ICS1 (ISOCHORISMATE SYNTHASE1)* expression (Figure 2) (Lu, 2009; Gangadharan et al., 2013).

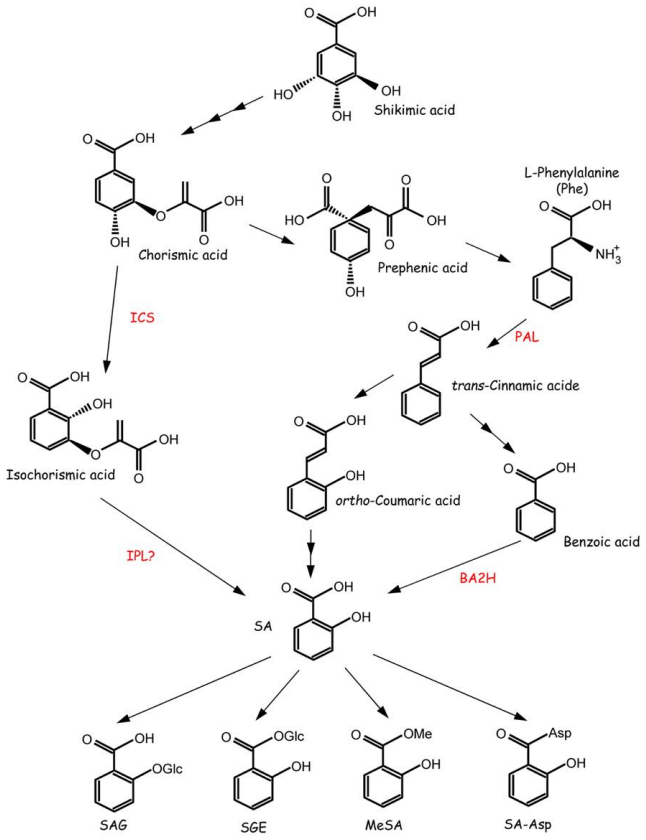


**Figure 2: Proposed biosynthesis of SA and its modification**

SA can be synthesized through the isochorismate synthase (ICS) or phenylalanine ammonia-lyase (PAL) pathway. BA2H: benzoic acid-2-hydroxylase, IPL: isochorismate pyruvate-lyase, SAG: salicylic acid 2-O-β-glucoside, SGE: salicyloyl glucose ester, MeSA: methyl salicylate, SA-Asp: salicyloyl-L-aspartic acid. Illustration was adapted from Miura and Tada (2014).

SA biosynthesis can occur via the ICS- or the PAL (PHENYLALANINE AMMONIA LYASE)-pathway (Figure 2). The precursor of both pathways is chorismic acid which is the end product of the shikimate pathway and is synthesized in the plastid. The ICS pathway appears to be the major route to synthesize SA in plants upon pathogen infection and is also used by bacteria to produce SA (Wildermuth et al., 2001; Zheng et al., 2015). Chorismic acid is converted to IC (isochormismic acid) by isochorismate synthase (ICS). Isochorismate pyruvate lyase (IPL) may catalyze the conversion of IC to SA, but this remains unclear because no plant gene encoding a protein with IPL activity has been identified yet (Fragnière et al., 2011; Miura and Tada, 2014). PAL is a key regulator of the phenylpropanoid pathway and is induced by various biotic and abiotic stress conditions. It deaminates phenylalanine, leading to the production of trans-cinnamic acid, which is a precursor for the biosynthesis of diverse phenolic compounds (Dempsey et al., 2011; Shine et al., 2016). SA can be formed from cinnamate via free benzoic acid or through *o*-coumaric acid (Chen et al., 2009). To avoid high concentrations of free SA after defence responses, SA can be modified in different derivatives, such as SAG (salicylic acid 2-O-β-glucoside), SGE (salicyloyl glucose ester), MeSA (methyl salicylate)

or SA-Asp (salicyloyl-L-aspartic acid) (Figure 2). SAG is an inactive storage form of SA and can be transported actively from the cytosol into the vacuole (Vlot et al., 2009). The functions of the minor glycosylated conjugate SGE remain unknown. The biologically inactive methyl salicylate (MeSA) is directly produced from SA in plants and subsequently released as a volatile compound after pathogen or herbivory damage (Shulaev et al., 1997; Groux et al., 2014). It has been shown that MeSA plays a role as a signal in tobacco plants, inducing resistance in systemic uninfected plant parts (Park et al., 2007). The most noted SA-amino acid (AA) conjugate is salicyloyl-L-aspartate (SA-Asp)(Ponzio et al., 2013). It is the dominant stable SA-AA conjugate, as only SA-Asp conjugates have been detected in plants. Exogenous treatment with SA-Asp did not induce *PR1* expression indicating that SA-Asp might be an inactive form of SA (Dempsey et al., 2011).

Beside SA, jasmonic acid (JA) belongs to the main signalling molecules in plant defence regulation against biotic stresses and against necrotrophic pathogens in particular (Proietti et al., 2013). JA is an oxylipin and is synthesized via the octadecanoid pathway (Santino et al., 2013). Its metabolic derivatives, known as jasmonates, are important plant signal molecules and include the isoleucin conjugate JA-Ile and the volatile methyl-jasmonate (MeJA). JA-Ile is the active form of JA and seems to play the major role in jasmonate signalling (Bektas and Eulgem, 2014). MeJA is an ubiquitous JA-derivate and the reaction is catalyzed by S-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (Seo et al., 2001). The volatile and lipophilic MeJA plays a role in defence and many diverse developmental pathways and is produced after necrotrophic pathogen attack for internal defence and as a signalling compound to other plants (Cheong and Choi, 2003; Wasternack and Hause, 2013).

The gaseous hormone ethylene (ET) is known to regulate multiple physiological and developmental processes in plants, such as senescence, fruit ripening and organ abscission (Ent and Pieterse, 2012). ET plays an important role in the primary response to pathogen attack. Mutants disrupted in ET signalling display enhanced susceptibility to necrotrophic pathogens (Shin et al., 2014; Broekgaarden et al., 2015).

The phytohormone abscisic acid (ABA) is an isoprenoid compound and is synthesized from β-carotene via several enzymatic steps and appears to be regulated through a calcium-dependent phosphorylation pathway (Dong et al., 2015). ABA is critical for plant growth and development and is associated with seed dormancy (Gonzalez et al., 2015; Zhao et al., 2015). It plays an essential role in defence regulation against abiotic stresses, including cold stress, drought responses and osmotic stress (Yoshida et al., 2015; Eremina et al., 2016; Sah et al., 2016). Drought stress triggers an increase of ABA, which initiates a signalling cascade to close stomata and reduce water loss (Savchenko et al., 2014). Also during the early stages of pathogen attack, ABA can

positively regulate plant defence by the mediation of stomatal closure against invaders. If the pathogen evades the first line of defence, callose deposition can be induced by ABA (Alazem and Lin, 2015). In contrast, ABA deficiency promotes the activity and nuclear localization of the R proteins SNC1 (SUPPRESSOR OF NPR1-1, CONSTITUTIVE 1) and RPS4 (RESISTANCE TO PSEUDOMONAS SYRINGAE 4), which mediate ETI against *Pseudomonas syringae* (*P. syringae*) (Mang et al., 2012).

## 1.3 Phytohormones – crosstalk in plant resistance

To adapt to environmental changes, plants often rely on elaborate signalling networks regulated by phytohormones interacting with each other. In nature, plants have to cope with various stresses at once, such as defending themselves from pathogens while handling a drought period. Each stress elicits a complex cellular and molecular response system in order to prevent damage and ensure survival, leading often to the impairment of growth and yield (Nguyen et al., 2016).

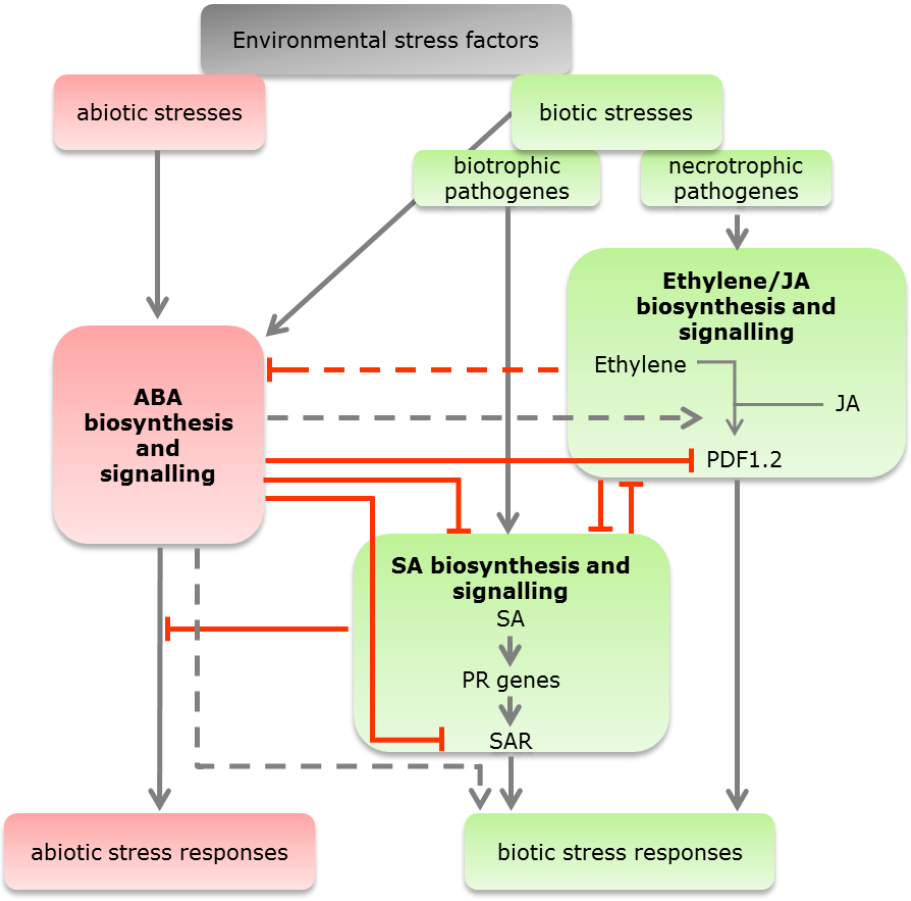


**Figure 3: The crosstalk of plant hormones in regulating the interaction in biotic and abiotic stress.**

This figure shows crosstalk occurring between hormones and other regulatory components when biotic and abiotic stresses occur concurrently. Grey arrows show induction or positive regulation, while red bars show inhibition or repression. Events characteristic of abiotic stress responses are shown in pink, while those

characteristic of biotic stress responses are shown in green. Dashed lines indicate indirect impact. ABA: abscisic acid, JA: jasmonic acid, PDF1.2: plant defensin 1.2, SA: salicylic acid, PR: pathogenesis-related, SAR: systemic acquired resistance. Figure modified from Atkinson and Urwin (2012).

Defence responses to biotic aggressors are regulated by antagonistic responses between the SA and JA pathways (Schmiesing et al., 2016) (Figure 3). ET mainly acts synergistically with JA (Häffner et al., 2015) (Figure 3). Both SA and JA biosynthesis are induced by biotic stress, whereby SA is mainly associated with the defence against biotrophic and hemibiotrophic pathogens and JA is induced by necrotrophic pathogens and herbivores (Proietti et al., 2013; Yang et al., 2015). These two important phytohormones act antagonistically and synergistically whereby ET is required for different processes in both pathways (Caarls et al., 2015). The regulation of the JA-responsive *PLANT DEFENSIN1.2* (*PDF1.2)* requires both JA and ET activation (Figure 3) whereby the induction of the SA-dependent *PR1* is supported by ET (Eshraghi et al., 2014; Broekgaarden et al., 2015). The SA- and JA-signalling interact mainly antagonistic (Caarls et al., 2015; Yang et al., 2015). Mutants, which are impaired in SA accumulation, show enhanced gene expression responses to JA (Gupta et al., 2000). Vice versa, SA treatment suppresses the gene expression of JA-responsive marker genes like *PDF1.2* and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*) (Leon-Reyes et al., 2010; Caarls et al., 2015). In *Arabidopsis*, SA-signalling is activated upon infection with *P. syringae* and this leads to a suppression of JA-dependent defence, thus making plants more susceptible to necrotrophic pathogens, including the fungus *Alternaria brassicicola* (Spoel et al., 2007; Wittek et al., 2015). To avoid the SA-dependent defence response, *P. syringae* produces a JA-mimic that leads to a suppression of the SA signalling in the plant (Geng et al., 2014). Studies on tobacco plants impaired in the SA- or JA- pathway suggest that JA may modulate early components of the SA pathway, but it is unknown how JA regulates SA biosynthesis and resistance in compatible interactions (Zhu et al., 2014).

ABA biosynthesis is mainly triggered by abiotic stresses, leading to an ABA signalling and stress response. It is assumed that ABA acts both synergistically and antagonistically with biotic stress signalling, whereby ABA acts mostly as a negative regulator of JA- and SA-mediated disease resistance (Yasuda et al., 2008; Proietti et al., 2013; Nguyen et al., 2016). ABA is therefore likely to be central in the fine-tuning of stress responses to simultaneous biotic and abiotic factors. ABA antagonizes JA and ET defence signalling by inhibiting the induction of JA/ET-dependent defence genes such as *PDF1.2* (Yang et al., 2013). ABA signalling, in turn, is negatively regulated by ET-dependent induction of various transcription factors (Asselbergh et al., 2008). It is known that ABA treatment represses systemic acquired resistance (SAR), which is a SA-dependent long-lasting defence mechanism against a variety of microorganisms. The SAR signalling pathway is

negative regulated by ABA both upstream and downstream of SA by suppressing BTH-induced *PR1* gene expression but also the *PR2* expression induced by BTH (Yasuda et al., 2008). ABA also restrains the accumulation of crucial defence compounds (Ding et al., 2016). In turn, induction of SAR leads to the suppression of abiotic stress responses (Yasuda et al., 2008). Although ABA is an inhibitor of JA and SA signalling, it can also have a positive effect on biotic stress responses (Asselbergh et al., 2008; Mang et al., 2012). As mentioned above, ABA induces stomatal closure as a biotic defence strategy and this requires intact SA signalling (Savchenko et al., 2014). Together, the involvement of ABA likely depends on the time of infection and the nature of the attacker (Alazem and Lin, 2015).

This complex network of interactions allows plants to respond in a highly specific fashion to the exact combination of environmental stresses encountered.

## 1.4 Systemic acquired resistance

Systemic acquired resistance (SAR) is an induced defence mechanism that confers a long-lasting protection against a broad spectrum of microorganisms. It occurs in the systemic healthy tissue of locally infected plants (Durrant and Dong, 2004; Kumar, 2014; Gao et al., 2015). The initial infection leads to an elevated SA level and the production of various mobile immune signals (Fu and Dong, 2013). This long-distance signalling involves networking between multiple vascular-translocated signalling molecules which then lead to SA-accumulation in the uninfected parts of the plant (Hayashi et al., 2013). There, the enhanced SA level causes the secretion of PR proteins like PR1 that induce a defence response in the systemic healthy tissue against further pathogen infections (Niu et al., 2016). During SAR, transcriptional reprogramming takes place (Shah and Zeier, 2013), which leads to a defence initialisation against recurrent pathogen attack (Bernsdorff et al., 2015). Genome-wide microarrays revealed changes in the gene expression in systemic healthy leaf tissue of locally infected plants (Gruner et al., 2013). The SAR regulator FLAVIN-DEPENDENT MONOOXYGENASE1 (FMO1) is essential for this transcriptional reprogramming during SAR which is also dependent on the transcription cofactor NPR1 and its associated transcription factors (Gruner et al., 2013; Kuai et al., 2015). Studies revealed that genes which are positively regulated during SAR are associated with SA defence, signal transduction and transport (Gruner et al., 2013). Genes which are negatively regulated during SAR are mostly activated via the JA/ET-defence pathway. Also, genes associated with cell wall remodelling and biosynthesis of constitutively produced secondary metabolites are negatively regulated during SAR in systemic healthy leaf tissue (Gruner et al., 2013). Elevated SA levels in systemic uninfected leaves also lead to chromatin modifications, DNA methylation or changes in small interfering RNAs that prime immune-related genes for increased expression after

subsequent pathogen attack and thus establish immune memory (Jaskiewicz et al., 2011; Luna et al., 2012). This epigenetic modification is given to the next generation and leads to a higher defence potential against pathogen attack in this generation (Traw et al., 2007; Jaskiewicz et al., 2011; Luna et al., 2012). This is called trans-generational SAR (Luna et al., 2012).

One of the main regulators of SAR and SA-dependent defence responses is the lipase-like protein ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1). EDS1 controls PTI and is essential for ETI in response to TIR-NBS-LRR R proteins, which belong to a large group of resistance proteins (Vlot et al., 2009; Bhattacharjee et al., 2011; Rietz et al., 2011; Zheng et al., 2015). In plants, the TIR (toll interleukin 1 receptor) domain is found almost exclusively in nucleotide-binding site (NBS) leucine-rich repeat (LRR) resistance proteins (Steinbrenner et al., 2012; Nandety et al., 2013). TIR-NBS-LRR (TNL) receptors converge genetically on EDS1 to trigger resistance. Most of the R proteins belong either to the TNL class or to the N-terminal coiled-coil (CC)-NBS-LRR (CNL) class (Marone et al., 2013; Sha, 2014). In contrast to TNL receptors, CNL receptors require NONRACE SPECIFIC DISEASE RESISTANCE1 (NDR1) and not EDS1 for ETI activation (Aarts et al., 1998; Jones and Dangl, 2006).

During PTI and ETI as well as SAR, EDS1 interacts with PAD4 (PHYTOLAXIN DEFICIENT4) and SAG101 (SENESCENCE ASSOSIATED GENE101) (Feys et al., 2001; Rietz et al., 2011; Wagner et al., 2013). Both genes encode lipase-like proteins that are structurally related to EDS1 and function in PTI and TNL-mediated ETI. *SAG101* encodes an acyl hydrolase involved in senescence (He and Gan, 2002). EDS1 together with PAD4 and SAG101 stimulates the accumulation of SA, whereby EDS1 is needed for PAD4 and SAG101 accumulation (Feys et al., 2001; Rietz et al., 2011; Wagner et al., 2013). SA treatment restores SA-mediated defence in *eds1* and *pad4* mutants and leads to an increased gene expression of *EDS1* and *PAD4* in wildtype plants. Together, the data indicate that EDS1 and PAD4 are acting upstream of SA and are part of an SA-dependent positive feedback loop (Glazebrook et al., 1997; Falk et al., 1999; Feys et al., 2005). In the cytoplasm, EDS1 forms homodimers, whereas it forms heteromeric complexes with PAD4 in the nucleus and cytoplasm. The EDS1-SAG101 complex is heteromeric and localised only to the nucleus (Feys et al., 2001; Wagner et al., 2013). The combination and coordination of nuclear and cytoplasmic trafficking of EDS1 is essential for the full plant disease resistance response (García et al., 2010). Hence, the different complexes have distinct but interactive and cooperative functions. Cytoplasm-localised EDS1 leads to pathogen-induced cell death, whereas nuclear EDS1 triggers transcriptional reprogramming leading to downstream defence responses (García et al., 2010; Kunz et al., 2016).

Amongst others, EDS1 is involved in the recognition process of the TNL receptor RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4) in response to the *P. syringae* effector AvrRps4 (Bhattacharjee et al., 2011; Kunz et al., 2016). EDS1 interacts with AvrRps4 as well as with RPS4 in the nucleus and these interactions are suggested to be required for EDS1-mediated transcriptional reprogramming towards defence (Bhattacharjee et al., 2011; Heidrich et al., 2011). Accumulation of the RPS4-EDS1 complex in the cytoplasm leads to SA accumulation and HR, indicating a compartment-specific RPS4-EDS1 defence branch (Heidrich et al., 2011). *EDS1* is not required for resistance conferred by *RPM1* (RESISTANCE TO PSEUDOMONAS SYRINGAE pathovar MACULICOLA1) or *RPS2* (RESISTANCE TO PSEUDOMONAS SYRINGAE2), both CNL receptors (Wagner et al., 2013; Wu et al., 2014). Nevertheless, *eds1* and *pad4* mutant plants are SAR-defective in response to local activation of RPM1 or RPS2 (Aarts et al., 1998; Rietz et al., 2011; Breitenbach et al., 2014), and functional EDS1-PAD4 signalling in the primary infected leaf is required for systemic signalling (Rietz et al., 2011). Thus, EDS1 is not only important in local resistance during PTI and TNL-triggered ETI, but is also required for systemic signalling and SAR (Vlot et al., 2008a). Studies in mutants lacking EDS1 revealed that EDS1 is required for both SAR signal generation in the primary infected leaves in response to RPM1 and SAR signal perception in systemic uninfected tissues (Breitenbach et al., 2014). The fact that the *eds1* mutant is defective in SAR although RPM1-mediated ETI responses are not altered allows SAR-specific studies resting upon EDS1.

## 1.5 The induction of systemic acquired resistance

Biological SAR can be activated by pathogens eliciting PTI or ETI at inoculation sites or by pathogen-derived molecules (PAMPs). SAR can also be induced chemically, e.g. by SA or one of its functional analogues, which can give indications of the mechanism of SAR under constant conditions.

It is well known that ETI can trigger SAR through both local and systemic synthesis of SA. Yet, it is not ascertained how avirulent pathogens induce the biosynthesis of SA locally and systemically (Fu and Dong, 2013). Grafting experiments in tobacco have shown that SA accumulation in the systemic rather than local infected tissue is required for SAR and thus that the initial ETI-produced mobile signal likely is not SA (Vernooij et al., 1994). SA is required for the induction of SAR, but SAR can also be induced by exposing the plant to the chemical SA-analogue benzo(1,2,3)-thiadiazole-7-carbothioic acid-*S*-methyl ester (BTH) (Lawton et al., 1996; Hien Dao et al., 2009). BTH is a strong SAR inducer, triggering the expression of a similar gene set as biological induced SAR (Gruner et al., 2013). In contrast, the gene response by SA is less similar to that of biological induced SAR than BTH.

As mentioned above (1.1) ETI and PTI also trigger the induction of reactive oxygen species (ROS). ROS is a hypernym of different species including superoxide radicals, singlet oxygen, hydroxyl radicals, and hydrogen peroxide. Pathogen infection leads to the rapid release of hydrogen peroxide ($H_2O_2$) which is mainly produced by the NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOGS (RBOHs) localised at the plasma membrane (Wang et al., 2013; Morales et al., 2016). $H_2O_2$ can provide a direct antimicrobial effect and induce cell wall strengthening to inhibit pathogen invasion (Kärkönen and Kuchitsu, 2015). The impact of ROS on the plant is depending on the concentration (Wang et al., 2014). It can either damage the plant by tissue necrosis or function as useful membrane-permeable signals during defence responses (Baxter et al., 2014; Wendehenne et al., 2014). ROS are also associated with systemic signalling and therefore connected to SAR (Peleg-Grossman et al., 2010; Wang et al., 2014). Due to their high reactivity potential, it is unlikely that ROS themselves travel systemically (Mittler, 2002). However, it is possible that ROS trigger a cascade of cell-to-cell communication that distribute throughout the plant tissue (Baxter et al., 2014). Not only ROS are produced during pathogen attack, also the toxic volatile NO is released (Mur et al., 2013). NO, like ROS has an antimicrobial effect but is toxic in high concentrations (Wang et al., 2014). During plant defence and especially during HR, NO and ROS act synergistically (Wendehenne et al., 2014). Due to its gaseous form, NO can diffuse through membranes and therefore act rapidly as a signal transducer (Matsumoto and Gow, 2011). Similar to $H_2O_2$, NO is also involved in the induction of defence-related genes and in mediating the establishment of SAR (Wang et al., 2014). NPR1 as a key regulator of SA–mediated signalling during SAR is regulated by NO/GSNO (S-nitrosoglutathione) via S-nitrosylation (Tada et al., 2008; Salgado et al., 2013). GSNO is assumed to act as mobile reservoir of NO and regulates the NO concentration in the cell (Farnese et al., 2016). The translocation of NPR1 to the nucleus is supported by NO and GSNO promotes the TGA-binding of NPR1. Therefore, NO/GSNO appear as redox regulator of the NPR1/TGA1 system in local infected and systemic uninfected tissue (Mur et al., 2013; Wendehenne et al., 2014). Studies in tobacco revealed that SA-induced SAR is attenuated by NO inhibitors or NO scavengers. This indicates that NO is required for the full function of SA as a SAR inducer (Song and Goodman, 2001). Wang et al. suggested that the lack of NO affected SAR signal generation or perception (Wang et al., 2014). To test this, petiole (leaf stem) exudates of NO-accumulating and biosynthetic mutants were compared to those of wild-type to analyse the composition of the phloem sap. The presence of SAR signals can be determined in petiole exudates from leaves undergoing ETI. If infiltrated into healthy leaves, SAR signal-containing petiole exudates trigger defence gene expression or systemic immunity. Reciprocally, such analyses can be used to investigate if a particular mutant responds to SAR signals in SAR signal-

containing petiole exudates from wild type plants. In Wang et al (2014), petiole exudates of leaves infiltrated with *Pseudomonas syringae* pv. *tomato* (*Pst*) activating the CC-NB-LRR–type R protein RPS2 (*Pst AvrRpt2*), were used to induce SAR in naive plants. The data suggest that NO is involved in generation/emission of SAR signals, but not in the perception of SAR signals (Wang et al., 2014). It was also shown that NO and ROS induced SAR in a concentration-dependent manner. The data suggested that that SAR is abolished at too high or too low concentrations of NO and ROS (Rusterucci et al., 2007; Wang et al., 2014). In line with this hypothesis, mutants which accumulate elevated levels of NO are compromised in SAR (Wang et al., 2014). Taken together, NO and ROS play a major role in one of two possible signalling branches of SAR which will be discussed in detail in section 1.7 The two branches of systemic acquired resistance.

## 1.6 Signals involved in systemic acquired resistance

In recent years, several plant-derived substances have been proposed to participate in long-distance signalling in plant defence against pathogens and herbivores (Huber and Bauerle, 2016). Presumably mobile signals in SAR are the methyl ester of SA (MeSA), the putative lipid transfer proteins DEFECTIVE IN INDUCED RESISTANCE1 (DIR1) and DIR1-like, the dicarboxylic acid azelaic acid (AzA), a derivative of glycerol-3-phosphate (G3P), the diterpenoid dehydroabietinal (DA), and the Lys catabolite Pipecolic acid (Pip) (Maldonado et al., 2002; Park et al., 2007; Jung et al., 2009; Chanda et al., 2011; Chaturvedi et al., 2012; Návarová et al., 2012; Champigny et al., 2013). These compounds are produced upon an initial/local infection and are suggested to be transported to the distal leaves to induce SAR.

Various publications in the last years suggest MeSA as the mobile SAR signal (Park et al., 2007; Park et al., 2009; Liu et al., 2011a; Liu et al., 2011b). In tobacco, reduced expression of *SALICYLIC ACID-BINDING PROTEIN2 (SABP2)*, encoding the enzyme that converts the biologically inactive MeSA to the active SA, compromised the accumulation of SA and the development of SAR (Park et al., 2007). Grafting experiments revealed that the conversion of MeSA to SA is required in the systemic tissue to generate SAR (Park et al., 2007). In *Arabidopsis*, studies indicate that for SAR to occur locally induced SA has to be converted to MeSA by the SA methyltransferase BENZOIC ACID/SA CARBOXYLMETHYLTRANSFERASE1 (BSMT1) (Liu et al., 2010; Gao et al., 2014a). However, further studies revealed that mutants lacking the SA methyltransferase BSMT1 are not always compromised in SAR (Attaran et al., 2009; Liu et al., 2011a). SAR-deficient *dir1-1* mutants showed increased levels of MeSA and *BSMT1* transcripts, leading to the suggestion that MeSA might be influenced by additional factors during SAR (Liu et al., 2011b). Liu et al. also showed that MeSA-dependent SAR induction in *Arabidopsis* is assumed to be light-dependent (Liu et al., 2011a; Liu et al., 2011b). The extent of light

exposure after the first infection defines whether MeSA and its metabolizing enzymes are required for SAR. It seems that SAR induction requires MeSA if the primary infection occurs in the late daytime, but not if the primary infection occurs in the early daytime (Liu et al., 2011a; Liu et al., 2011b). Taken together it seems that MeSA is crucial for SAR where MeSA's role in SAR might be dependent on light exposure (Park et al., 2007; Attaran et al., 2009; Park et al., 2009; Dempsey and Klessig, 2012).

The lipid transfer protein DIR1 has been associated with SAR since 2002 (Maldonado et al., 2002). DIR1 is able to bind with high affinity to monoacylated phospholipids (Lascombe et al., 2008). The protein further contains proline-rich regions potentially involved in protein-protein interactions (Maldonado et al., 2002; Lascombe et al., 2008; Dempsey and Klessig, 2012). DIR1 is found in all vascular cell types including phloem sieve elements and companion cells suggesting that DIR1 functions as chaperone-protein for long-distance SAR signal(s) (Champigny et al., 2011; Chaturvedi et al., 2012; Dempsey and Klessig, 2012). Recent studies analysed phloem exudates of SAR-induced *dir1* mutant plants which ectopically expressed *DIR1-GFP* after estrogen treatment. The data show that DIR1 can move via the phloem to distant leaves during pathogen-induced SAR (Champigny et al., 2013; Carella et al., 2016). Champigny et al (2013) also identified a partially functionally redundant DIR1 paralogue, DIR1-like which is assumed to be capable of participating in SAR. Mutants lacking in *DIR1* gene expression are SAR-deficient and fail to systemically express *PR* genes although the local immune response is not altered (Maldonado et al., 2002). ETI and PTI induce local SA accumulation in *dir1* mutant plants that is comparable to that in wildtype plants. Also, systemic SA accumulation is not abolished in *dir1* during the establishment of SAR (Maldonado et al., 2002). Thus, DIR1 is not required for the accumulation of SA either locally or systemically. Although petiole exudates from infected *dir1* do not show SAR-inducing activity in wildtype plants, petiole exudates from wild type plants can induce *PR1* expression in *dir1* (Maldonado et al., 2002). Therefore, DIR1 is necessary for SAR signal generation, but not for the perception or propagation of a mobile signal in distant leaves (Maldonado et al., 2002). Further studies revealed that DIR1-mediated SAR signalling is required in *Arabidopsis* and cucumber and bioinformatic analyses provided evidence that *DIR1* orthologs also exist in tobacco, tomato, cucumber, and soybean (Isaacs et al., 2016). This leads to the conclusion that DIR1-mediated SAR signalling might be conserved in higher plants.

Studies on petiole exudates of avirulent pathogen-treated *Arabidopsis* also identified AzA as a putative mobile signal for SAR (Jung et al., 2009). AzA is a C9 dicarboxylic acid and is synthesised in the chloroplast by lipid peroxidation of C18 membrane lipids via the intermediate 9-oxononanoic acid (Zoeller et al., 2012; Wang et al., 2014; Wittek et al., 2014). Studies revealed that AzA promotes disease resistance through priming of SA

signalling, rather than directly activating defence responses (Jung et al., 2009). It was discovered, that DIR1 is required for AzA-related resistance and that AzA induces the *AZELAIC ACID INDUCED1 (AZI1)* which is required for AzA- and biologically-induced SAR and encodes a putative lipid transfer protein (Jung et al., 2009). Recent studies showed that the transcript abundances of *PR* genes increased significantly in local and systemic leaves of wild-type and *AZI1* overexpressing plants challenged with avirulent *Pst* (Wang et al., 2016). Transcript accumulations of *PR* genes in *azi1* mutants were decreased in local and systemic leaves after local infection (Jung et al., 2009; Wang et al., 2016). This indicates that AZI1 is an essential lipid transfer protein for SAR in *Arabidopsis*. In 2015, Cecchini et al. showed that AZI1 and its closest paralog EARLY *ARABIDOPSIS* ALUMINIUM INDUCED1 (EARLI1) are necessary for the systemic movement and uptake of AZA in *Arabidopsis* (Cecchini et al., 2015). AZI1 and EARLI1 do not seem to act as mobile signals, but are both needed locally for proper SAR establishment (Cecchini et al., 2015). Metabolomic studies in distal leaves of *azi* mutants compared to wildtype plants showed that some soluble carbohydrates might function as signal substances in the systemic immunity of *Arabidopsis* (Wang et al., 2016). The transcript abundances of sugar signalling genes, were changed significantly in distal leaves, indicating sugar-related genes are involved in regulation of the systemic immunity mediated by AZI1 (Wang et al., 2016). The stability of AZI1 is dependent on the PLASMODESMATA LOCALISING PROTEIN1 (PDLP1) and PDLP5 (Lim et al., 2016). They regulate the appearance of AzA in petiole exudates similar to pathogen infection and might therefore be important for the systemic movement of AzA (Cecchini et al., 2015; Lim et al., 2016). PDLP1 and PDLP5 play also a role in the systemic mobility of DIR1 (Carella et al., 2015). It is known that DIR1 and AZI1 interact with themselves and with each other and regulate the accumulation of G3P.

Within 6h after an initial/primary infection, G3P is produced through the activity of GLY1 (SFD1, SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1) (Chanda et al., 2011). GLY1 reduces dihydroxyacetone to G3P and is required for SAR (Fu and Dong, 2013; Gao et al., 2014a). G3P can also be synthesized through phosphorylation of glycerol by the glycerokinase GLI1 (Lu et al., 2001). Mutants defective in GLY1 or GLI1 show SA- and AzA- accumulation comparable to wildtype but are compromised in SAR (Yu et al., 2013). SAR could be restored in these mutants by exogenous application of G3P, but G3P application alone on wildtype plants did not induce SA biosynthesis or SAR. This indicates that G3P itself is an essential but not sufficient mobile signal for SAR (Fu and Dong, 2013). Studies in mutants lacking GLY1 and GLI1 revealed that the translocation of G3P to the distal leaves is dependent on DIR1, whereby DIR1 movement requires G3P (Chanda et al., 2011). Similar to DIR1 also AZI1 is essential for G3P biosynthesis, suggesting that G3P and DIR1/AZI1 regulate SAR via a feedback loop (Yu et al., 2013).

Further studies on petiole exudates of avirulent pathogen-treated *Arabidopsis* leaves identified DA as a putative mobile SAR signal (Chaturvedi et al., 2012). Studies with labelled DA revealed that DA is rapidly translocated to the distal untreated leaves during SAR. DA-induced resistance requires SA biosynthesis and signalling as well as NPR1, FMO1 and DIR1. Local DA treatment induces SAR in *Arabidopsis*, tobacco and tomato and enhances *PR1* transcript levels locally and systemically. The efficiency of DA in inducing SAR increases when DA is applied together with AzA, suggesting a synergistic effect of DA and AzA during SAR induction (Chaturvedi et al., 2012).

Pip is a non-protein amino acid and discussed as a potential long-distance SAR signal. It was identified in petiole exudates of avirulent pathogen-treated *Arabidopsis* leaves (Návarová et al., 2012). Due to its chemical characteristics, Pip as a water-soluble amino acid would be able to travel via the phloem suggesting a possible transport of Pip from the inoculated leaves via the phloem to the distal tissue. The Pip-deficient mutant *ald1* is SAR-deficient and incapable of SA accumulation in the distal leaf tissue, indicating a substantial role of Pip during SAR (Song et al., 2004; Jing et al., 2011; Návarová et al., 2012). ALD1 is the aminotransferase required for the Lys-derived biosynthesis of Pip (Návarová et al., 2012). SAR can be restored in *ald1* by exogenous supply of Pip, suggesting that Pip accumulation is required for SAR activation (Návarová et al., 2012; Bernsdorff et al., 2015). After the initial infection, Pip levels in the systemic uninfected tissue increased rapidly and enhanced Pip accumulation was measured earlier than systemic accumulation of SA (Návarová et al., 2012). Therefore, Pip is assumed to function as an initial trigger for signal amplification which then leads to the establishment of SAR. In turn, Pip-induced resistance might occur in a SA-independent manner, indicating that Pip and SA act both synergistically and independently from each other to mediate *PR* gene expression and plant basal resistance to *P. syringae* (Gruner et al., 2013; Shah and Zeier, 2013; Bernsdorff et al., 2015).

Most of these plant-derived substances act together during SAR. They all share their requirement for SA, an essential downstream component of the SAR pathway. Together with NO-derived and ROS-derived signalling (1.5), new insights in the overlapping pathways leading to SAR can be identified.

## 1.7 The two branches of systemic acquired resistance

SAR-related chemical and protein signals mentioned above (1.5, 1.6) can now be placed in one of two main branches that comprise the SAR pathway (Figure 4) (Wang et al., 2014; Gao et al., 2014b; Gao et al., 2015). SA and its signalling component NPR1 compose one branch whereas the other branch depends on the free radicals NO and ROS. Local SA-application cannot confer SAR on mutants defective in NO, ROS, or G3P

biosynthesis (Chanda et al., 2011; Wang et al., 2014; Gao et al., 2014b). In contrast, NO/ROS is not able to restore SAR in mutants defective in SA synthesis or signalling (Wang et al., 2014). Hence, SAR signalling components of one branch cannot complement molecular defects in the other branch, providing the evidence of the co-existence of the two branches and their necessity in SAR.

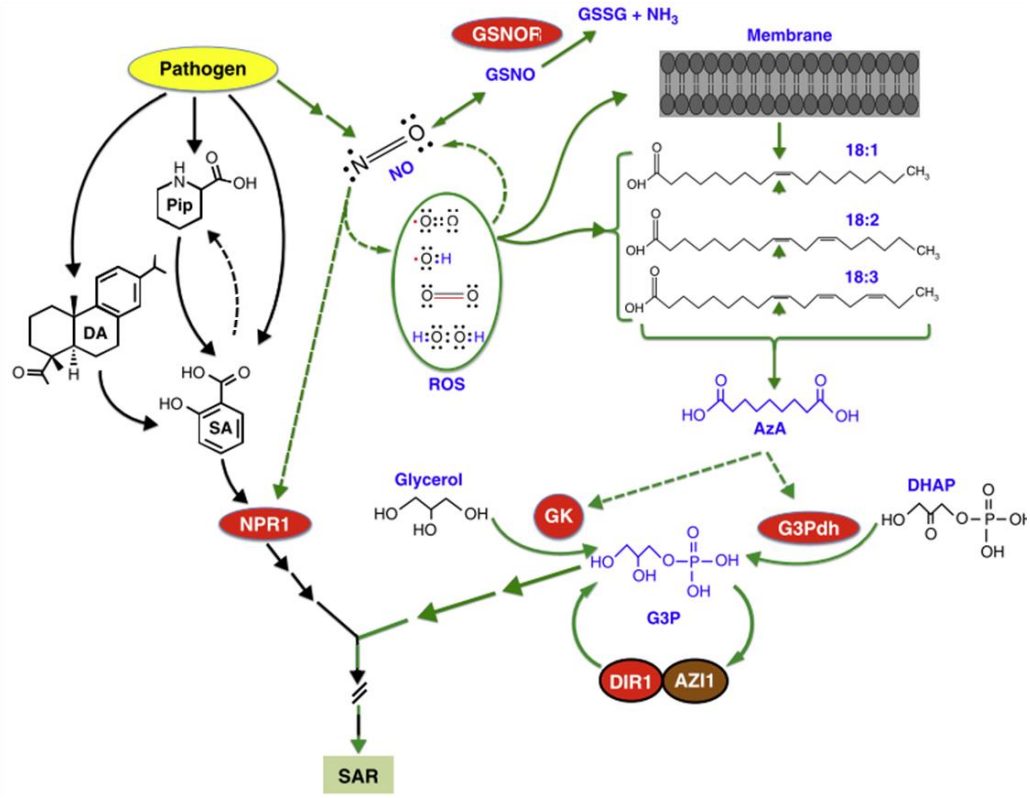


**Figure 4: The two branches of SAR - Pathways, chemicals, and proteins involved in SAR.**

The SA-branch is marked in black arrows whereas the NO/ROS-branch is highlighted in green arrows. SA: Salicylic acid, NO: Nitric oxide, ROS: Reactive oxygen species, FAs: Fatty acids, AzA: Azelaic acid, G3P: Glycerol-3-phosphate, GK: Glycerol kinase, G3Pdh: G3P dehydrogenase, DIR1: DEFECTIVE IN INDUCED RESISTANCE1, AZI1: AZELAIC ACID INSENSITIVE1, GSNO: S-nitrosoglutathione, GSSG: Glutathione disulfide, GSNOR: S-NITROSOGLUTATHIONE REDUCTASE, NPR1: NONEXPRESSOR OF *PR* GENES1, SAR: Systemic acquired resistance. Figure adapted from Wendehenne et al. (2014).

Pathogen attack can induce SA accumulation, which is essential for DA, Pip, AzA, and G3P to induce SAR. In the absence of pathogen infection, only exogenous application of DA and Pip induce SA biosynthesis (Gao et al., 2015). That indicates that Pip and DA are related to the SA-branch (Figure 4) (Chaturvedi et al., 2012; Návarová et al., 2012; Gao et al., 2015). The induction of DA leads to an increase of SA in the uninfected tissue and to a NPR1-dependent defence pathway as described above (1.2)(Chaturvedi et al., 2012). Because SAR cannot be induced by DA in SA accumulation/signalling-deficient

*sid2* or *npr1* mutant plants, DA can be seen as an activator of SA-mediated SAR. Due to the fact that Pip supports systemic SA accumulation, Pip was suggested to function as an initial trigger of SAR (Návarová et al., 2012). It is likely that Pip and SA signalling might act in a positive feedback amplification mechanism during SAR (Bernsdorff et al., 2015). Upon primary pathogen infection, a moderate SAR might be induced in the absence of SA when Pip is fully functional (Bernsdorff et al., 2015). Studies in mutants lacking SA biosynthesis and Pip accumulation, SAR is fully abolished, leading to the suggestion that Pip might act as a SA signal intensifier (Bernsdorff et al., 2015).

Initial pathogen challenge also leads to the accumulation of cellular NO. To regulate NO accumulation, it can be metabolised into the storage molecule GSNO (S-nitrosoglutathione) by reacting with glutathione (GSH), which is an important antioxidant in plants (Rusterucci et al., 2007). The NO donor GSNO can be reduced to GSSG (glutathione disulfide) and NH3 by GSNOR (S-nitrosoglutathione reductase) (Corpas et al., 2013). ROS acts directly downstream of NO and operates with NO in a feedback loop (Wang et al., 2014). ROS can catalyse the oxidation of unsaturated fatty acids, which leads to the generation of AzA (1.6) (Yu et al., 2013). AzA induces the transcription of G3P biosynthetic genes glycerol kinase and G3P dehydrogenase (Yu et al., 2013). This places G3P downstream of AzA, which is confirmed by the fact that SAR cannot be restored by AzA in G3P-compromised mutants (Wang et al., 2014; Wendehenne et al., 2014; El-Shetehy et al., 2015; Gao et al., 2015). The lipid transfer proteins DIR1 and AZI1 are essential for G3P accumulation and are required for G3P-induced SAR (Yu et al., 2013). Reduced G3P results in decreased *AZI1* and *DIR1* transcript accumulation suggesting G3P to act in a feedback loop together with the DIR1/AZI1 complex (1.6)(Yu et al., 2013).

It is important for the plant to find a balance between activation and inhibition of cellular processes of the two branches without harming itself. Therefore, the parallel signalling of SA and NO/ROS are co-regulated mutually. It is known that the two branches crosstalk at the nitrosylation of NPR1 and that EDS1 positively regulates SA and AzA (Tada et al., 2008; Wittek et al., 2014; Gao et al., 2015). Both branches are required for a fully functional SAR, but it probably needs a fine balance of the coordination of these pathways.

## 1.8 Lectins and their role in plant defence

Lectins are found in many different species and in many different organs and tissues (Dias et al., 2015). Therefore, they are expected to play fundamental biological roles in plants. Murdock and Shade (2002) proposed that plant lectins play a role in the general defence against a multitude of plant attackers and are also used as storage proteins that

can be mobilized for plant growth and development (Murdock and Shade, 2002). Lectins and lectin-like proteins are putative carbohydrate-binding proteins containing at least one non-catalytic conserved sugar-binding domain to bind glycoproteins, glycolipids, or polysaccharides with high affinity (Lannoo and Van Damme, 2014; De Schutter and Van Damme, 2015). Some lectins are also able to bind groups of carbohydrates or to interact with liposomes containing natural or artificial glycolipids (Szoka et al., 1981; Berg et al., 2002; Benvegnu et al., 2014). Lectins occur most abundantly in seeds and vegetative storage tissues. Because of their binding affinity, they have the capability to serve as recognition molecules within a cell, between cells, or between organisms (Chrispeels and Raikhel, 1991; Lannoo and Van Damme, 2014). Analyses at the level of the genome and the transcriptome have shown that lectin sequences are ubiquitous in the plant kingdom (Lannoo and Van Damme, 2014). Various plant lectins are highly toxic for phytophagous insects (Dung et al., 2015; Macedo et al., 2015) and other herbivores (Harley and Beevers, 1986; Vasconcelos and Oliveira, 2004). Some lectins show antibacterial activity correlated with PTI and bacteria-mediated stomatal closure (Singh et al., 2013; Carvalho et al., 2015)

The *Arabidopsis* genome contains more than 200 lectin genes, which can be divided into 12 families (Chandra et al., 2006; Jiang et al., 2010). The overall structure of the carbohydrate-binding site(s) is conserved in each lectin family. Besides legume-like lectins, which are mainly focused on in this work (1.9 Legume lectin like proteins related to systemic acquired resistance, Figure 5), jacalin-related lectins, chitin-binding lectins, phloem lectins, ricin b-like lectins and GNA-lectins have been connected to plant defence (Peumans et al., 2000; Jiang et al., 2010). Jacalin-like lectins contain one or more mannose-binding jacalin-like lectin domains (Fernandez-del-Carmen et al., 2013). These lectins might play a role in the SA- and JA-mediated defence signalling pathways (Xiang et al., 2011). It was shown that overexpression of a wheat jacalin-related lectin leads to the expression of SA- and JA-dependent defence genes like *PR1, PR2, NPR1, COI1, ERF1, PR3* and *PDF1.2* as well as elevated free SA and JA levels. Furthermore, silencing of the same wheat jacalin-related lectin resulted in weakened plant resistance to pathogenic fungi (Xiang et al., 2011).

Some chitin-binding plant lectins in red kidney bean (*Phaseolus vulgaris*) (Ye et al., 2001) and stinging nettle (*Urtica dioica*) (Lerner and Raikhel, 1992) were shown to negatively affect growth and development of pathogenic fungi (Van Damme et al., 2004; Hasan et al., 2014). Phloem lectins are also called the phloem proteins 2 (PP2 proteins) and may play a role in defence against phloem-feeding insects (Dinant et al., 2003). Ricin is a lectin from the seeds of the castor bean plant. The ricin b-like lectins are able to bind terminal galactose residues on cell surfaces (Soga et al., 2015). Together with the ricin a-hydrolase, the lectin is highly toxic for insects and higher animals, including

mammals (May et al., 2013). Finally, G-type lectins, also named GNA-lectins (*Galanthus nivalis* agglutinin) specifically bind to mannose. In *Arabidopsis* 32 different G-type LecRLKs (lectin receptor-like kinases) are known, whereas in rice 100 G-type LecRLKs are found (Vaid et al., 2012). Recently, Kim et al. (2015) showed also in pepper plants that a GNA-lectin is required for plant cell death and for defence responses. Overexpression of the same pepper GNA-lectin in *Arabidopsis* enhanced plant resistance to *Pseudomonas syringae* and *AtPDF1.2* expression (Kim et al., 2015).

Notably, some plant lectins may have an antiviral role by binding to glycoproteins in virions and inhibiting their replication (Keyaerts et al., 2007; Takebe et al., 2013).

Taken together, lectins can affect processes in plants to support plant defence. Some also inhibit the pathogen directly by preventing their reproduction and development.

## 1.9 Legume lectin like proteins related to systemic acquired resistance

The legume lectin-like protein family comprises one of the largest lectin families and consists of 18 members in *Arabidopsis* (Armijo et al., 2013)(Figure 5). Legume lectin-like lectins have putative binding capacity in principal to glucose, k-acetylglucosamine, mannose, or galactose and are transcriptionally induced by pathogens or elicitor treatments (Lyou et al., 2009; Singh et al., 2012; Lannoo and Van Damme, 2014). Molecular masses of the subunits of leguminous lectins average around 30-kDa. They may assemble to the fully active lectin either as dimers or tetramers without further processing. Legume lectins can be subdivided into two categories: single-chain lectins are composed of subunits equal in size, and two-chain lectins characterized by different subunit types (Rudiger and Gabius, 2001). Comparative analyses on apoplast-enriched extracts from wild type and *eds1-2* mutant plants (2.1) that were induced for SAR signal generation, led to the identification of new potential SAR regulatory proteins, including LEGUME LECTIN-LIKE PROTEIN1 (LLP1) (Figure 5)(Breitenbach et al., 2014). In addition, also LECTIN1 (LEC1) (Figure 5) was detected in apoplast-enriched extracts, linking both LLP1 and LEC1 to SAR (Breitenbach, 2012). By BLAST sequence search, a close homologue of LEC1, LECTIN2 (LEC2) was found which showed 89% nucleotide sequence homology with LEC1 and therefore is included in this study. LEC2 was detected on one 2D gel set comparing the apoplast-enriched extracts from *AvrRpm1-HA*-expressing Col-0 and *eds1-2* mutant plants (Breitenbach et al., 2014).

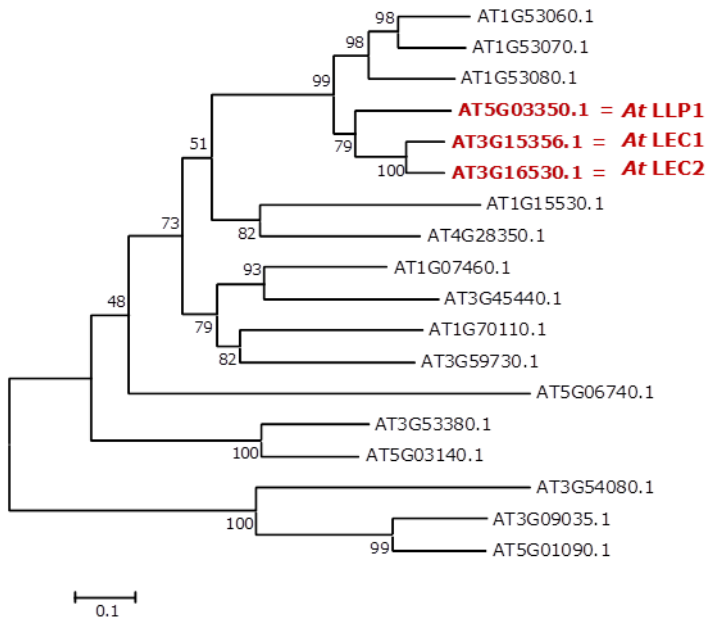


**Figure 5: Phylogenetic tree based on the similarity of the coding sequences of the 18 members of the legume lectin-like protein family in *Arabidopsis thaliana.***

The phylogenetic tree was built using Molecular Evolutionary Genetics Analysis Software (MEGA) by Heiko Breitenbach (Breitenbach, 2012). Lectins focused on in this work are highlighted in red (*At*5G03350 = *AtLLP1*, *At*3G15356 = *AtLEC1*, *At*3G16530 = *AtLEC2*) Illustration is modified from Breitenbach (2012).

The gene expression of *LLP1* can be induced by virulent and avirulent *P. syringae* and by the SA functional analogue BTH (Breitenbach, 2012; Armijo et al., 2013; Breitenbach et al., 2014). Also, *LLP1* is induced early after an SA treatment and this induction is dependent on NPR1 (Armijo et al., 2013). LLP1 is a N-glycosylated protein tightly bound to the apoplastic side of the plasma membrane (Armijo et al., 2013). Studies in *Arabidopsis thaliana* suggest that LLP1 promotes systemic but not local resistance (Breitenbach et al., 2014). Local defence responses against *Pst AvrRpm1, Pst AvrRps4* or virulent *Pseudomonas* do not differ in *llp1* mutants compared to those in wild-type plants (Breitenbach et al., 2014), although the LLP1 protein might be involved in the HR cell death process triggered by *Pst AvrRpm1* (Armijo et al., 2013). The systemic *LLP1* transcript accumulation in *Pst AvrRpm1*-infected wild-type plants is 4-fold higher compared to that in mock-treated plants. It could be shown that this induction is dependent on *EDS1* and thus likely related to SAR (Breitenbach et al., 2014). Mutants lacking in *LLP1* are SAR defective but show modestly induced *PR1* expression in the systemic uninfected tissue of locally infected plants. This suggests that systemic immune signalling is not abolished in *llp1* mutants but compromised (Breitenbach et al., 2014). Furthermore, mutant plants defective in *LLP1* respond to exogenous SA application with

elevated resistance and *PR1* expression. Hence, the local response to SA is not affected by LLP1, suggesting that LLP1 does not act downstream of SA (Breitenbach et al., 2014). Because *Pst AvrRpm1*-induced SA accumulation also is normal in *llp1* mutant plants, the data suggest that LLP1 acts in parallel with SA. Overexpression of *LLP1* did not trigger a significant defence response to *Pst* leading to the suggestion that LLP1 might cooperate with additional components, including SA (Armijo et al., 2013; Breitenbach et al., 2014).

LEC1 was first described 2009 by Lyou et al. (2009) in *Arabidopsis*. Mutation in *LEC1* compromises SAR but not local resistance to *P. syringae* (Breitenbach, 2012). *LEC1* is highly expressed in rosette leaves, primary inflorescence and roots of mature plants. *LEC1* gene expression is induced in young plants within 3-6 hours after treatment with chitin, MeJA or ethylene (Lyou et al., 2009). Chitin-induced transcript accumulation of LEC1 was also observed in JA- and ET-insensitive mutants. This leads to the suggestion that the induction of LEC1 by chitin might be JA/ET-independent (Lyou et al., 2009).

Chitin treatment of *Arabidopsis thaliana* also induces the transcript accumulation of the *LEC1* homologue *LEC2* (Zhang et al., 2002). The rapid and strong induction of *LEC2* is dependent on the chitin concentration and chitin size (Ramonell et al., 2002; Zhang et al., 2002). During pathogen infection, *LEC1* and *LEC2* transcripts were also found after treating the plants with oligogalacturonides, elicitors released from the plant cell wall (Casasoli et al., 2008). Oligogalacturonides act as a *DAMP* signal to trigger plant immunity (Boller and Felix, 2009). Furthermore, *LEC2* expression is altered in a microarray after treatment of the *Arabidopsis* plants with the aphid *Myzus persicae*, indicating a role of LEC2 in plant-insect interaction (Zhu-Salzman et al., 2004; Couldridge et al., 2007).

### 1.10 Aim of this work

The aim of this thesis is to characterize the role of the lectins LLP1, LEC1, and LEC2 in the plant immune system. The main focus will be on SA-dependent defence, including systemic acquired resistance (Breitenbach, 2012), and on a possible role of LLP1, LEC1, and LEC2 in phytohormone crosstalk affecting SAR.

The legume lectin-like proteins LLP1 and LEC1 each appear to be essential for SAR, although their expression is regulated by different phytohormones that respectively activate (SA) and antagonize (JA) SAR. Therefore, this work will first assess if and how the expression of *LLP1, LEC1* and *LEC2* is regulated by SA, JA, and ABA, which similarly to JA is believed to antagonize SAR. This will be done in wildtype *Arabidopsis* plants and in the *eds1-2* mutant which is defective for SAR and supports reduced accumulation of LLP1 in the apoplast of *AvrRpm1*-expressing plants. This analysis will provide insights into the relationship of LEC1, LEC2 and LLP1 to EDS1 in different defence-related

phytohormone signalling pathways. This type of analyses will further be optimized by generating tools to investigate the *in planta* expression pattern of the lectin genes under different stress responses.

Next, this work aims to assess if LLP1, LEC1, and LEC2 interact in SAR. Therefore, phytopathological experiments with transgenic plants undergoing RNAi-mediated silencing of *LEC1, LEC2* and *LLP1* or silencing of *LEC1* and *LEC2* will be performed. This should clarify a possible interplay of LLP1, LEC1 and LEC2 also in relation to EDS1 during local and systemic SA-dependent defence responses.

Furthermore, this work aims to ascertain a possible role of the lectins in JA- and ABA-dependent immune responses. To this end, the above-introduced RNAi lines will be challenged with biotic and abiotic triggers that are associated with JA and ABA signalling. Together with the above-introduced analyses of the role of the lectins in SA-dependent immune responses, these experiments will provide insight into a possible role of LLP1, LEC1, and LEC2 in cross talk between phytohormone signalling pathways.

Finally, this work aims to find putative protein binding partners of LLP1, LEC1 and LEC2 that might act as co-regulators of SAR. To this end, protein-protein interactions will be examined using the yeast-two-hybrid system in collaboration with Dr. Pascal Falter-Braun (Institute of Network Biology, HelmholtzZentrum München, Germany) at the Technical University of Munich.

The hypothesis is that the lectins play an essential role in plant defence. Here, it will be investigated if they act as regulators, inhibitors or mediators in different phytohormone pathways. As proteins with putative carbohydrate binding capacity, it is also possible that the lectins influence each other or additional defence proteins.

# 2. Materials

## 2.1 Plant Material

Throughout all experiments the Arabidopsis (*Arabidopsis thaliana, A. thaliana*) ecotype Col-0 was used as wild-type (WT). The mutants *eds1-2*, *llp1-1*, *llp1-3* have been described previously (Bartsch et al., 2006; Breitenbach et al., 2014). The mutant lines *jar1* (Staswick et al., 1998)(gift from Dr. Anton Schaeffner), *med4-2* (Vlot, Liu et al. 2008), *npr1* (Cao, Glazebrook et al. 1997), and *sid2* (Wildermuth, Dewdney et al. 2001) were used for gene expression analysis. The Transfer-DNA (T-DNA) insertion line SALK_030762 (*lec1*) and SALK_204573 (*xth25*) were obtained from the Nottingham Arabidopsis Stock Centre (Scholl et al., 2000). Seeds of selected homozygous plants were used for further experiments. For protein localization, *Nicotiana benthamiana* was used. All seeds were stored at room temperature at a dark and dry place.

## 2.2 Microorganisms and their culture conditions

*Escherichia coli* One Shot® TOP10 (Invitrogen™) and *Agrobacterium tumefaciens* GV3101::pMP90 were used in cloning procedures and cultured on LB medium (Table 4) at 37°C and 28°C, respectively. The virulent strain *Pseudomonas syringae pv tomato* (*DC3000* or *Pst*) and the avirulent strains *Pseudomonas syringae pv tomato* carrying either the type III effector *AvrRpm1* (*Pst AvrRpm1*), *AvrRps4* (*Pst AvrRps4*) or *AvrRpt2* (*Pst AvrRpt2*) were used for different experiments and cultured at 28°C on NYGA medium (Table 4). The necrotrophic fungus *Alternaria brassicicola* was grown on oat medium (Table 4) for formation of mitospores at room temperature and light. The fungal stock plates were kept at a dark place at room temperature on malt medium (Table 4). For Yeast-2-Hybrid Assay, yeast strains Y8800 and Y8930 (James et al., 1996) were obtained from Dr. Pascal Falter-Braun (Institute of Network Biology, HelmholtzZentrum München, Germany) and were cultivated and incubated on YEPD (Table 4) plates at 28-30°C.

## 2.3 Primers

Primers were applied in various cloning strategies and in the characterization of gene expression. The working concentration of the primers was 5µM for PCR and 10µM for qPCR. The annealing temperatures of the primers and the identified fragment sizes of the resulting products are specified for every primer pair in Table S1 through Table S5 that are presented in the supplemental part of this thesis (8.1).

## 2.4 Vectors

For all cloning strategies, pENTR™/D-TOPO® (Invitrogen, Karlsruhe, Germany) was used as the Gateway entry vector. For the construction of artificial microRNA constructs, the vector pRS300 served as the PCR target (Schwab et al., 2006). For RNAi-mediated gene silencing, the donor vector pHANNIBAL and the binary expression vector pART27 were used for cloning and plant transformation (Wesley et al., 2001). pHANNIBAL and pART27 were obtained from Daniel F. Klessig, Boyce Thompson Institute, Cornell University, Ithaca, New York, USA. For the expression of genes-of-interest driven by their native promoter in plants, the binary Gateway® cloning vector pBGWFS7,0 (Karimi et al., 2002) was used. For this purpose, the GUS sequence was removed from pBGWFS7,0 using the restriction enzyme NruI (pBGWFS7,0ΔGUS). Depending on the primer-sequence and the resulting PCR-product, pBGWFS7,0ΔGUS links an EGFP-tag to the C-terminal end of the encoded protein. For transient expression in tobacco, the RNA silencing suppressor vector *p35S::p19* (Lindbo, 2007) was used. The vectors pDEST-AD and pDEST-DB (Dreze et al., 2010) used for Y2H Assay were obtained from Pascal Falter-Braun, Institute of Network Biology, HelmholtzZentrum München, Germany.

## 2.5 Antibiotics

Table 1 illustrates the different antibiotics, their final working concentration and their use in various applications.

**Table 1: Antibiotics, their working concentrations and purpose**

| Name | Working concentration | Source | Purpose |
|---|---|---|---|
| Ampicillin | 50 µg/ml | Roth, Karlsruhe, Germany | selection of pHANNIBAL, pDEST-AD and pDEST-DB |
| Gentamycin | 25 µg/ml | Roche, Mannheim, Germany | selection of *Agrobacterium tumefaciens* GV3101::pMP90 |
| Kanamycin | 50 µg/ml | Roth, Karlsruhe, Germany | selection of pENTR™/D-TOPO® and *Pseudomonas syringae pv tomato* (virulent and avirulent strains) |
| Rifampycin | 100 µg/ml | Duchefa Biochemie, Germany | selection of *Agrobacterium tumefaciens* GV3101::pMP90 and *Pseudomonas syringae pv tomato* (virulent and avirulent strains) |

| | | | |
|---|---|---|---|
| Spectinomycin | 100 µg/ml | Sigma, Taufkirchen, Germany | selection of pART27 and pBGWFS7,0ΔGUS |
| Streptomycin | 50 µg/ml | Sigma, Taufkirchen, Germany | selection of pART27 and pBGWFS7,0ΔGUS |
| Carbenicillin | 250 µg/ml | Roth, Karlsruhe, Germany | against Gram-negative bacteria |
| Cefotaxim | 100 µg/ml | AppliChem, Darmstadt, Germany | against Gram-positive and Gram-negative bacteria |

All stock solutions except for Rifampycin were dissolved in water (ddest.). Rifampycin was dissolved in 100% Dimethylsulfoxide (DMSO). Stock solutions were filter sterilized through 0.22 µm sterile filters (Millipore, Billerica, MA, United States) and stored as aliquots at -20°C.

## 2.6 Enzymes used for cDNA-synthesis, restriction and Gateway® cloning

To produce cDNA from isolated plant RNA of *A. thaliana*, SuperScript™II Transcriptase and RNaseOut™ (Invitrogen, Karlsruhe, Germany) were used. Restriction applications and ligations during the cloning procedures were realised by FastDigest Enzymes and T4 DNA Ligase, respectively (Fermentas, St. Leon-Rot, Germany). FastAP™ Thermosensitive Alkaline Phosphatase (Fermentas, St. Leon-Rot, Germany) was used to dephosphorylate DNA to prevent re-circularization during ligation. For Gateway® cloning, Gateway® LR Clonase™ Enzyme Mix (Invitrogen, Karlsruhe, Germany) was used.

## 2.7 DNA-Polymerases

The proof-reading PCR enzyme Phusion® High Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) was used in cloning procedures, and the Mango *Taq*™ DNA-polymerase (Bioline, Luckenwalde, Germany) was used in colony-PCRs. For qPCR analysis, SensiMix™ SYBR Low-ROX Kit (Bioline, Luckenwalde, Germany) was used.

## 2.8 Chemicals

Only high purity grade chemicals were used from Sigma Aldrich GmbH (Taufkirchen, Germany, from here onwards named Sigma), Carl Roth GmbH (Karlsruhe, Germany), Merck KGaA (Darmstadt, Germany) or from other sources as indicated in the tables.

## 2.9 Media, buffer and solutions

The following tables illustrate the names of solutions used, their compositions and the source of each reagent.

**Table 2: Solutions used for plant RNA extraction**

| Name | Composition | Source |
|---|---|---|
| TriReagent, per 100ml (adjust pH 5.0 before adding phenol) [1] | 3.05 g Ammoniumrhodanide (Ammoniumthiocyanate)<br>9.44 g Guanidinthiocyanat<br>5 ml Glycerol<br>3 M Na-Acetate pH 5.2 | Roth, Karlsruhe, Germany<br>Merck, Darmstadt, Germany<br>Roth, Karlsruhe, Germany<br>Merck, Darmstadt, Germany |
| further solvent | Phenol<br>Chloroform<br>2-Propanol<br>Ethanol | Roth, Karlsruhe, Germany<br>Merck, Darmstadt, Germany<br>Roth, Karlsruhe, Germany<br>Merck, Darmstadt, Germany |

**Table 3: Solutions used for gel electrophorese**

| Name | Composition | Source |
|---|---|---|
| 1X TAE buffer (Tris-Acetate-EDTA) | 40mM Tris acetate<br>1mM EDTA | Roth, Karlsruhe, Germany<br>Roth, Karlsruhe, Germany |
| further solvent | Agarose<br>6X loading dye<br>ethidium bromide | Biozym, Hessisch Oldendorf, Germany<br>Fermentas, St Leon-Rot, Germany<br>Roth, Karlsruhe, Germany |

[1] Buffer composition for the phenol-chloroform-based RNA extraction was produced according to Logemann J, Schell J, Willmitzer L. 1987. Improved method for the isolation of RNA from plant tissues. *Anal Biochem* 163: 16-20

**Table 4: Media composition used for cultivation of bacteria, fungi, plants and yeast**

| Name | Composition | Source |
|---|---|---|
| NYGA, per liter, pH 7.0 [2,3] | 5g Bacto-proteose Peptone<br>3g yeast extract<br>20ml Glycerol | Roth, Karlsruhe, Germany<br>Roth, Karlsruhe, Germany<br>Roth, Karlsruhe, Germany |
| Culture media, Luria broth medium (LB),<br>per liter, pH 7.4 [2,3] | 10g Tryptone<br>5g yeast extract<br>10g NaCl | Roth, Karlsruhe, Germany<br>Roth, Karlsruhe, Germany<br>Roth, Karlsruhe, Germany |
| Oat media, per liter | 20g oat flakes<br>15g Agar-Agar | Alnatura, Darmstadt, Germany<br>Merck, Darmstadt, Germany |
| Malt media, per liter | 30g malt extract<br>15g Agar-Agar | Merck, Darmstadt, Germany<br>Merck, Darmstadt, Germany |
| Sterile plant growth medium (MS), per liter, pH 5.0 [3,4] | 4.302g Murashige & Skoog<br>10g Sucrose<br>0.5g MES | Duchefa Biochemie, Germany<br>Duchefa Biochemie, Germany<br>Roth, Karlsruhe, Germany |
| YEPD media, per liter [5,6] | 10g Yeast extract<br>20g Bacto-proteose Peptone | Roth, Karlsruhe, Germany<br>Roth, Karlsruhe, Germany |
| Yeast Synthetic complete (Sc) media, per liter, pH 5.9 [6] | 5.2 g amino acid powder<br>6.8 g yeast nitrogen base<br>20 g $(NH_4)_2SO_4$ | Table 7<br>Sigma-Aldrich, Steinheim, Germany<br>Merck, Darmstadt, Germany |

[2] For agar plates, 18g of Agar-Agar (Merck, Darmstadt, Germany) was added after pH adjustment.

[3] After autoclaving, the media was cooled down to ~55°C to add selective antibiotics.

[4] For agar plates, 8g of Agar-Agar (Merck, Darmstadt, Germany) were added before autoclaving.

[5] After autoclaving and before pouring, add 100ml of autoclaved 40% (w/v) glucose (Merck, Darmstadt, Germany) and 30ml of 65 mM adenine solution

[6] Adjust pH with 10M NaOH (Merck, Darmstadt, Germany) before autoclaving. For agar plates, add 20g of Agar-Agar (Merck, Darmstadt, Germany)

**Table 5: Solutions used in infiltration experiments**

| Name | Composition | Source |
|---|---|---|
| MOCK and dilution - solution, pH 7.0 | 10mM $MgCl_2$ | Merck, Darmstadt, Germany |
| Bacteria isolation solution | 10mM $MgCl_2$<br>0.01% Silwet | Merck, Darmstadt, Germany<br>Lehle Seeds, Texas, USA |
| Monopotassium phosphate buffer, (MKP – buffer), pH 6 | 62mM $KH_2PO_4$<br>0.01% Glucose<br>0.01% Tween® 20 Detergent | Merck, Darmstadt, Germany<br>Merck, Darmstadt, Germany<br>Merck, Darmstadt, Germany |
| Infiltration buffer for transient tobacco transformation, pH 5,7 | 10mM $MgCl_2$<br>10mM MES-KOH | Merck, Darmstadt, Germany<br>Roth, Karlsruhe, Germany |

**Table 6: Solutions used for various plant treatments**

| Name | Content | Source |
|---|---|---|
| Basta® [7] | 200g/l Glufosinate-ammonium | Hoechst AG, Hattersheim, Germany |
| BION® (BTH) [8] | BTH - Pflanzenaktivator | Ciba-Geigy AG, Frankfurt, Germany |
| methyljasmonate (MeJA) [9] | Jasmonsäuremethylester 95% | Sigma-Aldrich, Steinheim, Germany |
| salicylic-acid (SA) [9] | SA ≥ 99%, p.a., ACS | Roth, Karlsruhe, Germany |
| abscisic acid 98% (ABA) [9] | (±)-Abscisic acid, 98% 100mg 100MG | Acros Organics, New Jersey, USA |

[7] Plants were sprayed with 0,8% BASTA® (dissolved in water) to select transgenic plants expressing genes-of-interest driven by their native promoter.

[8] BION® contains benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), a salicylic acid analogue. For experiments BION® was dissolved in bidest $H_2O$.

[9] MeJA, SA and ABA were first dissolved in 100%MeOH and then diluted in bidest $H_2O$.

**Table 7: Solutions and chemicals used for the Yeast-2-Hybrid Assay**

| Name | Content | Source |
|---|---|---|
| Tris-EDTA-Lithiumacetat (TE/LiAc) | 10mM Tris HCl<br>1mM EDTA<br>900mM LiAc | Roth, Karlsruhe, Germany<br>Sigma-Aldrich, Steinheim, Germany<br>Sigma-Aldrich, Steinheim, Germany |
| Tris-EDTA-Lithiumacetat-Polyethylengelycol (TE/LiAc/PEG) | 10mM Tris HCl<br>1mM EDTA<br>100mM LiAc<br>35,2 % (w/v) PEG 3350 | Roth, Karlsruhe, Germany<br>Sigma-Aldrich, Steinheim, Germany<br>Sigma-Aldrich, Steinheim, Germany |
| HDE-I-Puffer | 0,05 M Glucose<br>2,5mM Tris HCl<br>10mM EDTA<br>2mg/l Lysozym[10] | Merck, Darmstadt, Germany<br>Roth, Karlsruhe, Germany<br>Sigma-Aldrich, Steinheim, Germany<br>Biochemica, Sauerlach, Germany |
| HDE-II-Puffer | 0,2 N NaOH<br>1 % (w/v) SDS | Merck, Darmstadt, Germany |
| Amino acid powder | 3g of each of the following amino acids: alanine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine | SERVA GmbH, Heidelberg, Germany and Sigma-Aldrich, Steinheim, Germany |
| Sodiumacetat | 3 M NaAc pH5.5 | Merck, Darmstadt, Germany |

[10] Lysozym was added just before use.

**Table 8: Selective Amino Acids used in the Y2H Assay**

| Selective amino acids | Stock solutions | storage | Source |
|---|---|---|---|
| Histidine-HCl | 100mM | dark, room temp | SERVA, Heidelberg, Germany |
| Leucine | 100mM | room temp | SERVA, Heidelberg, Germany |
| Adenine sulfate | 65mM | room temp | SERVA, Heidelberg, Germany |
| Tryptophan | 40mM | dark, 4°C | SERVA, Heidelberg, Germany |

## 2.10 Kits

To isolate plasmid DNA from bacteria, QIAprep® Spin Miniprep Kit, No. 27104 (Qiagen GmbH, Hilden, Germany) was used. QIAquick® Gel Extraction Kit, No. 28704 (Qiagen GmbH, Hilden, Germany) was used for the extraction of DNA from agarose-gels. PCR products for cloning were purified with QIAquick® PCR Purification Kit, No. 28104 (Qiagen GmbH, Hilden, Germany). For qPCR, SensiMix™ SYBR Low-ROX Kit, No. QT625-05 was used (Bioline GmbB, Luckenwalde, Germany). The pENTR™ Direction TOPO® Cloning Kit, No. K2400-20 (Invitrogen, Karlsruhe, Germany) was used for generating the entry vectors.

# 3. Methods

## 3.1 Surface sterilization of *Arabidopsis* seeds

To select transgenic plants on MS-plates (first generation), *A. thaliana* seeds were sterilized by first rotating them in 70% Ethanol for one minute. Afterwards the ethanol was decanted and the seeds were incubated in 10% DanKlorix (Colgate-Palmolive, Hamburg, Germany) solution for 15 minutes. Subsequently, the DanKlorix solution was decanted and the seeds were washed with autoclaved bidest $H_2O$ for four times. 0,1% of Agar was given on top of the seeds and sown on sterile plates by pipetting. For all other experiments and selection of further generations that required sterilized seeds, *A. thaliana* seeds were rotated in 70% Ethanol for 15 minutes. The ethanol was decanted, and the seeds were washed with 100% Ethanol for two times. The seeds were dried on filter paper before sowing on sterile media (2.9, Table 4).

## 3.2 Plant growth conditions

For experiments, *A. thaliana* plants were grown on breeding soil (Floraton 1, Floragard, Oldenburg, Germany) mixed with silica sand in a ratio of 5:1. Plants that were used for seed collection were grown on a 1:1 soil mixture (Floraton 1, Floragard, Oldenburg, Germany and Einheitserde Classic, Einheitserde- und Humuswerke, Gebr. Patzer GmbH, Sinntal-Jossen, Germany) mixed with silica sand in a ratio of 5:1. In both cases, seeds were placed on well-watered soil and subsequently covered with wrapping film and stratified for 2 days at 4°C to synchronize germination. Seeds sown on sterile media (3.1) were stratified for 2 days at 4°C and kept under long term condition for 10 to 12 days before transferring the seedlings to breeding soil. *A. thaliana* Plants were kept either under short-day conditions (18°C/22°C (night/day), 70% relative humidity, and 100 µE m-2 s-1 light for 10-h days) or 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) depending on the experiment.

For transient tobacco transformation, *Nicotiana benthamiana* was grown on soil (Einheitserde Classic, Einheitserde- und Humuswerke, Gebr. Patzer GmbH, Sinntal-Jossen, Germany) mixed with perlite (Knauf, Iphofen, Germany) in a ratio of 5:1 and poured in 4-well plant pots. Seeds were placed on wet soil and transferred to the plant chamber. After two weeks, plants were individualized and transferred to a bigger pot. *N. benthamiana* was grown in 14 hours light – 10 hours dark cycles (20°C/25°C (night/day), 70% relative humidity, and approx. 200 µE $m^{-2}$ $s^{-1}$ light during the day).

### 3.3 Bacterial strains and culture conditions

*Escherichia coli* One Shot® TOP10 (Invitrogen™) was cultured on LB medium (2.9, Table 4) at 37°C (Memmert 500, Nuremberg, Germany). *Agrobacterium tumefaciens* strain GV3101::pMP90 was cultured at 28°C (MMM-Friocell 111, Munich, Germany) on LB medium. For liquid cultivation, *E. coli* and *A. tumefaciens* bacteria were shaken at 37°C and 28°C, respectively, at ~200 rpm (New Brunswick scientific Co., Inc. G25, New Jersey, USA). Selective antibiotics were added to solid or liquid media depending on the plasmid contained in the bacteria (2.5, Table 1). *Pseudomonas syringae* pathovar *tomato* (referred to as DC3000 or *Pst*) and *Pst* carrying *AvrRpm1, AvrRpt2* or *AvrRps4* were maintained on NYGA medium containing selective antibiotics (2.5, Table 1). The bacteria were grown at 28°C 2X O/N. For the Yeast-2-Hybrid assay, Y8800 MATa and Y8930 MAT$\alpha$, two different haploid yeast strains of opposite mating types were used. Both strains are derived from PJ69-4 (James et al., 1996; Vidal et al., 1996; Yu et al., 2008) and were cultured on YEPD (2.9, Table 4) plates 2X O/N at 28°C.

### 3.4 RNA and DNA extraction and cDNA-synthesis

Frozen plant material was ground with a mortar and pestle in liquid nitrogen. RNA was subsequently isolated by the phenol-extraction method as described (Logemann et al., 1987). DNA was isolated from frozen and ground plant material by adding 500µl chloroform and 500µl lysis buffer (CTAB+1% β-mercaptoethanol) to 50-100mg of plant material. The mixture was shaken at 1400 rpm at 8°C for 15 minutes. Subsequently, the samples were centrifuged at 14000 rpm at 4°C for 10 minutes. The supernatant (approx. 600µl) was added to a new reaction tube, which was supplemented with 100µl phytopure (GE Healthcare, Buckinghamshire, UK) and 500µl chlorophorm. Samples were shaken at 1400 rpm at 8°C for 15 minutes and centrifuged at 14000 rpm at 4°C for 10 minutes. The supernatant (approx. 500µl) was added to a new reaction tube, which was supplemented with 250µl isopropanol and incubated on ice for 10 minutes to precipitate the DNA. Subsequently, the samples were centrifuged at 14000 rpm at 4°C for 10 minutes. Supernatant was decanted and 1 ml of 70% ethanol/0.1M sodium acetate was added to the pellet. Additionally, the samples were incubated for 5 minutes at room temperature. The pellet was washed twice (once with 80% ethanol and once with 100% ethanol) and subsequently dried. In order to resolve the pellet, 30µl of purified water (Licrosolv, Merck, Darmstadt Germany) was added. The samples were then shaken at 800rpm at 8°C for 20 minutes.

Quality and concentration of the RNA and DNA samples were determined measuring the absorption at 260 nm and 280 nm using the Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The $A_{260nm}/A_{280nm}$ ratio was used to assess

the purity of total RNA or DNA and to detect the presence of protein, phenolics or other contaminants that absorb at or near 280nm. A ratio of approximately 1.8 to 2.0 is generally accepted for pure RNA or DNA. The $A_{260nm}/A_{230nm}$ ratio is a second purity measure, which should commonly be in the range of 2.0-2.2. An appreciably lower ratio may indicate the presence of contaminants absorbing at 230 nm. RNA integrity was further analysed by using 1% agarose gel electrophoresis (3.6).

Synthesis of cDNA from RNA was performed by using Superscript™II Reverse Transcriptase following the manufacturer's instructions (Invitrogen, California, USA).

### 3.5 Polymerase Chain Reaction and quantitative real-time-PCR analysis

Polymerase chain reaction (PCR) is a method that allows the exponential amplification of defined DNA sequences within a double stranded DNA (dsDNA) molecule *in vitro*. Amplification of genes-of-interest was performed by a standard PCR protocol consisting of three repeating sections, including denaturing of the dsDNA, annealing of the primers to the single-stranded DNA, and elongation of the new DNA strand. Depending on the DNA polymerases Mango *Taq*™ or Phusion® High Fidelity (2.7), dsDNA was first denatured at 94°C or 98°C, respectively. The annealing temperature of the primers depended on length and base pair composition of the primers used for amplification (8.2). Finally the elongation was carried out at 70 or 72°C depending on the DNA-Polymerase used (2.7). The duration of elongation depended on the expected length of the PCR product. PCR was executed in a MJ Research PTC-200 Peltier Thermal Cycler.

Quantitative real-time-PCR (qPCR) is a form of PCR, where quantification is accomplished by fluorescence measurements which are recorded during a PCR cycle. A cyanin-dye, in this case SYBR Green I, which is a major component in SensiMix™ SYBR Low-Rox Kit (2.6) binds to dsDNA and the resulting DNA-dye-complex emits green light ($\lambda_{max}$ = 520 nm). The fluorescence increases directly proportionally to the amount of PCR products, which enables the target quantification. cDNA was used as a template for the qPCR, in order to quantify the transcript accumulation of genes-of-interest with the primers in Table S4. To conduct the qPCR, the 7500 Real Time PCR System from Applied Biosystems (Darmstadt, Germany) and SensiMix™ SYBR Low-Rox Kit (2.6) were used according to the manufacturer's instructions.

### 3.6 Agarose gel electrophoresis

The separation of nucleic acids according to fragment size was done in 1% or 1.5% agarose gels in 1X TAE buffer (2.9, Table 3). Samples were supplemented with 6X loading dye to a final dye concentration of 1X. For the detection of the DNA, 0.05 µg/ml ethidium bromide was added to the gel which intercalates between double stranded DNA

and fluoresces when irradiated with ultraviolet light of wavelengths between 254nm and 366nm. After gels were run at a voltage between 70 of 100 V, depending on the size of the gel and the size of the fragment, nucleic acids were visualized with UV light (302nm). Subsequently, the gels were photographed and documented using the BIO-Print M1 gel documentation system from Vilber Lourmat (Eberhardzell, Germany).

### 3.7 Purification of PCR products and DNA reaction mixtures

If necessary for downstream experiments, DNA was purified, for example after PCR with the QIAquick PCR purification Kit (Qiagen) according to the manufacturer´s instructions. The concentration of the DNA was subsequently examined by measuring the absorption at 260 nm and the purity by measuring the absorption at 280 nm and 230 nm, calculating the ratios of $A_{260nm}/A_{230nm}$ and $A_{260nm}/A_{280nm}$ with the Nanodrop ND-1000 spectrophotometer (3.4) (NanoDrop Technologies, Wilmington, USA). Occasionally, DNA bands were cut out of agarose gels under UV light with a scalpel and transferred to a 2 ml reaction tube. DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer´s instructions. The concentration and purity of the DNA was analysed with the Nanodrop ND-1000 spectrophotometer as described above.

### 3.8 Gateway Cloning

For all cloning strategies, the Gateway® vector pENTR™/D-TOPO® (Invitrogen, Karlsruhe, Germany) was used as the entry vector, following the user manual from the pENTR™ Direction TOPO® Cloning Kit (2.10). 2µl of the TOPO® Cloning reaction was used to transform One Shot® chemically competent *E.coli* (3.3). To transfer the gene of interest from the entry construct into the Gateway™ destination vector to generate an expression clone, a LR recombination reaction using Gateway® LR Clonase™ II enzyme mix (2.6) was performed according to the manufacturer´s instructions. To transform One Shot® chemically competent *E.coli* (3.3) or electrocompetent *A. tumefaciens* (3.3), 2µl of the LR Cloning reaction was used.

### 3.9 Transformation of competent *E. coli* and *A. tumefaciens* GV3101

Chemically competent *E. coli* One Shot® TOP TEN cells were transformed by heat shock. To this end, an aliquot of 50µl of competent cells was thawed on ice for 10 minutes and mixed with 2µl (1 – 20 ng) of plasmid DNA by gentle tapping. The mixture was incubated on ice for 20 minutes. Afterwards, the cells were transformed by incubation at 42°C for 30 seconds with subsequent immediate cooling on ice. After 2 min on ice, the cells were suspended in 500µl LB media (2.9, Table 4) pre-warmed at 37°C, and shaken (250 rpm) for one hour at 37°C. Subsequently, 50 – 200µl were plated on LB plates with

appropriate antibiotics and incubated O/N at 37°C. Single colonies were picked and resuspended in 50µl bidest. $H_2O$. Colony PCR was performed by using 1µl of each bacterial suspension as template for normal PCR (3.5) to ascertain positive colonies. For further experiments, 45µl of the rest-suspension was put in 2ml of LB media (2.9, Table 4) with appropriate antibiotics and shaken (250 rpm) O/N at 37°C. Subsequently, 700µl were mixed with 700µl of 30% glycerol, immediately frozen in liquid nitrogen, and kept at -80°C.

For electroporation of *A. tumefaciens*, an aliquot of 50µl of electro competent cells, obtained from Birgit Geist (Institute for biochemical plant pathology, HelmholtzZentrum München, Germany), was thawed on ice for 10 minutes, mixed with 1µl (80 – 100 ng) of plasmid DNA and transferred to a chilled 1mm Gene Pulser cuvette (Bio-Rad, Munich, Germany). An electric pulse of 25 µF capacitance, 1.25 V and 400 Ω resistance was applied. Afterwards, cells were immediately suspended in 2 ml LB medium and shaken (250 rpm) for one hour at 28°C. Subsequently, 50 – 200µl were plated on LB plates with appropriate antibiotics (2.5, Table 1) and incubated at 28°C 2X O/N. Single colonies were checked by Colony PCR and positive colonies were further propagated and stored as described above.

### 3.10 Identification of homozygous knock out plant lines

The T-DNA knock out (KO) lines of *A. thaliana,* obtained from the Nottingham Arabidopsis Stock Centre (2.1) were tested to identify homozygous KO mutants. To screen the plants for chromosomal T-DNA insertion, an established PCR- based method was applied (Figure 6; http://signal.salk.edu/tdnaprimers.2.html).

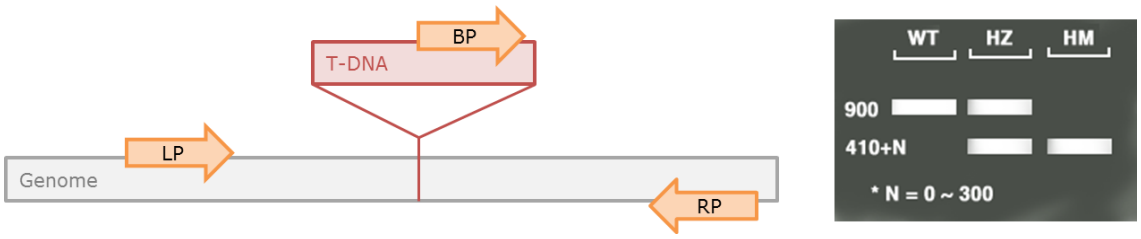


**Figure 6: PCR-based strategy to identify homozygous KO mutants**

With the primers LP (left genomic primer) and RP (right genomic primer), PCR product size of wild-type plants (WT) with no insertion is about 900-1100bp. PCR product size of homozygous (HM) lines, where the insertion is on both chromosomes, is 410+N bp - from BP (T-DNA border primer) to RP. Whereas heterozygous lines (HZ) have the insertion of one of the pair chromosomes and the resulting PCR product will show both sizes (from LP to RP and BP to RP). N, difference of the actual insertion site and the flanking sequence position, usually 0 - 300 bases; Figure was modified Breitenbach (2012).

Primers used for screening the *lec1* mutants were T1 (BP), HB-P2 (LP) and HB-P3 (RP) (8.2, Table S2), whereas T1 (BP), HB-P4 (LP) and T2 (RP) (8.2, Table S2) were used to screen *llp1-3*. The primer T1 (BP), T3 (LP) and T4 (RP) were used for screening *xth25* (8.2, Table S2). Two PCR reactions were performed on DNA that was isolated from four to six plants of every line. As illustrated in Figure 6, the LP and RP primers were used to identify the wild-type genomic fragment, whereas the BP and RP primers identified the T-DNA insert. The PCR products were separated via electrophoresis on a 1% agarose gel and the occurring bands were evaluated. If there was only a PCR product with primer pair LP and RP, the plant was considered as wild-type and if both primer pairs yielded PCR products, the plant was identified as heterozygous (Figure 6). Plants that only showed a PCR product with the primer pair BP and RP were homozygous KO mutant plants and used for further analysis.

### 3.11 Generation of transgenic lines

All vector constructs generated during this PhD project were cloned into pENTR/dTOPO (2.4), transformed in *E.coli* TOP10 (2.2) and incubated O/N at 37°C on LB plates (2.9, Table 4) with appropriate antibiotics (2.5, Table 1). Single colonies were picked from the plates and tested by PCR (3.5) for positive insertion of the requested sequence and cultivated O/N in liquid LB media (2.9, Table 4) with antibiotics (2.5, Table 1). After plasmids were isolated (2.10) they were sent for sequencing of the cloned insert to MWG Operon. Only accurate plasmids containing fully correct insert sequences were used for further cloning as described in detail in the subsequent sections. 'Silent' mutations that do not affect the encoded amino acid sequence were allowed.

Sequencing was done with the M13 forward and M13 reverse primers that anneal close to the 5' and 3' ends of the respective DNA inserts in pENTR/dTOPO. If insert sequences were longer than 800 bases, additional sequencing reactions with the other primers in Table S3 were performed.

#### 3.11.1 Multiple silencing by RNA-Interference

The RNAi constructs targeting *LEC1* and *LEC2* (double silencing construct) and *LLP1*, *LEC1* and *LEC2* (triple silencing construct) are described in detail in Breitenbach (2012). In short, 272- to 319-nucleotide fragments of the coding sequences of each target gene were amplified by PCR using cDNA that was generated with RNA from untreated Col-0 WT plants as template in the first PCR step. For the double silencing construct, the primer sets HB-P17/HB-P18 and HB-P19/HB-P20 (8.2, Table S5) were used. The resulting products were merged in a further PCR with the primer pair HB-P17/HB-P20 (8.2, Table S5). Templates for the triple silencing construct were first generated by using the primer sets HB-P21/HB-P22, HB-P23/HB-P24 and HB-P25/HB-P26 (8.2, Table S5). To

merge the resulting fragments, PCR was performed with the primer pair HB-P21/P26 (8.2, Table S5). For both, double and triple silencing constructs, the resulting 591- and 882-nucleotide fragments were cloned into pENTR/dTOPO (2.4) and sequenced. The sequenced fragments were subsequently cloned in pHANNIBAL (2.4) in the sense and antisense orientations using XhoI/Kpn I and BamHI/HindIII (2.6), respectively. The RNAi cassettes from pHANNIBAL were transferred to the binary vector pART27 (2.4) using NotI (2.6). The pART27 vectors containing the RNAi:*LEC1/LEC2* or RNAi:*LEC1/LEC2/LLP1* cassettes were transformed into *Agrobacterium tumefaciens* strain GV3101 (2.4). The resulting *A. tumefaciens* strains were used to transform Col-0 and *eds1-2* mutant plants with the double and triple silencing constructs (as described in 3.11.3 Transformation of *A. thaliana*).

### 3.11.2 Native promoter constructs

Constructs to generate transgenic plants expressing *LEC1*, *LEC2* or *LLP1* from their own native promoters were generated via Gateway Cloning (2.6, 3.8). Constructs where GREEN FLUORESCENT PROTEIN (GFP) was tagged to the protein were used for protein localization as described in 3.17. Furthermore, constructs were generated driving the expression of *GFP* from the native promoter of *LEC1*, *LEC2* or *LLP1*, respectively. The full DNA sequences of all constructs are shown in Table S7 (8.3). Approximately 2 kilo bases (Kb) upstream of the respective transcription start sites of *LEC1*, *LEC2* or *LLP1* were defined as the respective native promoter regions. The relatively large promoter size was chosen to increase the probability that all promoter elements would be present, including the core promoter region, the proximal promoter and the distal promoter elements, which was set at ~2000kb to the transcriptional start site.

Genomic DNA was isolated (3.4) from Col-0 plants as template and PCR (3.5) was performed using the proof-reading Phusion® High Fidelity DNA Polymerase (2.7). The primer sets listed in Table 9 were used for amplifying the respective native promoters or the native promoters with the attached protein coding sequences. If the *GFP* tag from pBGWFS7,0ΔGUS was to be merged to the coding sequence, the reverse primer was adapted not to contain the stop codon of the respective gene.

**Table 9: Cloning procedure: primer sets, resulting constructs and expected PCR target sizes.** The sequences of the indicated primers can be found in table Table S7 in the supplementary material (8.3).

| Primer Set | resulting construct | PCR target size |
|---|---|---|
| C1/C3 | pLEC1:*GFP* | 2256nt |
| C1/C4 | pLEC1:*LEC1-GFP* | 3069nt |
| C1/C5 | pLEC1:*LEC1* | 3072nt |

| | | |
|---|---|---|
| C6/C8 | pLEC2:*GFP* | 1643nt |
| C6/C9 | pLEC2:*LEC2-GFP* | 2471nt |
| C6/C10 | pLEC2:*LEC2* | 2474nt |
| C11/C13 | pLLP1:*GFP* | 1875nt |
| C11/C14 | pLLP1:*LLP1-GFP* | 2697nt |
| C11/C15 | pLLP1:*LLP1* | 2700nt |

The resulting PCR fragments were cloned into pENTR/dTOPO (2.4) and sequenced. Accurate insert sequences were transferred to pBGWFS7,0ΔGUS (2.4) via Gateway® cloning (3.8). All pBGWFS7,0ΔGUS vectors containing the native promoter constructs were transformed into *A. tumefaciens* strain GV3101 (3.3, 3.9).

### 3.11.3 Transformation of *A. thaliana*

For generating transgenic plants, *A.thaliana* was grown under long-day conditions and first floral stems were cut after 6 weeks to increase flower development (Clough and Bent, 1998). One week after cutting, the newly developed flowers were 'dipped' in suspensions containing *A. tumefaciens* with selected constructs. Floral dipping was performed as previously described (Logemann et al., 2006). An overview of all plant transformations is given in Table S6.

### 3.11.4 Selection of transgenic plants

For the selection of the silencing lines (3.11.1), seeds from the respective transformed plants were surface sterilized (3) and selected on MS medium (2.9, Table 4) containing the selective antibiotic Kanamycin (2.5, Table 1). The plates were kept at 4°C for 2 days for stratification and afterwards kept under long-day conditions (3.2). After two to three weeks, plants carrying a transgene developed healthy true leaves, whereas plants not carrying a transgene died or cotyledons were severely yellowed. Healthy looking, green seedlings of the first generation (T1) were transferred to soil and grown in long-day conditions (3.2). Leaves of 4-week-old plants were harvested and RNA was isolated (3.4). After cDNA-synthesis (3.4), qPCR was performed (3.5) for analysing the expression levels of *LEC1*, *LEC2* or *LLP1*. Transformants which showed a significantly reduced expression level of the targeted genes were kept for further seed production. Seeds from single plants in the second generation (T2) were harvested, surface sterilized with EtOH (3) and sown on MS plates as described above. After two weeks, segregation analysis was used for identification of single insertion lines (3:1 ratio) in the second generation. 24 seedlings per single insertion line were transferred to soil and grown in long-day conditions (3.2). Seeds of single plants were subsequently sown on MS plates as described and seedlings were counted two weeks later. Single insertion lines with a

100% survival rate were considered homozygous for the respective RNAi transgene. One to two independent and homozygous single insertion lines were selected from each transformation for further characterization.

### 3.12 The induction of SAR in *A. thaliana*

Systemic acquired resistance (SAR, 1.4) is a mechanism of induced defence and occurs in the systemic healthy tissue of locally infected plants. SAR experiments were conducted with wild-type and different transgenic and mutant plants that were grown in short-day conditions (3.2). For primary infection, the first two true leaves (Figure 7, red arrows) of 4-week-old plants were inoculated as described below. Three days later, a secondary infection was conducted in the third and fourth true leaves (Figure 7, yellow arrows), which were harvested four days after the secondary infection to monitor the growth of the bacterial pathogen used.

For each inoculation, the appropriate *P. syringae* strain was grown O/N at 28°C on a NYGA medium plate (2.9, Table 4) containing selective antibiotics (2.5, Table 1). For inoculation, cells were scraped off the O/N culture with an inoculation loop and resuspended in 10mM $MgCl_2$, pH 7,0. By measuring the optical density at a wavelength of 600 nm using a photometer (Ultrospec 3100 pro, GE Healthcare, Munich, Germany), the required concentration was calculated assuming an $OD_{600}$ of 0.2 contains $10^8$ CFU/ml (colony-forming unit/ml) of bacteria (Marion Wenig and Finni Häußler, personal communication). For primary infection, the first two true leaves were inoculated either with the suspension containing 1 x $10^6$ CFU/ml (diluted in 10mM $MgCl_2$) of *Pst AvrRpm1* or with the mock solution (10mM $MgCl_2$) as control by infiltration with a needleless syringe. Three days after the primary infection, the next two true leaves were inoculated with 1 x $10^5$ CFU/ml of DC3000.

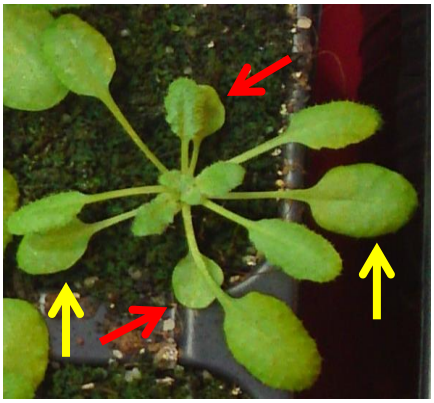

**Figure 7: SAR assay**

1° leaves (red arrows) of the different genotypes were infiltrated with *Pst AvrRpm1* or with 10mM $MgCl_2$ as a control. After 3 days 2° leaves (systemic, yellow arrows) were infiltrated with DC3000. After another 4 days the 2° infected leaves were harvested.

Another 4 days later, the secondary infected leaves (Figure 7, yellow arrows), were harvested and growth of the bacteria was analysed. To this end, bacteria were extracted from 6mm leaf discs from the secondary infected leaves. Per genotype and primary treatment, four samples were harvested containing leaf discs from three independent leaves each. These were shaken at 600 rpm at 25 °C in 500µL 10mM $MgCl_2$ with 0,01% Silwet. After 1h, the resulting suspension was diluted in 10mM $MgCl_2$ in five serial 10X steps. Subsequently, 20µl of each dilution was spotted onto NYGA plates containing antibiotics (2.5, Table 1) and placed for 2 days at 28°C. Afterwards single colonies were counted optically in the dilution level which showed between 5 and 70 single colonies. Due to the dilution level and regarding the diameter of the leaf disc, CFU/cm² of bacteria was ascertained.

### 3.13 Growth curve analysis of *Pseudomonas syringae pv. tomato* (*Pst*)

Growth curve analyses were performed with different bacterial strains (3.3). For each inoculation the appropriate *Pst* strain was grown O/N at 28°C on a NYGA medium plate containing appropriate antibiotics (2.5, Table 1). On the day of the inoculation, cells were removed from the plates and resuspended in 10mM $MgCl_2$, pH 7.0. Leaves of 4-week-old short-day-grown *A.thaliana* plants were infiltrated with the appropriate *Pst* strain in a suspension containing 1 x $10^5$ CFU/ml. Subsequently, the *in planta* growth of the bacteria was measured at four different time points. Two hours (t=0) after infiltration, the first samples from the infiltrated leaves were taken whereby the surface of the leaf area was sterilized by dipping them for 10s into 70% ethanol followed by 10s in bidest $H_2O$. For additional time points: 2, 4 and 7 days after the infection, surface sterilization of the harvested leaves was not performed as no differences were noted in the bacterial titers with or without surface sterilization (Marion Wenig and Corina Vlot, personal communication). For analysis of *Pst* growth in leaves, bacteria were extracted from three 6mm leaf discs per sample (in triplicate per genotype) and counted 2 days after harvesting as described in 3.12.

### 3.14 Treatment of *A.thaliana* with different phytohormones

**16-day-old Col-0 and *eds1-2* plants grown in short-day conditions were sprayed until drop-off with 1mM and/or 100µM of BTH or SA or with 100µM of MeJA or ABA (2.9,**

**Table 6) in water (BTH) or 0.1% MeOH (all others). As controls, water was used for comparison with the BTH treatment and 0,1% of Methanol (MeOH, mock) was used for comparison with the SA, MeJA and ABA treatments. All spray treatments were performed in the presence of 0,01% Tween-20 (2.9,**

Table 6). The aerial part of 8 plants per experimental replicate was harvested 2 hours after infection (hpi), 6hpi, 24hpi and 48hpi and analysed for the transcript accumulation of *LEC1*, *LEC2*, and *LLP1* relative to that of *AtTUBULIN* by qRT-PCR (3.4, 3.5).

### 3.15 Abiotic stress of *A.thaliana* with NaCl

Seeds of different transgenic and non-transgenic *A. thaliana* lines were surface-sterilized (3) and placed on MS plates (2.9, Table 4) with Carbenicilin and Cefotaxime (2.4) and different concentrations of NaCl (0mM, 50mM and 100mM). Plates were stratified for 2 days at 4°C to synchronize germination and subsequently kept under long-day conditions (3.2). 10 days after stratification, 10-12 plants per replicate were harvested and analysed for *AtABI1* (*ABA-INSENSITIVE1*), *AtRAB18* (*RESPONSIVE TO ABA 18*) and *AtRD29B* (*RESPONSIVE TO DESICCATION 29B*) transcript accumulation relative to that of *AtUBIQUITIN* by qRT-PCR (3.4, 3.5).

### 3.16 *Alternaria brassicicola* infection protocol

**To investigate whether the silencing of the lectins might influence the JA pathway, 4-week-old plants that were grown in long-day conditions (3.2) were infected with the necrotrophic fungus *Alternaria brassicicola.* Two to three weeks before the infection, ~1cm² pieces of the fungus were cut out from the stock plates and placed on oat media. On the day of the infection, mycelium was removed from the oat plates and solved in MKP buffer (2.9,**

Table 6) until a concentration of 200 mitospores per µl was achieved as monitored with an optical microscope. For the infection, 3µl (600 spores) of the solution were dropped onto the third and fourth true leaves of 10 plants per line. Lesion sizes were measured optically with a ruler 5 days after the infection.

### 3.17 Transient tobacco transformation

The *Agrobacterium tumefaciens* strains (2.2) hosting a GFP-encoding plant expression construct (2.4) or *p35S::p19* (2.4) were inoculated in 5 ml LB with appropriate antibiotics (2.5) and grown two days at 28°C until a $OD_{600}$ of 1,7-2,0 was achieved. Each

bacterial solution was centrifuged at 3500xg for 10min. After discarding the supernatant, the pellet was washed by resuspending it in 1ml infiltration buffer (2.9, Table 5) and was centrifuged at 3500xg for 10min. The supernatant was discarded and the pellet was resuspended in infiltration buffer (2.9, Table 5) until $OD_{600}$ of 1.6 was achieved. The resulting suspension was incubated at room temperature for 3h. *A. tumefaciens* cultures containing *GFP* expression constructs were mixed 1:1 immediately before infiltration with cultures containing the p19 silencing suppression vector. For infiltration, two to three bigger lower leaves of 5-week-old *N.benthamiana* (2.1) were syringe infiltrated. Five days after infiltration, 0,5mm leave discs were punched out of the infiltrated leaf areas and analysed with a confocal fluorescence microscope (TCS SP8 X, Leica Microsystems GmbH, Wetzlar, Germany).

### 3.18 Yeast-2-Hybrid assay

To look for potential protein-protein interactions between LLP1, LEC1, and/or LEC2 or between these and other proteins, yeast-two-hybrid analyses (Y2H). For cloning the coding sequences of *LLP1, LEC1* and *LEC2,* PCR was performed with the proof-reading Phusion® High Fidelity DNA Polymerase (2.7) using the primer pairs C12/C15, C2/C5 and C7/C10 (8.2), respectively. cDNA that was generated using RNA from infected Col-0 plants was used as template for the PCRs.

The resulting PCR fragments were cloned into pENTR/dTOPO as described in 3.8. Sequenced and accurate inserts were cloned via Gateway® cloning (3.8) in AD-Y and DB-X vectors (2.4). The Y8800 MATa and Y8930 MAT$\alpha$ yeast strains (2.2) were transformed by heat shock (3.9) with the cloned AD-Y and DB-X constructs, respectively. The transformation and cultivation of yeast was further performed exactly according to (Dreze et al 2010b). The Y2H-Assay was accomplished in cooperation with Dr. Pascal Falter-Braun (Institute of Network Biology, HelmholtzZentrum München, Germany).

### 3.19 Statistical analysis and IT tools

All statistics were done with the Student's t-test (Excel, Microsoft Office 2007 Microsoft) to determine significantly different data sets.

Microscopy images were edited with the image processing application of the attendant microscope (Leica TCS SP8) and overlays were generated with ImageJ1 (ImageJ, NIH, Bethesda, USA).

The reference manager Mendeley (Mendeley Ltd., London, UK) was used in this thesis for citation and bibliography.

# 4. Results

## 4.1 Regulation of *LEC1, LEC2,* and *LLP1* by phytohormones

*LEC1* was first identified in microarray-based screening experiments for MeJA-responsive genes (Schenk et al., 2000; Jung et al., 2007a; Jung et al., 2007b). It is known that *LEC1* is highly expressed in the rosette leaves and roots of mature plants. Lyou et al. (2009) confirmed that *LEC1* is induced by JA and also reported that *LEC1* is repressed by SA in young plants (Lyou et al., 2009). Zhang et al. (2002) showed that *LEC2* is induced by long-chain polymeric chitin and suggested *LEC2* to play a role in the plant defence response (Zhang et al., 2002). The expression of *LLP1* is induced by *P. syringae* infection and by SA and its functional analogue BTH (Armijo et al., 2013; Breitenbach et al., 2014). *LLP1* is known to play a role in plant defence and in particular in SAR (Breitenbach, 2012; Breitenbach et al., 2014).

**To gain more insights into the regulation of *LEC1*, *LEC2*, and *LLP1* by different phytohormones, 16-day-old Col-0 and *eds1*-2 *Arabidopsis* plants were sprayed with phytohormones, including the JA-derivate MeJA, SA and its functional analogue BTH, and ABA (2.9,**

Table 6). The transcript accumulation of *LEC1*, *LEC2*, and *LLP1* was measured at 2hpi, 6hpi and 24hpi (3.14). Henceforth, the focus is on gene expression changes that were reproducible between three biologically independent experiments.

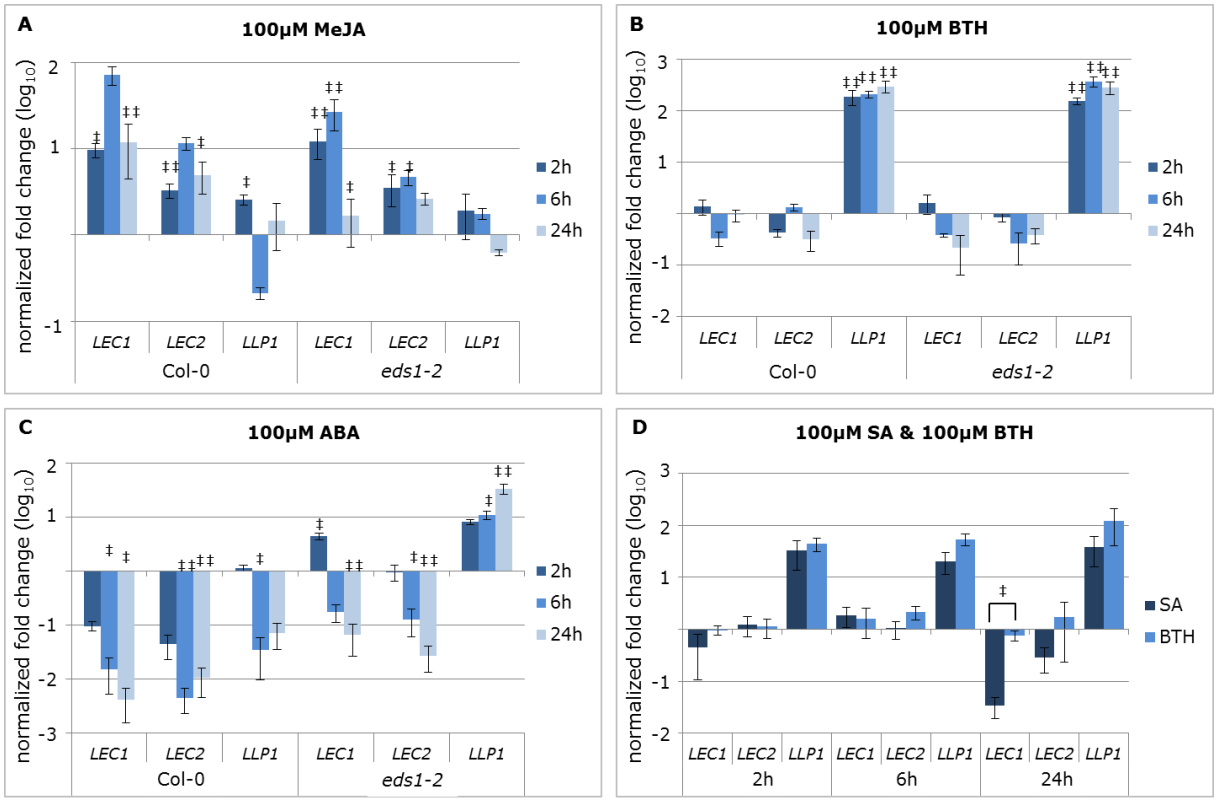


**Figure 8: Transcript accumulation of *LEC1*, *LEC2* and *LLP1* in Col-0 (A-D) and *eds1-2* (A-C) after different phytohormone treatments**

16-day-old plants were sprayed with 100µM MeJA, 100µM BTH, 100µM ABA or 100µM SA. Aerial parts were harvested 2hpi, 6hpi and 24hpi to monitor the gene expression of *LEC1*, *LEC2* and *LLP1* compared to MOCK treatment and relative to that of *AtTUBULIN*. Bars represent the average ± standard deviation of three replicates. Asterisks indicate reproducible significant differences to MOCK-treated plants (A-C) or in between the different treatments (D) in at least two experiments (‡ reproducible in two experiments, ‡‡ reproducible in three experiments). Experiment was repeated three times. (Figure 8) shows the data from one representative experiment; the replicate experiments are shown in Figure S1.

After treatment of the plants with MeJA, gene expression of *LEC1* and *LEC2* was induced at 2hpi in Col-0 plants and remained elevated until at least 24hpi (Figure 8A). The expression pattern of *LEC1* and *LEC2* in response to MeJA was not significantly different in *eds1-2* compared to Col-0 plants. That leads to the suggestion that the induction of *LEC1* and *LEC2* by MeJA was *EDS1*-independent. Transcript accumulation of *LLP1* after MeJA treatment was only moderately elevated compared to mock at 2hpi in Col-0, suggesting that the gene expression of *LLP1* was less responsive to MeJA than that of *LEC1* and *LEC2* (Figure 8A).

In contrast to MeJA, the SA-analogue BTH did not induce the gene expression of *LEC1* and *LEC2* in either Col-0 or *eds1-2* (Figure 8B). Transcript accumulation of *LLP1* was elevated by BTH from 2hpi to 24hpi at a similar level in both Col-0 and *eds1-2* plants (Figure 8B). The transcriptional regulation of *LLP1* at 2hpi was comparable to that at 6hpi

and 24hpi in Col-0. Similarly, in *eds1-2* the transcript pattern of *LLP1* did not differ from 2hpi to 24hpi. In 2013, Gruner et al. detected differential regulation of genes by BTH compared to SA (Gruner et al., 2013). To confirm that BTH activates *LLP1* in a similar manner as SA, the transcript accumulation of *LLP1* in response to BTH and SA was compared in Col-0 plants (Figure 8D). The *LLP1* expression pattern in Col-0 plants in response to BTH as compared to mock shown in Figure 8D was comparable to that in Figure 8B. *LEC1* and *LEC2* were not induced at 2hpi, 6hpi or 24hpi in Col-0 by BTH or SA (Figure 8D). Gene expression of *LLP1* was induced by BTH and SA in a similar manner at 2hpi, 6hpi and 24hpi. The data indicate that *LEC1, LEC2,* and *LLP1* were regulated by BTH and SA in a similar manner (Figure 8D). Hence, BTH was used throughout the following experiments as SA substitute.

After treatment with ABA, *LEC1* and *LEC2* transcript accumulation was significantly reduced as compared to mock in Col-0 from 6hpi to 24hpi (Figure 8C). In *eds1-2*, *LEC1* was slightly induced 2hpi but transcript accumulations of *LEC1* and *LEC2* were reduced at 6hpi and 24hpi. It seems that the reduction of *LEC1* and *LEC2* at 2hpi and 6hpi in Col-0 is stronger than that in *eds1-2*. This was confirmed in all replicate experiments at 6hpi for *LEC1* and *LEC2* but not at 2hpi for *LEC2* (8.1, Figure S1). Hence, the regulation of *LEC1* and *LEC2* by ABA seems to be partially dependent on EDS1. Gene expression of *LLP1* was downregulated by ABA in Col-0 at 6hpi and 24hpi. In contrast, elevated *LLP1* expression was observed in response to ABA in *eds1-2* from 6hpi to 24hpi (Figure 8C). This indicates that *LLP1* is downregulated by ABA but only in the presence of EDS1 (Figure 8C).

Taken together, the data indicate that *LEC1* and *LEC2* were upregulated by MeJA and downregulated by ABA (Figure 8A, Figure 8C). The transcriptional regulation of *LEC1* and *LEC2* by MeJA were EDS1-independent, whereas *LEC1* and *LEC2* regulation by ABA was partially EDS1-dependent. *LLP1* gene expression was induced by BTH and SA, which confirmed results from Breitenbach et al. (Figure 8B, Figure 8D)(Breitenbach et al., 2014). This induction was EDS1-independent, whereas the regulation of *LLP1* by ABA happened in an EDS-dependent manner. The data suggest that *LLP1* is negatively influenced by EDS1 under ABA treatment.

## 4.2 Expression of GFP-tagged LEC1, LEC2 and LLP1 driven by their own native promoters

The legume lectin-like protein LLP1 was found in apoplast-enriched extracts (Breitenbach et al., 2014) and was confirmed to be localized mainly on the apoplastic side of the plasma membrane (Armijo et al., 2013). To verify the localization of LLP1 and to localize its two closest homologs, LEC1 and LEC2, the constructs shown in 8.3 (Table S7) were generated to express *LEC1, LEC2* or *LLP1* from their respective native promoters

(3.11.2). These promoter:protein-GFP constructs should provide insight into the subcellular localization of the lectins. To check the construct for functionality, they were first transformed transiently in tobacco. To this end, fully expanded leaves of 5-week-old *Nicotiana benthamiana* plants were syringe-infiltrated with *Agrobacterium tumefaciens* containing the promoter:protein-GFP constructs (3.11.2, 3.17). The GFP signal was examined in leaf discs from the agroinfiltrated leaves by laser scanning microscopy (TCS SP8 X, Leica Microsystems GmbH, Wetzlar, Germany) 5 days after infiltration (3.17).

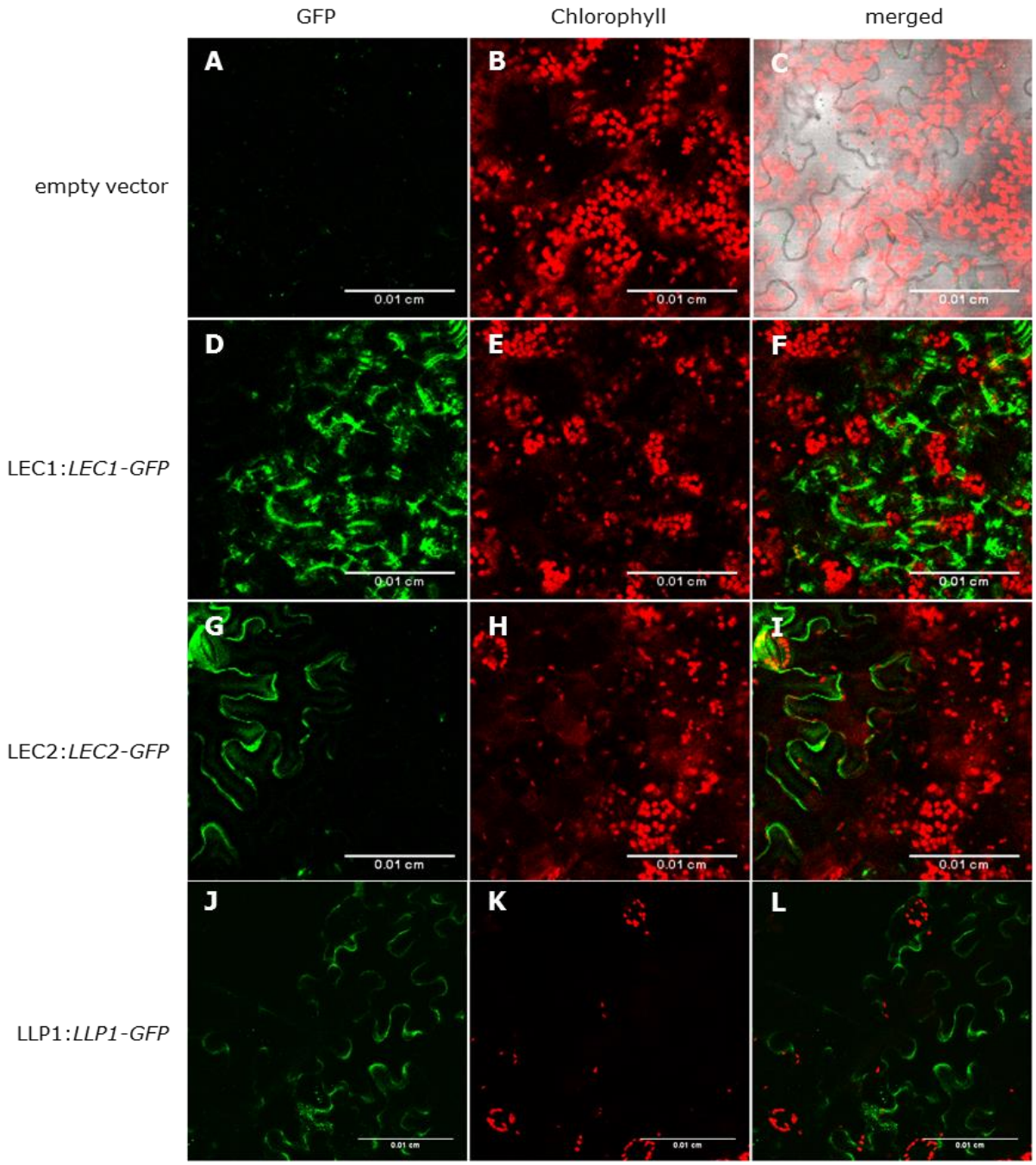


**Figure 9: GFP signal in transient transformed tobacco**

Transient Expression of empty vector (A-C) and the lectin-GFP fusions indicated to the left of the panels (D-L) 5 days after infiltration of *A. tumefaciens* carrying the respective promoter:protein-GFP constructs into tobacco leaves. (A, D, G, J) GFP fluorescence in green; (B, E, H, K) Chlorophyll fluorescence in red; (C, F, I, L) overlay of the two preceding images; C shows overlay with Bright field to show epidermal structure. Scale bars: 0,01cm.

The empty vector was used as control to exclude auto-fluorescence (Figure 9A-C) in the wavelength spectrum of GFP (Figure 9A). Chlorophyll was detected with a wavelength ratio of 654-784nm (Figure 9B) by using a multi-colour-laser. In Figure 9C both images were merged and bright field image was laid over to contour the epidermal structure of *Nicotiana benthamiana*. The GFP signal of LEC1:*LEC1-GFP* was mostly observed in parenchymal cells (GFP: Figure 9D, RFP/Chlorophyll: Figure 9E, Overlay: Figure 9F), suggesting that LEC1 is located not necessarily in the extracellular region of endodermal tobacco cells. Figure 9G-I show the GFP-signal in tobacco leaves infiltrated with LEC2:*LEC2-GFP* (GFP: Figure 9G, RFP/Chlorophyll: Figure 9H, Overlay: Figure 9I). Although it cannot be ascertained by this experiment, it seems that LEC2 might be located near the plasma membrane, which would correlate with the assumption that LEC2 is found in the apoplast (1.9). For LLP1 (GFP: Figure 9J, RFP/Chlorophyll: Figure 9K, Overlay: Figure 9L) it is known that it is mainly located on the apoplastic side of the plasma membrane (Armijo et al., 2013). Data shown in Figure 9J appear to confirm this finding.

Notably, only 20-30% of the agroinfiltrated leaf areas showed an evaluable GFP signal in *Nicotiana benthamiana*. This might be due to the use of the native gene promoters from *Arabidopsis,* which might not be equally efficient when ectopically expressed in *N. benthamiana.* Nevertheless, significant accumulation of GFP-tagged LEC1, LEC2, and LLP1 was observed, indicating that the native promoter regions that were chosen for *LEC1, LEC2* and *LLP1* were adequate for functional transcript accumulation.

## 4.3 Characterization of RNAi-mediated silencing lines targeting *LEC1, LEC2* and *LLP1*

Due to the close phylogenetic relationship between *LEC1, LEC2* and *LLP1* (1.9), the coding DNA sequences (CDS) were compared to assess the percentage of homology in base-pairs between the three loci (Table 10). The CDS is the region of a gene's DNA that is transcribed to generate mRNA, which is subsequently translated by the ribosomes to generate protein. If genes are phylogenetically distinct, which is not the case here; it is more expressive to compare the protein sequence, which is more conserved. *LEC1*, *LEC2* and *LLP1* have just one splicing isoform each and are phylogenetically close (Figure 5). Therefore, it is more informative to compare the CDS rather than the amino acid sequence.

**Table 10: Comparison of the coding sequences of *LEC1, LEC2* and *LLP1***

The coding sequences (CDS) were downloaded from TAIR (www.arabidopsis.org) and compared by Align Sequences Nucleotide BLAST (Zhang et al. 2000). Percent nucleotide identity is given.

| | ***LEC1*** | ***LEC2*** | ***LLP1*** |
|---|---|---|---|
| ***LEC1*** | - | 89% | 76% |
| ***LEC2*** | 89% | - | 77% |
| ***LLP1*** | 76% | 77% | - |

As described in Table 10, *LEC1, LEC2* and *LLP1* share a high similarity of their CDS of 76% (*LEC1* and *LLP1*), 77% (*LEC2* and *LLP1*) up to 89% (*LEC1* and *LEC2*). Because of their close relationship, it might be that these lectin genes fulfil similar functions during plant defence. It is possible that LEC1, LEC2 and/or LLP1 compensate for each other (i.e. are functionally redundant) or interact during stress response. Therefore, RNAi-mediated silencing lines were generated (3.11.1) to gain insight into a putative interplay between LLP1, LEC1 and LEC2 during SAR. Table 11 gives an overview of the multiple silencing constructs that were designed by Heiko Breitenbach and used in this study (Breitenbach, 2012).

**Table 11: Overview of RNAi-mediated silencing constructs**

Constructs to silence *LEC1* and *LEC2* (double silencing construct, 2) in Col-0 (C) as well as to silence *LEC1*, LEC2, and *LLP1* (triple silencing construct, 3) in Col-0 (C) and *eds1-2* (e) were designed and generated by Heiko Breitenbach (Breitenbach, 2012).

| **Name** | **Construct** | **Transformed into** |
|---|---|---|
| C2 | RNAi:*LEC1*/*LEC2* | Col-0 |
| C3 | RNAi:*LEC1*/*LEC2*/*LLP1* | Col-0 |
| e3 | RNAi:*LEC1*/*LEC2*/*LLP1* | *eds1-2* |

The constructs were used to generate transgenic *Arabidopsis thaliana* plants as described in 3.11. Independent homozygous lines of the third generation were selected as described in 3.11.4. In the background of Col-0 two independent single insertion lines were selected that carry the double silencing construct (C2 5-2, C2 6-2) or the triple silencing construct (C3 12-2, C3 13-1). For the triple silencing line in the background of *eds1-2,* one homozygous single insertion line was available for further experiments (e3 3-5).

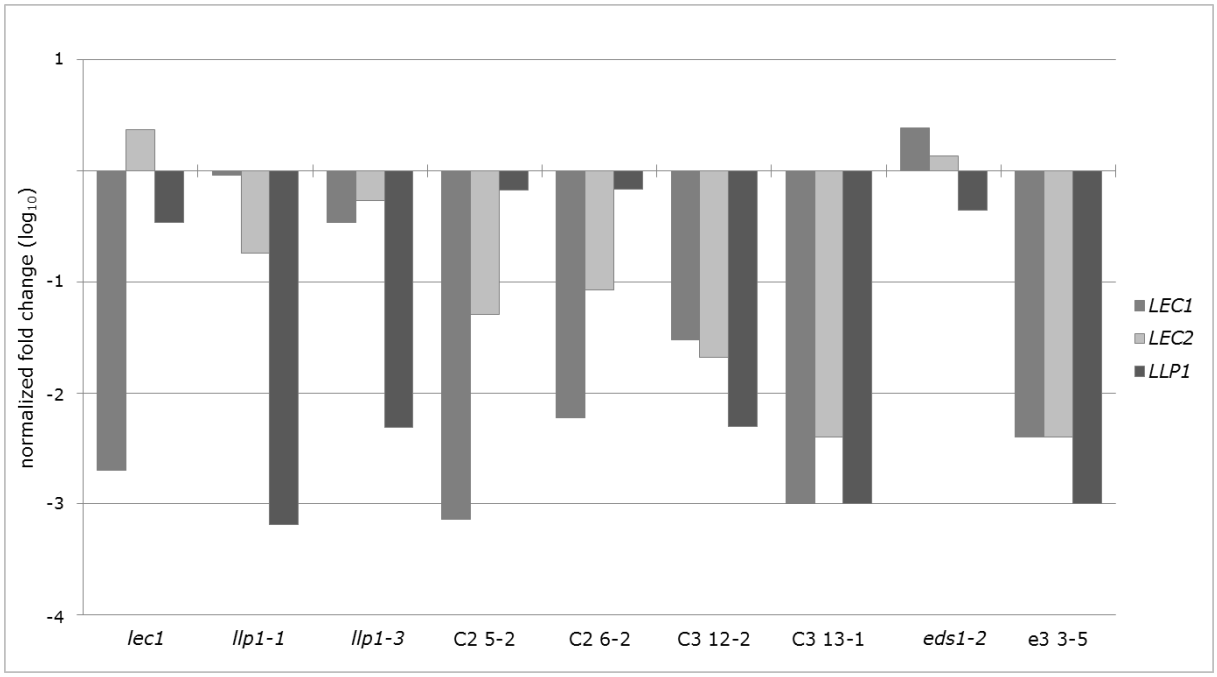


**Figure 10: RNAi-mediated silencing lines**

Transcript accumulation of *LEC1, LEC2* and *LLP1* in T-DNA insertion lines (*lec1, llp1-1* and *llp1*-3) and in RNAi lines targeting two or three legume-lectin like genes in the background of Col-0 (C2, C3) or *eds1-2* (e3). Transcript levels were normalized to that of *AtUBIQUITIN* and are shown relative to that in untreated Col-0 plants. Transcript levels were measured several times concomitantly to various experiments and with comparable results.

Leaves of 4-week-old *Arabidopsis* plants were analysed for basal transcript accumulation of *LEC1, LEC2* and *LLP1* (Figure 10). The Col-0 mutant lines *lec1, llp1-1,* and *llp1-3* carried T-DNA insertions closely behind the start codon of *LEC1* (*lec1*), *LLP1* (*llp1-1*) (Breitenbach, 2012; Breitenbach et al., 2014) or upstream of *LLP1* (*llp1-3*)(Breitenbach et al., 2014), and were included in this analysis. All T-DNA insertions reduced the transcript accumulation of the targeted gene by more than 100-fold (Figure 10). Beside the T-DNA insertion lines, also the transcript accumulations in the RNAi-mediated silencing lines were characterized. Compared to Col-0, the double silencing lines C2 5-2 and C2 6-2 (Table 11) showed reduced transcript accumulation of *LEC1* by more than 100-fold (Figure 10). The expression of *LEC2* was 10- to 20-fold reduced in C2 5-2 and C2 6-2, respectively, compared to the WT level. The double silencing lines showed WT expression levels of *LLP1*, confirming the specificity of the RNAi construct used. The transcript accumulation of *LLP1* in the triple silencing line C3 12-2 (Table 11) was more than 100-fold reduced compared to that in Col-0 (Figure 10). The gene expression of *LEC1* and *LEC2* in the same C3 line was down-regulated by 40- and 50-fold, respectively (Figure 10). The additional triple silencing line C3 13-1 exhibited more than a 300-fold

down regulation of the transcript accumulations of *LEC1, LEC2* and *LLP1* (Figure 10). Similarly, in the background of *eds1-2* the transcript levels of *LEC1, LEC2* and *LLP1* in the triple silencing line e3 3-5 were down-regulated more than 300-fold compared to its parental line (*eds1-2*).

Most of the experiments were performed with all of the lines introduced here. This diversity of different lines silencing one or more of the lectin genes *LEC1, LEC2* and *LLP1* will be conductive to gain a more detailed insight in the function and potential interactions of these genes during stress responses.

### 4.4 *Pseudomonas syringe* triggers local defence response

In order to characterize the defence response of the different T-DNA insertion lines and the RNAi-mediated silencing lines, the plants were exposed to different bacterial pathogens. To this end, leaves of 4-week-old plants were inoculated with *Pst* or *Pst* carrying different effectors (3.3) to analyse local resistance (3.13). *Pst* is a virulent strain inducing *EDS1*-dependent basal defences (Aarts et al., 1998; Breitenbach et al., 2014). The avirulent strains *Pst AvrRpm1* and *Pst AvrRpt2* induce ETI responses which are *EDS1*-independent (Aarts et al., 1998; Breitenbach, 2012). The ETI response to the avirulent strain *Pst AvrRps4* is *EDS1*-dependent (Aarts et al., 1998; Breitenbach, 2012).

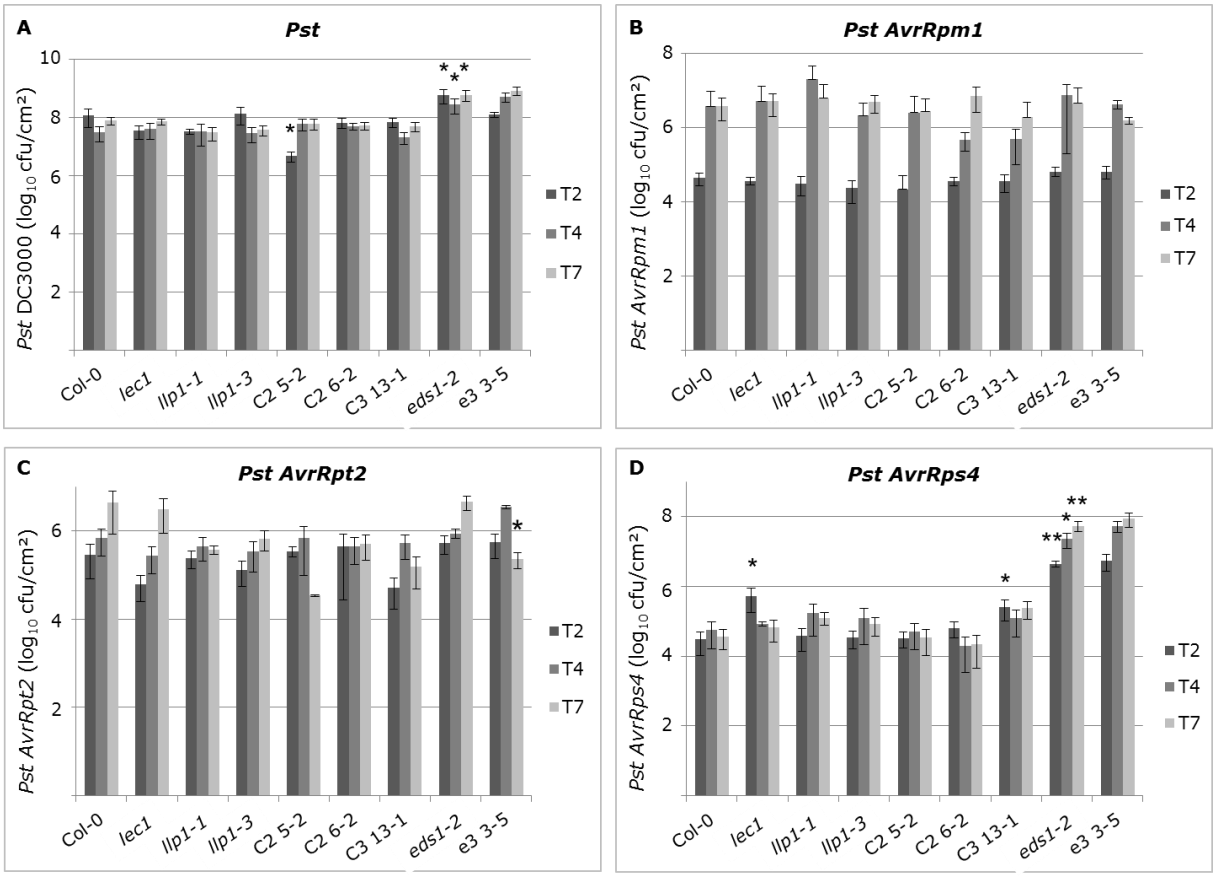


**Figure 11: Growth of different *Pseudomonas* strains in various *Arabidopsis* mutants and silenced plants**

4-week-old *Arabidopsis* plants were infected with (A) *Pst*, (B) *Pst AvrRpm1*, (C) *Pst AvrRpt2* and (D) *Pst AvrRps4.* Bacterial growth was measured at 2, 3 and 7 days after infection (T2, T4, and T7). Bars represent the average ± standard deviation of three replicates. Asterisks indicate reproducible significant differences compared to Col-0 plants for *lec1, llp1-1, llp1-3,* C2 5-2, C2 6-2, C3 13-1 and *eds1-2* and to *eds1-2* for e3 3-5 in at least two experiments (*p<0,05 **p<0,01, Student's t test). Experiments were repeated three times. Figure 11 shows the data from one representative experiment; the replicate experiments are shown in Figure S2.

The generally observed EDS1-dependent defence response to *Pst* (Aarts et al., 1998; Breitenbach et al., 2014) was supported here by higher *Pst* titres in *eds1-2* mutant compared to Col-0 plants (Figure 11A). This difference was observed from T2 onwards and remained stable over time, although no further growth of *Pst* was observed from T2 to T4 and T7, which might be due to the fact, that *Pst* was inoculated at a high concentration of 1 x $10^6$ CFU/ml. Hence, saturation was achieved early after the infection. The T-DNA insertion lines *lec1, llp1-1* and *llp1-3* mounted similar *Pst* titres as Col-0, suggesting that the defence response to *Pst* remained unchanged in these mutants compared to WT. The double silencer line C2 5-2 supported less *Pst* growth at T2 compared Col-0. This was, however, not reproducible in the second C2 line, C2 6-2, which behaved similarly to Col-0 at T2. Both double silencing lines behaved similarly to

Col-0 at T4 and T7. The *Pst* titre in the triple silencing line C3 13-1 was comparable to that in Col-0 at all three time points. The triple silencing line e3 3-5 behaved similarly to its parental line *eds1-2.* Taken together, the data show that all genotypes behaved similarly as their respective parental lines when infected with *Pst*. Therefore, it seems unlikely that LEC1, LEC2 and LLP1 play a role in basal defence against *Pst.*

*Pst AvrRpm1* growth was comparable in Col-0 and *eds1-2* (Figure 11B), which supported previous findings that ETI downstream from AvrRpm1 and its cognate R protein RPM1 is not dependent on EDS1 (Aarts et al., 1998). Bacterial titres and growth pattern of *Pst AvrRpm1* seemed similar in Col-0 and all transgenic lines, indicating that the defence response to *Pst AvrRpm1* was not regulated by LEC1, LEC2 or LLP1 (Figure 11B).

Bacterial growth of *Pst AvrRpt2* was similar in Col-0 and *eds1-2* (Figure 11C). Similar to AvrRpm1, the ETI response induced by AvrRpt2 is independent of *EDS1* (Aarts et al., 1998), and this is confirmed here (Figure 11C). The single T-DNA insertion lines *lec1, llp1-1* and *llp1-3* did not show a significant difference in the bacterial titers of *Pst AvrRpt2* compared to Col-0. Same was observed for the multiple silencing lines C2 5-2, C2 6-2 and C3 13-1 at T2, T4 and T7. The triple silencing line e3 3-5 showed a reduced bacterial titre at T7 compared to *eds1-2* in 2 out of 3 experiments. This suggests that the immune response to *Pst AvrRpt2* was accelerated in the absence of EDS1 and at least one or more lectins.

Bacterial growth of *Pst AvrRps4* was significantly higher in *eds1-2* mutant as compared to Col-0 plants (Figure 11D), which confirmed published data that the defence response induced by *AvrRps4* is dependent on EDS1 (Aarts et al., 1998). The enhanced *Pst AvrRps4* growth in the *eds1-2* mutant was not changed by silencing the expression of *LEC1*, *LEC2* and *LLP1* (compare *Pst AvrRps4* titres in *eds1-2* and e3 3-5 in 4.2, Figure 9D) The *lec1* mutant and the triple silencing line C3 13-1 supported higher bacterial titres than Col-0 plants at T2, which was observed in two and three experiments, respectively, out of three biologically independent replicates (Figure 11D and 8.1, Figure S2). However, the *Pst AvrRps4* titres in these plants remained significantly below the respective titre in the *eds1-2* mutant at T2. Also, *Pst AvrRps4* growth at T4 and T7 was similar in *lec1* and C3 13-1 compared to that in Col-0. Similarly, the single T-DNA insertion lines *llp1-1* and *llp1-3* as well as the double silencing lines C2 5-2 and C2 6-2 did not display an altered immune response to *Pst AvrRps4* as evidenced by similar bacterial growth patterns in at least two out of three experiments. Together, the data suggest that *EDS1*-dependent ETI in response to *Pst AvrRps4* was largely intact in all mutant and transgenic plants, although reduced expression of *LEC1* somewhat delayed the response.

Taken together, basal resistance and ETI responses appeared to be mostly intact in mutant lines and transgenic lines, suggesting that LEC1, LEC2 and LLP1 do not regulate local resistance to *P. syringae.* A putative influence of LEC1 on *Pst AvrRps4*-triggered ETI was observed by delayed resistance to *Pst AvrRps4* growth in *lec1* and C3 13-1 plants. The observed effect was reproducible in 3 out of 3 experiments, but transient and not observed in C2 plants, suggesting that the role of LEC1, if any, in AvrRps4-mediated ETI is moderate.

## 4.5 Local *PR1* induction initiated by *Pseudomonas syringe*

The induction of basal and distal defence responses involves the accumulation of various pathogenesis-related (PR) proteins which leads to a long lasting resistance against a broad spectrum of microorganisms (1.4). The SA-responsive *PR1* gene is induced in reaction to pathogen attack and is a common marker gene for SAR (1.5).

The transcript accumulation of *PR1* in leaves of uninfected 4-week-old plants was comparable between Col-0 wild type plants and the mutants and transgenic lines used in this study (Figure 12, T0).

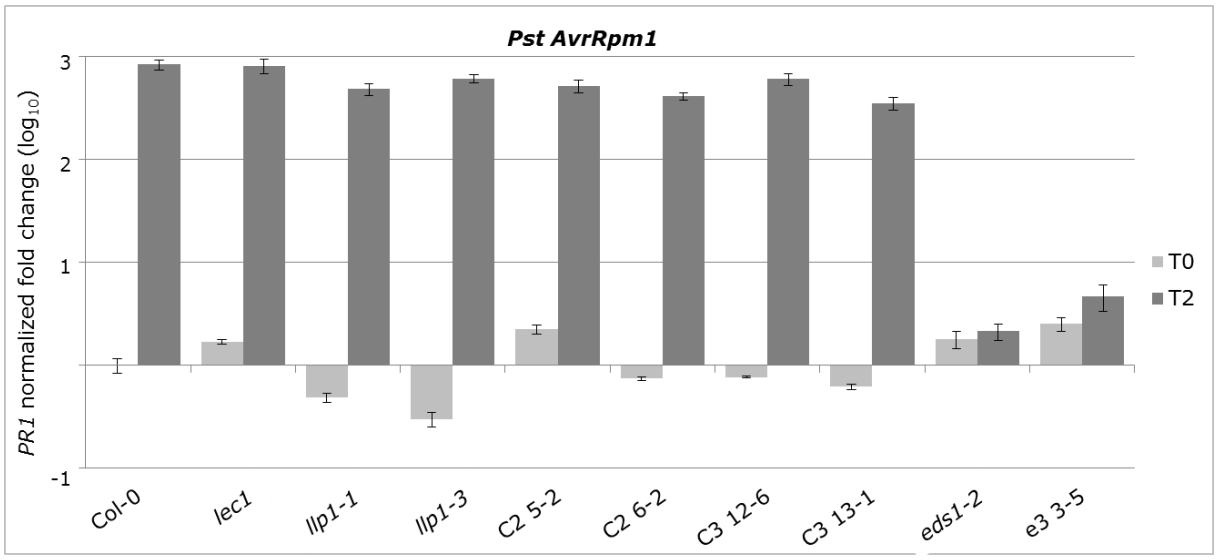


**Figure 12: *PR1* transcript level in uninfected and infected leaves**

*PR1* transcript accumulation was measured in uninfected (T0) leaves and two days after infection of leaves with *PstAvrRpm1* (T2) in the infected tissue. Transcript accumulation of *PR1* was normalized to that of *AtUBIQUITIN* and is shown relative to that in untreated Col-0 plants. Bars represent the average ± standard deviation of three replicates. Experiment was repeated twice with comparable results.

A primary infection was performed by infiltrating the first true leaves of 4-week-old *Arabidopsis* plants with *Pst AvrRpm1* to trigger a systemic immune response (3.12).

Locally induced resistance against *Pst AvrRpm1* (4.4) was accompanied by enhanced *PR1* transcript accumulation in the treated leaves 2 days after infection in Col-0, *lec1, llp1-1*, *llp1-3* and Col-0 transgenic lines C2 5-2, C2 6-2, C3 12-2 and C3 13-1 (Figure 12). Hence, LEC1, LEC2 and LLP1 do not play a crucial role in local *PR1* responses to *Pst AvrRpm1*. In contrast, this elevation of *PR1* transcript accumulation in response to *Pst AvrRpm1* was not observed in *eds1-2* and e3 3-5 plants. Although the local defence response to *Pst AvrRpm1* is *EDS1*-independent (Aarts et al., 1998)(4.4, Figure 11B), it is not yet known if the defence response of *eds1-2* to *Pst AvrRpm1* is linked to the expression of *PR1*. As shown in Figure S3, *PR1* transcript accumulation in *eds1-2* was comparable to Col-0 when it was measured 4 days after *Pst AvrRpm1* challenge. Therefore, it seems that although the defence response to *Pst AvrRpm1* is largely EDS1-independent, the *Pst AvrRpm1*-triggered induction of the defence gene *PR1* might be delayed in *eds1* mutant plants.

### 4.6 SAR induced by local *AvrRpm1* pretreatment

Systemic acquired resistance (SAR) is a long-lasting defence mechanism that occurs in the systemic uninfected tissue of local pre-infected plants (Vlot et al., 2017). This is associated with a mechanism of priming of defence (Conrath et al., 2015). Priming/SAR leads to a long-lasting protection of plants against a broad spectrum of harmful microbes, e.g. bacteria, fungi, or viruses (Shah, 2009; Vlot et al., 2009). During SAR establishment, SA accumulation leads to local defence followed by systemic, SA-dependent immunity (Vlot et al., 2009). To induce a SAR response, the first two true leaves of 4-week old plants were infected by syringe-infiltration with *Pst AvrRpm1.* Three days after the first infection, the third and fourth true leaves of the pre-treated plants were infiltrated with *Pst. Pst* growth was examined in the secondary infected leaves 4 days after infection (3.3).

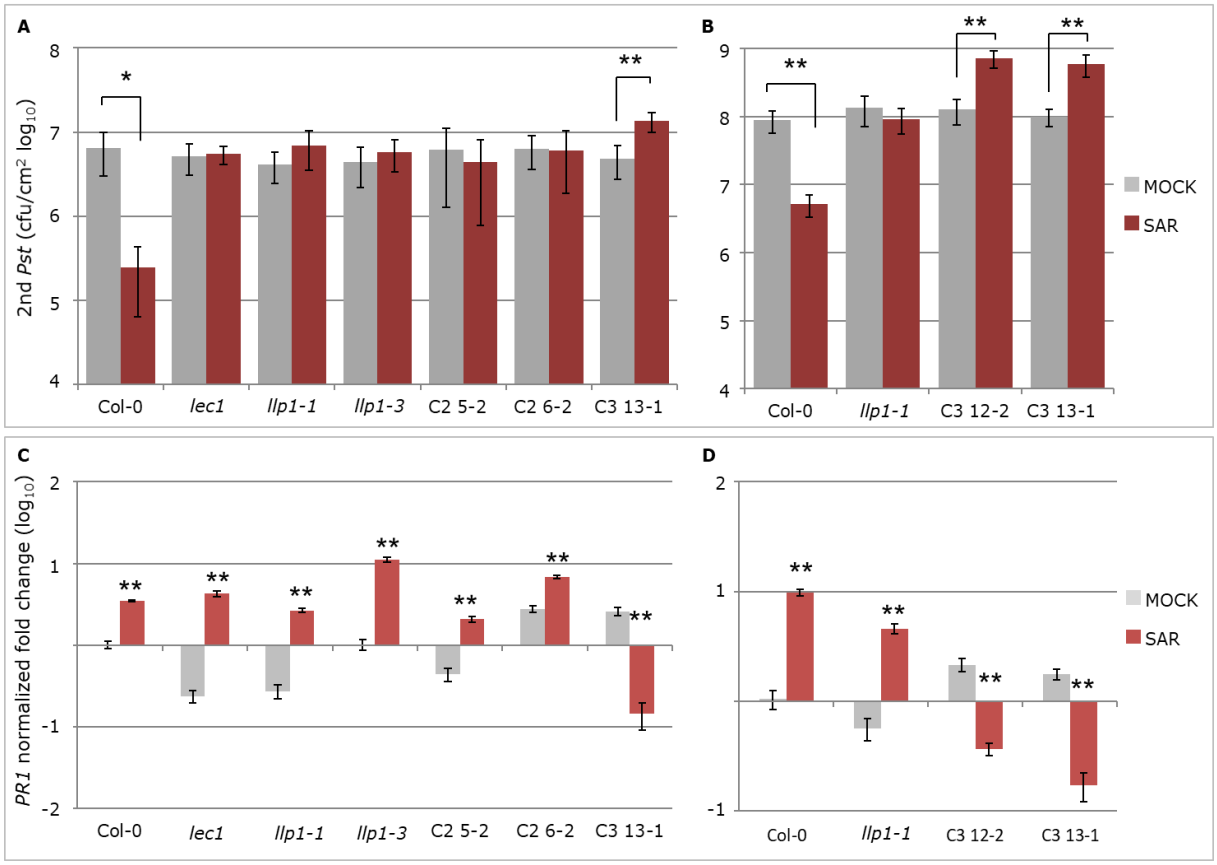


**Figure 13: Systemic defence responses in various *Arabidopsis* mutants and silenced plants**

(A, B) 2° *Pst* growth in systemic 2nd infected leaves of locally uninduced (MOCK-treated) and *Pst AvrRpm1*-induced (SAR) *Arabidopsis* plants. (C, D) *PR1* expression in systemic leaves of locally MOCK-treated (MOCK) and *Pst AvrRpm1*-treated (SAR) *Arabidopsis* plants. Transcript levels were normalized to that of *AtUBIQUITIN* and are shown relative to that in untreated Col-0 plants. Bars represent the average ± standard deviation of three replicates. Asterisks indicate statistically significant differences as compared to the mock control treatment (*p<0,05 **p<0,01, Student's t test). Experiments were repeated two (B, D) and four times (A, C) with comparable results.

After priming Col-0 with *Pst AvrRpm1* in the local tissue, the growth of a secondary *Pst* inoculum in the systemic tissue was reduced compared to that in MOCK-pretreated Col-0 plants (Figure 13A, Figure 13B). This indicates that SAR was induced in the Col-0 plants.

The same reduction of *Pst* growth in the systemic tissue after a local *Pst AvrRpm1* infection compared to mock was not observed in the T-DNA insertion lines *lec1, llp1-1* and *llp1-3* (Figure 13A, Figure 13B), indicating that these mutant lines were defective in SAR. The double silencing lines C2 5-2 and C2 6-2 also did not support reduced *Pst* growth in the systemic tissue after a local *Pst AvrRpm1* pretreatment, indicating that RNAi-mediated silencing of *LEC1* and *LEC2* compromised SAR. In the triple silencing lines C3 12-2 and C3 13-1 (Figure 13A, Figure 13B) the MOCK-pretreated plants showed *Pst* growth comparable to Col-0, but the *Pst AvrRpm1*-pretreated plants showed significantly

more growth of *Pst* in the systemic tissue compared to that in MOCK-pretreated plants. This indicates that silencing of *LEC1, LEC2* and *LLP1* did not only suppress SAR but enhanced the plant's susceptibility to *Pst*. The data suggest that LEC1, LEC2 and LLP1 act additively during SAR and that the interplay of all three fine-tunes systemic resistance. This hypothesis was supported by the *PR1* expression levels that were measured in the same experiments. In the systemic tissue of *PstAvrRpm1*-pretreated Col-0, elevated *PR1* expression was observed compared to that in MOCK-pretreated plants (Figure 13C, Figure 13D). The same was observed in *lec1, llp1-1, llp1-3* and the double silencing lines C2 5-2 and C2 6-2 (Figure 13C, Figure 13D). Thus, although the systemic responses in these plants did not restrict growth of a secondary *Pst* inoculum in the systemic leaves, partial SAR appeared to have been induced as illustrated by (in most cases) WT-like induction of the SAR marker gene *PR1* in the same tissues. In line with previous findings, the systemic *PR1* transcript accumulation in *Pst AvrRpm1*-pretreated *llp1-1* mutant plants appeared lower compared to that in WT plants (Breitenbach et al., 2014 and 4.3, Figure 10 C and D). The triple silencing lines C3 12-2 and C3 13-1 showed a down-regulation of *PR1* expression in the systemic tissue after challenging the local leaves with *PstAvrRpm1* compared to mock. This confirms that LEC1, LEC2 and LLP1 might act additively in SAR and that the same signalling mechanism that leads to systemic defence in SAR is turned into systemic susceptibility by the reduced transcript accumulation of *LEC1*, *LEC2* and *LLP1*.

It is known that EDS1 is a main regulator in SA-induced defence responses and required for SAR signalling. To investigate whether the SAR pattern of C3 12-2 and C3 13-1 is associated with EDS1, SAR was investigated in the triple silencing line e3 3-5 (4.3).

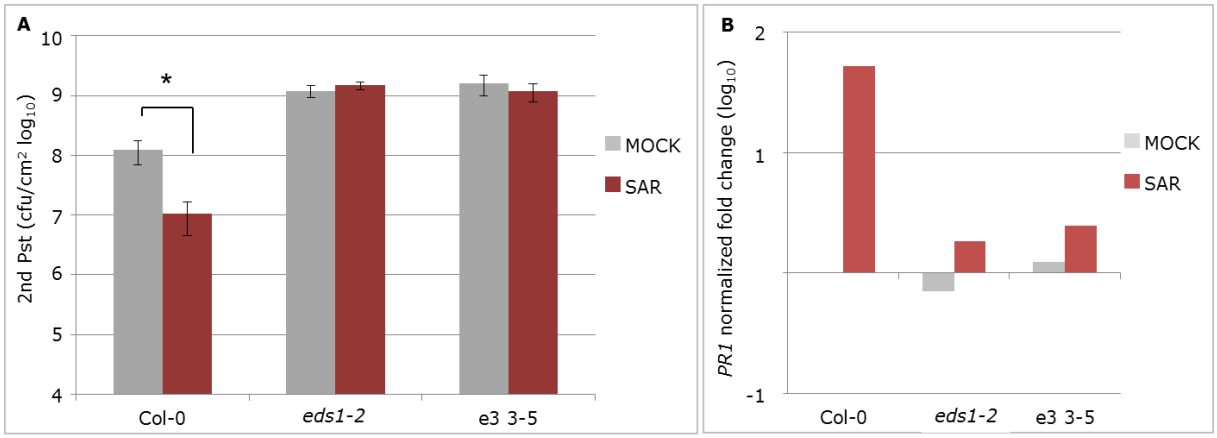


**Figure 14: Systemic defence responses and *PR1* expression in the *eds1-2* mutant and the *eds1-2* triple silencing line e3 3-5 compared to Col-0**

(A) 2° *Pst* growth in systemic 2nd infected leaves of locally uninduced (MOCK-treated) and *Pst AvrRpm1* induced (SAR) Col-0, *eds1-2* and e3 3-5 plants. (B) *PR1* expression in systemic leaves of locally MOCK-treated (MOCK)

and *Pst AvrRpm1*-treated *Arabidopsis* plants. Transcript levels were normalized to that of *AtUBIQUITIN* and are shown relative to that in untreated Col-0 plants. Bars represent the average ± standard deviation of three replicates. Asterisk in A indicates statistically significant difference as compared to the mock control treatment (*p<0,05, Student's t test). Experiments were repeated two times with comparable results.

In *eds1-2* and e3 3-5, *Pst* titres in the systemic tissue of locally MOCK-treated plants were higher compared to those in MOCK-treated Col-0 (Figure 14A). This supports the result in 4.4, Figure 11A, that the defence response to *Pst* is *EDS1*-dependent. Systemic *Pst* titres in *eds1-2* of locally *PstAvrRpm1*-treated plants were significantly higher compared to Col-0 and did not differ from those in locally MOCK-treated *eds1-2* plants, indicating that *eds1-2* is SAR-deficient, which confirmed published data (Truman et al., 2007; Breitenbach et al., 2014). Same could be observed for the triple silencing lines in the *eds1-2* mutant background e3 3-5 (Figure 14A). In Col-0, accumulation of *PR1* transcript was higher in the systemic tissue of SAR-induced compared to locally MOCK-treated plants 3 days after local infection (Figure 14B). In contrast, systemic *PR1* induction in *eds1-2* was negligible in SAR-induced compared to locally MOCK-treated plants confirming published data (Breitenbach, 2012; Breitenbach et al., 2014). The triple silencing line e3 3-5 showed similar systemic *PR1* expression in the untreated tissue of locally MOCK-treated and *PstAvrRpm1*-treated plants as its parental line *eds1-2 (*Figure 14B).

Taken together, these results suggest that LEC1, LEC2 and LLP1 work additively during SAR (Figure 13A, Figure 13B) and SAR signalling (Figure 13C, Figure 13D). It seems that EDS1 is required for an intact interplay of these lectins; otherwise the triple silencing line e3 3-5 would show similar results in SAR and systemic *PR1* induction as the triple silencing lines in the background of Col-0 (Figure 13A-D, Figure 14).

## 4.7 The role of *LEC1*, *LEC2* and *LLP1* in SA-induced stress responses

SA is known to play a crucial role in induced defence response against a variety of biotic and abiotic stresses through morphological, physiological and biochemical mechanisms. To gain further insight into a possible role of the lectins in SA signalling, the responses of the T-DNA insertion mutants *lec1, llp1-1,* and *llp1-3* and the RNAi-mediated silencing lines C2 5-2, C2 6-2, C3 12-2, C3 13-1 and e3 3-5 were compared to those in Col-0 and *eds1-2* plants in response to the exogenous application of SA.

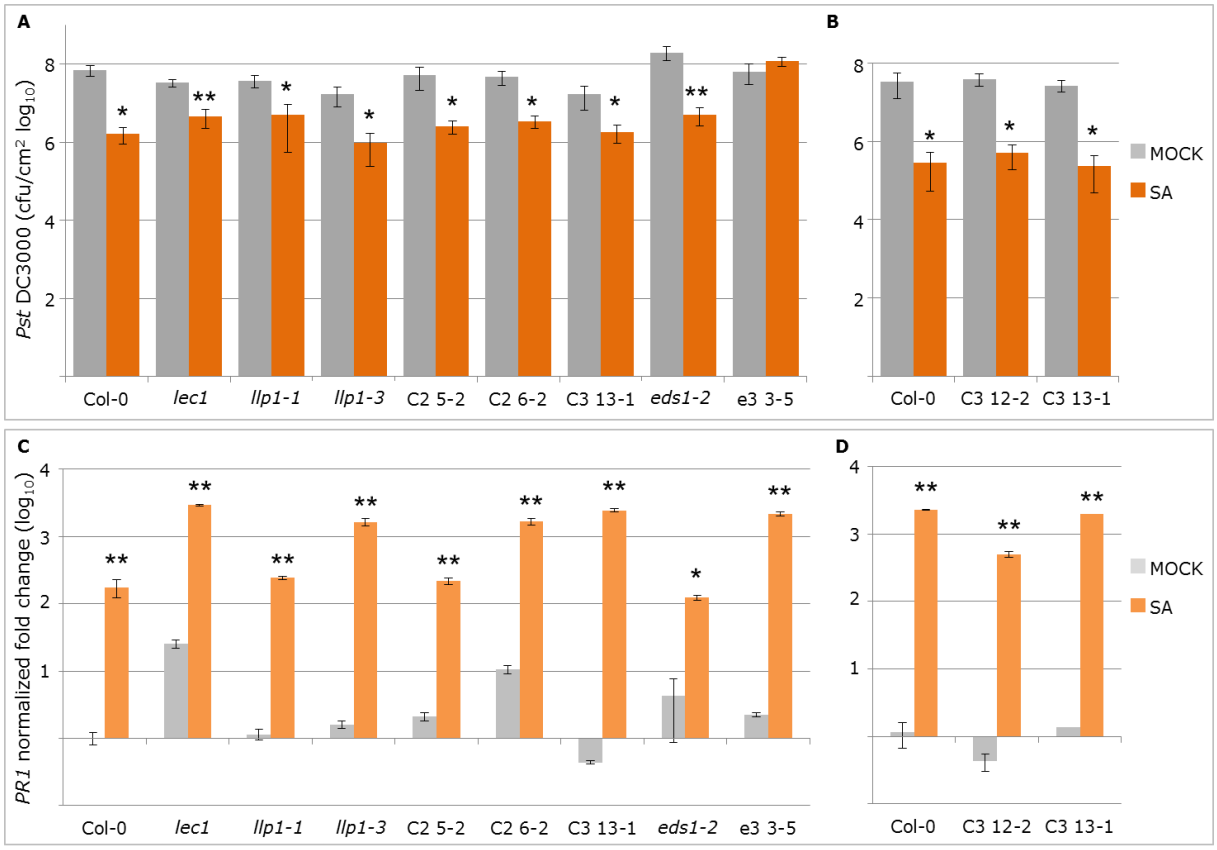


**Figure 15: SA-inducible resistance in different *Arabidopsis* mutants and silenced plants**

4-week-old plants were sprayed with 1mM of SA and 24hpi leaves of the treated plants were either infected with *Pst* (A, B) or harvested for qRT-PCR analysis(C, D). (A, B) The in planta *Pst* titres are shown at 2 dpi; (C, D) the transcript levels of *PR1* were normalized to that of *AtUBIQUITIN* and are shown relative to that in MOCK-treated Col-0 plants. Bars represent the average ± standard deviation of three replicates. Asterisks indicate significant differences from the MOCK-treated controls (*p<0,05 **p<0,01, Student's t test). These experiments were repeated two times (B, D) to at least three times (A, C) with similar results.

To this end, 4-week-old plants were sprayed with 1 mM of SA and 1 day later inoculated with *Pst. Pst* growth was reduced in SA-treated compared to mock-treated Col-0 plants (Figure 15A, Figure 15B), indicating that SA enhanced the resistance of these plants to *Pst* growth. The single T-DNA insertion lines *lec1, llp1-1* and *llp1-3* showed similarly reduced *Pst* growth in response to SA as compared to Col-0. Hence, SA-induced resistance was not altered in these mutant lines (Figure 15A). The double silencing lines C2 5-2 and C2 6-2 as well as the triple silencing lines C3 12-2 and C3 13-1 showed equally reduced *Pst* growth after SA-treatment compared to MOCK-treated plants, and this was comparable to the SA-induced *Pst* growth reduction in their parental line Col-0. Thus, SA-induced resistance against *Pst* was similar in the double and triple silencing lines compared to that in Col-0 plants (Figure 15A, Figure 15B). In line with previous findings, *eds1-2* responded normally to SA with reduced *Pst* growth, confirming published data, that EDS1 acts upstream of SA in SA-induced immunity (Falk et al., 1999). In

contrast, the *eds1-2* triple silencing line e3 3-5 showed defective immune response to *Pst* in SA-induced plants as to MOCK-treated plants, as illustrated by Figure 15A. The latter finding suggests that EDS1 requires *LEC1*, *LEC2* and/or *LLP1* for proper SA-related defence response.

SA-induced resistance in Col-0 was accompanied by enhanced *PR1* transcript accumulation in SA-treated leaves, which was measured before the *Pst* infection (Figure 15C, Figure 15D). Same could be observed in *lec1, llp1-1* and *llp1-3* confirming above results, that SA-induced defence response is not altered in these mutant lines. The double silencing lines C2 5-2 and C2 6-2 and the triple silencing lines C3 12-1 and C3 13-1 responded to SA treatment with normal *PR1* induction, which was comparable to that in Col-0. Similarly, *PR1* transcript accumulation in *eds1-2* was elevated to a comparable level as in Col-0 after SA treatment, confirming published data, that the local SA-related immune response is not compromised in *eds1-2* mutant plants (Falk et al., 1999; Feys and Parker, 2000). Furthermore, *PR1* was induced to a normal level after SA treatment of the triple silencing line e3 3-5 (Figure 15C), although resistance to *Pst* after SA treatment was not induced in these plants (Figure 15A). Together, *PR1* expression in SA-treated leaves was 100-fold or more elevated compared to that in MOCK-treated leaves in each of the mutant and transgenic lines tested (Figure 15C, Figure 15D). The data suggest that PR1 alone is not sufficient for an intact SA-mediated immune response in the absence of EDS1 and LEC1, LEC2 and LLP1. Hence, *EDS1* might require *LEC1, LEC2* and *LLP1* for a proper SA-dependent defence response that is partially independent of *PR1* transcript accumulation.

The data show that LLP1, LEC1 and LEC2 probably are not necessary for immune signalling downstream of SA in Col-0. However, it seems that for proper SA-induced defence, all three lectins were required in coherence with EDS1, independently of *PR1* gene expression.

## 4.8 Defence response triggered by the necrotrophic fungus *Alternaria brassicicola*

It is known that the SA- and JA/ET mediated pathways interact in a complex, mostly antagonistic crosstalk (Pieterse et al., 2001; Kunkel and Brooks, 2002; Glazebrook et al., 2003). As described in 4.1, JA-treatment induced the expression of *LEC1* and *LEC2* in Col-0 and *eds1-2*. It is known that resistance to *Alternaria brassicicola (A.brassicicola)* is dependent on JA and that SA is not required (Thomma et al., 1998). Also, *LEC1* expression is induced by MeJA (4.1, Figure 8A) and *A.brassicicola* (Mukherjee et al., 2010). Here, it shall be clarified if the lectins might play a role in the JA-dependent defence response of *Arabidopsis* plants to the fungal pathogen *A.brassicicola*. This was

investigated after the infection of 4-week-old wild type and lectin mutant plants by dropping 600 spores of the necrotrophic fungus *A.brassicicola* on leaves. The resulting lesions were measured 5dpi (3.16).

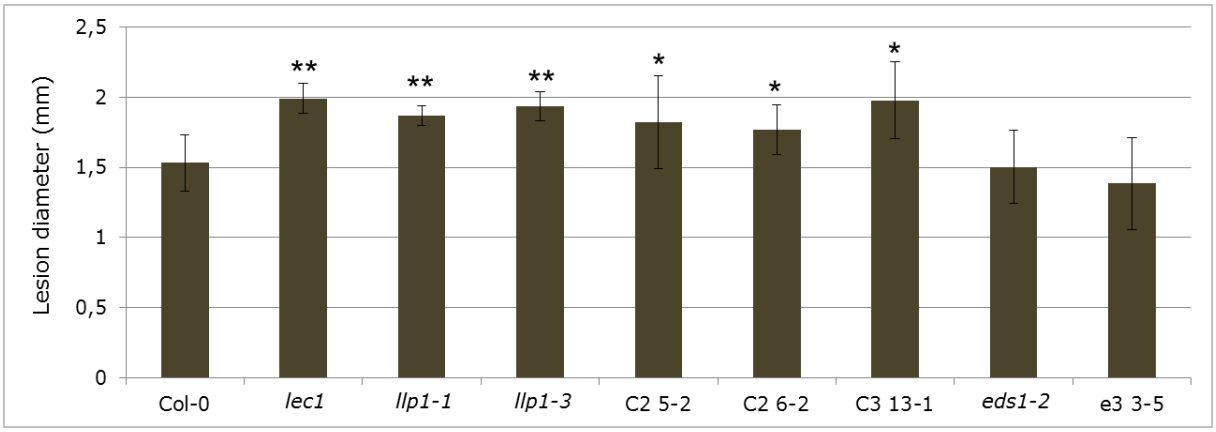


**Figure 16: Infection of various *Arabidopsis* mutants and silenced plants with *Alternaria brassicicola***

Lesion diameter on 20 leaves per plant line were measured 5 days after infection of 4 week old plants with *Alternaria brassicicola*. Bars represent the average ± standard deviation of 60 (*lec1, llp1-1, llp1-3*) and 120 (Col-0, C2 5-2, C2 6-2, C3 13-1, *eds1*-2, e3 3-5) lesions from 3 to 6 independent experiments. Asterisks indicate statistically significant differences as compared to Col-0 (*p<0,05 **p<0,01, Student's t test). (Preliminary data)

The T-DNA insertion lines *lec1, llp1-1* and *llp1-3* were more susceptible to *A.brassicicola* than Col-0 as they mounted significantly larger *A. brassicicola* lesions at 5dpi than Col-0 did (Figure 16). Similarly, the silencing lines C2 5-2, C2 6-2 and C3 13-1 supported the occurrence of larger *A. brassicicola* lesions as compared to Col-0 in response to the fungal infection, suggesting that all three lectins LEC1, LEC2 and LLP1 are necessary for proper defence against *A.brassicicola*. Col-0 supported a similar *A. brassicicola* lesion size as the *eds1-2* mutant, indicating that defence against *A.brassicicola* is independent of *EDS1*. The triple silencing line e3 3-5 mounted similarly sized *A. brassicicola* lesions as its parental line *eds1-2.* Hence, in *eds1-2* the absence of the three lectins is not sufficient to trigger the weaker immune response. Therefore, it seems that LEC1, LEC2 and LLP1 might be partially dependent on or regulated by *EDS1* for intact immunity to *A.brassicicola.* The fungal cell wall has several constituents, including chitin, and *LEC1* and *LEC2* are known to be induced by chitin (Ramonell et al., 2002; Zhang et al., 2002; Mukherjee et al., 2010; Egusa et al., 2015). Although lesion differences in between the lines appear to be minor, the data suggest that LLP1 and LEC1 and/or LEC2 play a role in defence in *Arabidopsis thaliana* against necrotrophic fungal pathogens.

### 4.9 Abiotic stress response induced by high salinity

Figure 8C showed that ABA, which is mainly associated with plant responses to abiotic stress, influenced the expression of *LEC1, LEC2,* and *LLP1.* Moreover, *LLP1* was negatively influenced by the SA signalling factor EDS1 under ABA treatment. These data suggested that the lectins may act on the interface between biotic and abiotic stress tolerance (4.1). Hence, stress experiments targeting the ABA-pathway were performed (3.15). To ascertain the putative role of LEC1, LEC2 and LLP1 in ABA-related abiotic stress responses, transcript accumulation of various ABA-related stress genes under osmotic stress (3.15) were monitored in the silencing lines (4.3, Table 11), their parental lines Col-0 and *eds1-2*, as well as in the T-DNA insertion lines *lec1, llp1-1* and *llp1-3*. To this end, sterile seedlings were grown on MS plates without stress (MS) or with 100mM NaCl and analysed 10 days after putting the seeded MS plates in plant chambers under long-day conditions for germination (days after germination; dag).

Transcript accumulations of *ABA-INSENSITIVE1* (*ABI1*), *RESPONSIVE TO ABA 18* (*RAB18*) and *RESPONSIVE TO DESICCATION 29B* (*RD29B*) were determined at 10 dag (Figure 17). *ABI1* is required for proper ABA responsiveness and negatively regulates ABA responses (Lu et al., 2015) whereas the stress-responsive gene *RAB18* is a common marker for salt stress and is linked to the ABA pathway (Singh et al., 2015). *RD29B* is an ABA-dependent and fast responsive gene to osmotic stress (Virlouvet et al., 2014).

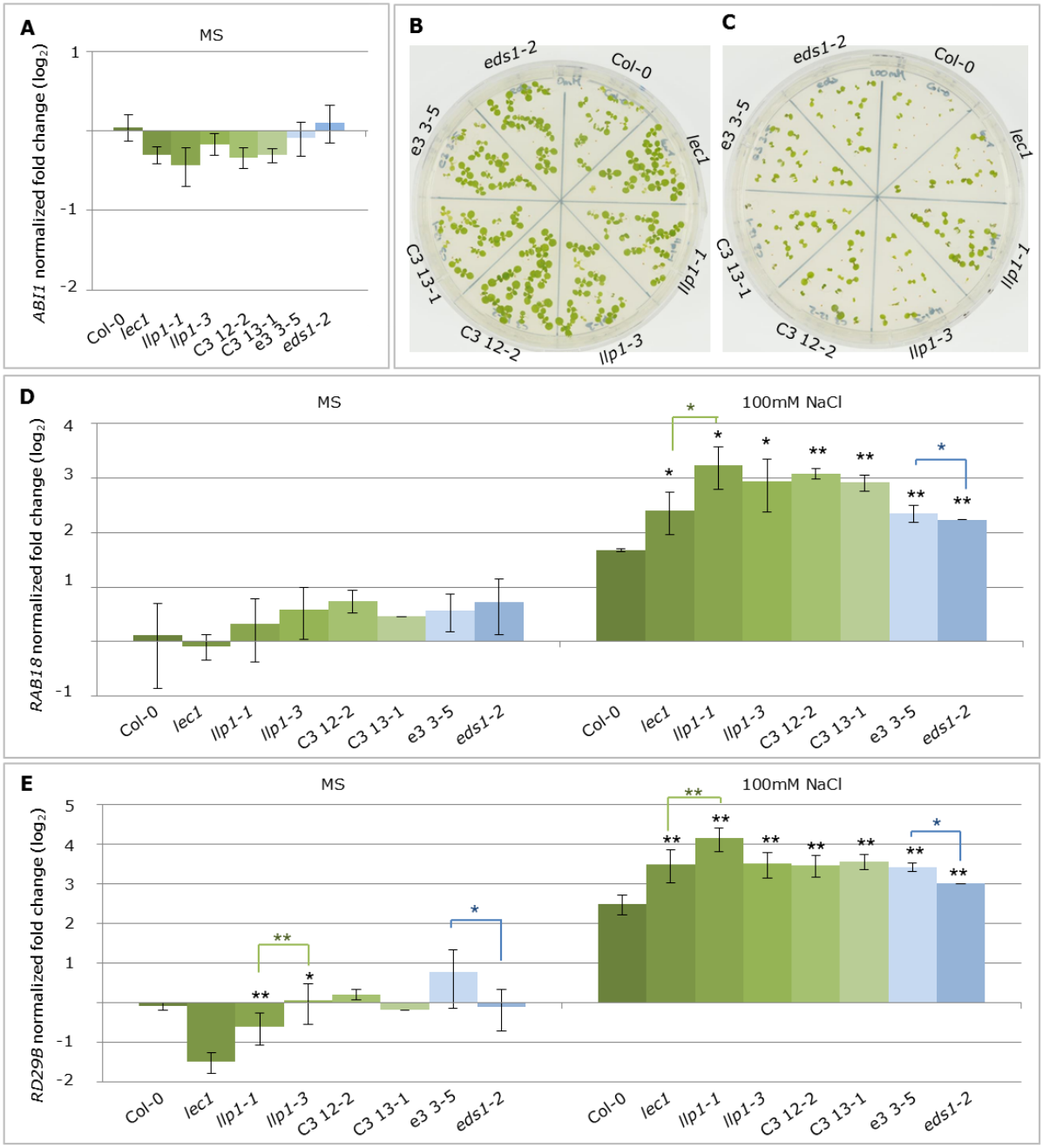


**Figure 17: Transcript accumulation of *RAB18* and *RD29B* in *Arabidopsis* mutants and silenced plants under salt stress**

10 seedlings of Col-0, *eds1-2*, *lec1*, *llp1-1* and *llp1-3*, as well as the RNAi-mediated silencing lines were germinated on media without osmotic stress (MS) (B) or in the presence of 100mM NaCl (C), harvested at 10 dag, and analysed for transcript accumulation of *ABI1* (A), *RAB18* (D) and *RD29B* (E) (B,C) Images were taken at 10 dag. (A,D,E) *ABI1* (A) *RAB18* (D) and *RD29B* (E) transcript levels were determined by qRT-PCR, normalized to that of *AtUBIQUITIN*, and are shown relative to that in untreated Col-0 plants. Bars represent the average ± standard deviation of three replicates. Black, green and blue asterisks indicate statistically significant differences to Col-0, *lec1*, and *eds1-2*, respectively (* $p<0.05$, ** $p<0.01$, student´s t-test). These experiments were repeated one (E) to three (A-D) times with comparable results.

*ABI1* expression was measured to study if the ABA pathway might be affected in different *Arabidopsis* mutants and silenced plants grown on MS plates (Figure 17A, B). Gene expression of *ABI1* in *lec1, llp1-1, llp1-3,* C3 12-2 and C3 13-1 was comparable to that of Col-0. Though slightly downregulated in *lec1, llp1-1,* C3 12-2 and C3 13-1 compared to Col-0, *ABI1* transcripts were detectable and the observed differences were not significant. The triple silencing line e3 3-5 and its parental line *eds1-2* showed similar *ABI1* expression compared to Col-0. Thus, the lack of one or more lectins in seedlings grown on MS plates without salt stress did not affect gene expression of *ABI1*, which is the key negative regulator in the ABA signalling pathway. Hence, basal ABA signalling is likely not affected by the various mutations and transgenes and is comparable to that of Col-0.

Compared to Col-0, the mutants *lec1, llp1-1, eds1-2* and the triple silencing lines C3 12-2, C3 13-1 and e3 3-5 growing on plates without stress (MS)(Figure 17B) showed no or little gene regulation of *RAB18* at 10 dag (Figure 17D). In contrast, 100mM of NaCl (Figure 17C) induced *RAB18* transcript accumulation in Col-0 compared to that in seedlings grown on MS (Figure 17D). Seedlings of the T-DNA insertion lines *lec1, llp1-1, llp1-3* and the triple silencing lines C3 12-2, C3 13-1 supported even higher transcript accumulation of *RAB18* when grown on 100mM NaCl compared to that in Col-0 grown under the same condition. It is thus possible that higher resistance of *Arabidopsis* which are defective in one or more lectins is related to an ABA-mediated immune response (4.9). Transcript accumulation of *RAB18* in *eds1-2* as well as in e3 3-5 seedlings grown on 100 mM NaCl was significantly higher compared to that in Col-0 grown under the same condition. Compared to its parental line, the triple silencing line e3 3-5 supported a significantly higher transcript accumulation of *RAB18*, confirming the above results that a lectin-mediated response to high salinity might occur in an *EDS1*-independent manner

*RD29B* transcript accumulation in *llp1-3*, C3 12-2, C3 13-1, e3 3-5 and *eds1-2* grown on MS plates without stress was comparable to that in Col-0 grown under the same conditions (Figure 17E). On plates without stress (MS), *RD29B* was down regulated in seedlings of the T-DNA insertion lines *lec1* and *llp1-1* 10dag compared to Col-0 (Figure 17E). *RD29B* was significantly more reduced in *lec1* compared to *llp1-1* under same growth condition. Col-0 plants grown on MS plates with 100mM NaCl (Figure 17C) showed elevated *RD29B* transcript accumulation compared to Col-0 plants grown on MS (Figure 17E). When grown on 100mM NaCl, transcript accumulation of *RAB18* was even higher in the T-DNA insertion lines *lec1, llp1-1, llp1-3* and the triple silencing lines C3 12-2, C3 13-1 compared to that in Col-0 grown under the same condition. Similarly, seedlings of *eds1-2* and e3 3-5 grown on 100mM NaCl showed significantly higher *RD29B* transcript accumulation compared to that in Col-0 grown under the same condition. Compared to *eds1-2*, e3 3-5 supported a significantly higher transcript accumulation of

*RD29B* which is comparable to that in the triple silencing lines in the background of Col-0 under same growth condition.

Taken together, these data suggest that the lectins might respond to high salinity in an ABA-related, but *EDS1*-independent manner, indicating a correlation between the lectins and abiotic stress.

## 4.10 Searching for interacting proteins of LEC1, LEC2 and LLP1 by Yeast-2-Hybrid

Lectins and lectin-like proteins are carbohydrate-binding proteins containing at least one non-catalytic conserved sugar binding domain to bind glycoproteins, glycolipids, or polysaccharides with high affinity (Lannoo and Van Damme, 2014; De Schutter and Van Damme, 2015). They occur most abundantly in seeds and vegetative storage tissues (Goldstein and Hayes, 1978). To identify potential co-signalling partners of LEC1, LEC2 and LLP1, Yeast-2-Hybrid assays (Y2H) were performed with the aim of identifying proteins that physically interact with LEC1, LEC2 and/or LLP1 and might thus transfer a lectin-perceived signal (3.18). Y2H is an applied genetic system utilized for the identification of protein-protein interactions in vivo. It employs the GAL4 transcriptional activator and takes advantage of the fact that GAL4 can be physically separated into two domains, one DNA binding domain (Gal4-BD) and one transcriptional activator domain (Gal4-AD) (Figure 18). GAL4 can be re-activated once the two domains are brought into proximity. In a canonical Y2H screen two fusion proteins are required. The protein-of-interest (here: one of the lectins) fused to Gal4-BD (X) and the library protein fused to Gal4-AD (Y). Interaction between the proteins brings the Gal4-BD and Gal4-AD close enough together to allow transcription of a downstream reporter gene that subsequently allows growth of the corresponding yeast strains on media that are selective for the reporter (Figure 18B). The assays described below were performed in cooperation with Dr. Pascal Falter-Braun at the TUM.

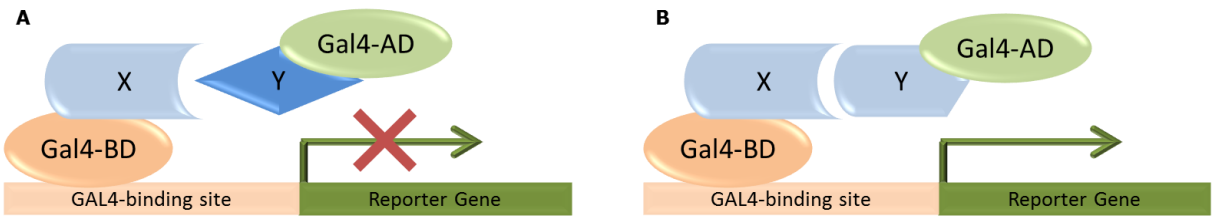


**Figure 18: Schematic Y2H system**

The protein of interest X, is fused to the DNA binding domain (Gal4-BD). The potential interacting protein Y is fused to the activation domain (Gal4-AD). The Gal4-BD-X fusion protein, binds to the GAL-4-binding site. (A) When interaction of X and Y is not given, subsequent transcription of a reporter gene is not possible. (B) The

interaction of X with Y reconstitutes a functional transcription factor, leading to further recruitment of RNA polymerase II and subsequent transcription of a reporter gene.

Three essential experimental stages, which together yield highly reliable interactions, are content of the Y2H pipeline. These include primary screening, secondary phenotyping, and verification (3.18) (Dreze et al., 2010). In the primary screen, yeast strains containing DB-X (DNA-binding domain to a protein X) and AD-Y (activation domain to a protein Y) inserts are mated and positive colonies are picked from selective plates. At the secondary phenotyping stage, those positive colonies are further put onto two types of selective plates to eliminate false positives. Resulting colonies are then picked from the plates and analysed by DNA sequencing to identify the putative binding partners. At the last step, these binding partners are then verified using fresh archival yeast stocks on fresh assay plates to ensure robust high-quality Y2H interactions. That verification step is done four times and only interactors showing a positive result at least three times are accepted as verified Y2H interactions (Dreze et al., 2010).

Here, primary screening and secondary phenotyping of putative binding partners was performed twice with similar results, but verification was not successful in both runs. In this assay, full length coding sequences of *LEC1, LEC2* and *LLP1* were cloned in a DB-vector (2.4) and therefore LEC1, LEC2 and LLP1 served as bait (X, Figure 18) for finding binding partners (3.18). Positive yeast colonies were picked and directly used for phenotyping. Hence, the DB-X and AD-Y inserts of positive colonies were amplified by yeast colony PCR according to 3.18 for subsequent ORF identification by end-read sequencing (Dreze et al., 2010). The candidate lectin interactors were identified by BLAST sequence search. Only putative interactors that were found in both runs are listed in Table 12.

**Table 12:** Yeast-2-Hybrid assay: putative binding partners of LEC1, LEC2, and LLP1 that were found in two independent Y2H runs. Data were found by Align Sequences Nucleotide BLAST (Zhang et al., 2000).

| **Bait X** | **Interactor** | ORF identification | Interactor located in | Description of Interactor |
|---|---|---|---|---|
| LEC1 | AT4G34138 | 100% | chloroplast, intracellular membrane-bounded organelle | UDP-glucosyl transferase 73B1 (UGT73B1) |
| | AT5G57340 | 100% | nucleus | hypothetical protein |
| | | 70-85% | | UDP-glucosyl transferase B2/B3/B4/B5 |
| LEC2 | AT5G57550 | 100% | extracellular region, apoplast | probable xyloglucan endotransglucosylase/hydrolase protein 25 (XTH25) |
| LLP1 | AT5G57550 | 100% | extracellular region, apoplast | probable xyloglucan endotransglucosylase/hydrolase protein 25 (XTH25) |
| | AT5G57340 | 100% | nucleus | hypothetical protein |
| | AT1G52870 | 100% | chloroplast, integral component of membrane | peroxisomal membrane Mpv17/PMP22 family protein |

For LEC1, three putative binding partners were found, whereby UGT73B1, also named *At*GT-2 is known to play a role in the ABA pathway as it shows ABA glucosyltransferase activity as an immediate early response to SA (Uquillas et al., 2004). AT5G57340, a hypothetical protein shows interaction with LEC1 and LLP1. For AT5G57340 microarray data suggest an induction after Auxin, ABA and MeJA treatment in seedlings (Luhua et al., 2013).

LEC2 as well as LLP1 show interaction with At5g57550, which encodes a predicted xyloglucan transferase/hydrolase XTH25, also known as XTR3. It is predicted to be involved in plant-type cell wall biogenesis, including biosynthetic process, formation and synthesis of cellulose- and pectin-containing cell walls. It has a predicted xyloglucan:xyloglucosyl transferase activity and is the only putative interactor which is similarly to LEC1, LEC2, and LLP1 predicted to localize to the apoplast. Therefore, XTH25 was included in further experiments.

LLP1 also showed a possible binding to AT1G52870, which belongs to the Mpv17/PMP22 family. PMP22 seems to be involved in pore-forming activity and may contribute to the unspecific permeability of membranes (Iida et al., 2001; Brosius et al., 2002).

### 4.11 SAR in mutant line lacking putative binding partner of LEC2 and LLP1

Xyloglucan endotransglucosylase/hydrolases (XTH) play important roles in cell wall loosening, synthesis and restructuring, enabling cell expansion (Rose, 2002). Especially in cell wall metabolism in response to abiotic stress the accumulation of XTHs is elevated (Le Gall et al., 2015). There are three *XTH* genes (*XTH4*, *XTH7* and *XTH31*) known to be downregulated during SAR (Gruner et al., 2013). XTH25 might act as a binding partner for LEC2 and/or LLP1 (4.10) and is predicted to be located in the extracellular region. To investigate if XTH25 might play a role in the systemic defence response, SAR assays were performed with *xth25* mutant plants (2.1).

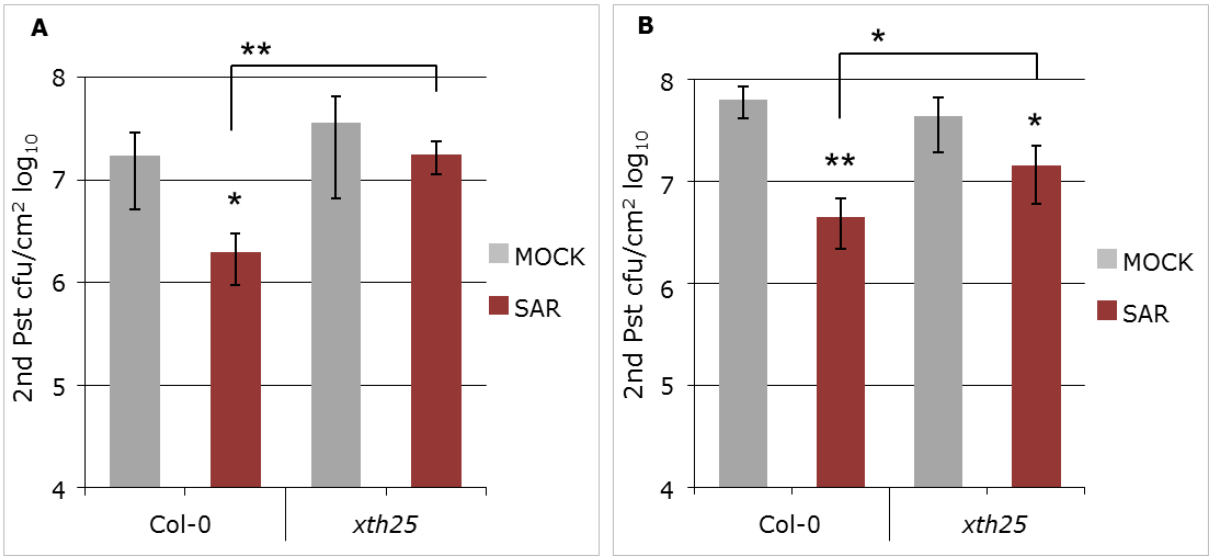


**Figure 19: Systemic defence response in Col-0 and *xth25***

(A, B) 2° *Pst* growth in uninduced (MOCK) plants and in *Pst AvrRpm1* induced (SAR) *Arabidopsis* Col-0 and *xth25*. Bars represent the average ± standard deviation of three replicates. Asterisks indicate statistically significant differences as compared to control treatment (*p<0,05 **p<0,01, Student's t test). Experiments were repeated three times, results were reproducible for (A) once and for (B) two times.

*Pst* growth was reduced upon a secondary infection of the systemic tissue compared to the MOCK-treated Col-0 plants after priming Col-0 with *Pst AvrRpm1* in the local tissue (Figure 19A, Figure 19B). This SAR response observed in Col-0 WT plants seemed to be abolished in *xth25* (Figure 19A), but this was observed only once. In further experiments (Figure 19B) SAR was compromised in *xth25* compared to that in Col-0. Hence, XTH25 might contribute to SAR.

### 4.12 Gene expression of *LEC1, LEC2* and *LLP1* in various mutant lines

As described previously in 4.1, the regulation of *LEC1, LEC2* and *LLP1* was influenced by different phytohormones. Furthermore, it was shown that LEC1, LEC2 and LLP1 are required for SAR (4.6) and together with EDS1 for SA-induced defence (4.7). Besides the immune response to biotrophic pathogens, experiments with the fungus *A. brassicicola* revealed that *LEC1, LEC2* and *LLP1* might also be involved in plant defence against necrotrophic pathogens (4.8). In addition, it seems that also plant responses to abiotic stress are affected by *LEC1, LEC2* and *LLP1* (4.9). To clarify if the stress response of the transgenic lines is a pathogen response or due to natural variation, basal expression levels of the lectins were measured in various T-DNA insertion lines.

The T-DNA insertion lines used lack in the defence-related proteins EDS1, NPR1, SID2, JAR1, ABI1, AZI1, GLY1 or XTH25 (2.1). As described in 1.4, EDS1 is an important key regulator of SA-dependent defence responses and essential for SAR signalling. NPR1 functions downstream of SA signalling and accumulation (Cao et al., 1997; Durrant and Dong, 2004; Vlot et al., 2009; Spoel and Dong, 2012) and is essential for SA-mediated defence gene induction (Mou et al., 2003; Pieterse and Van Loon, 2004; Spoel et al., 2009). SID2 is essential for functional pathogen-induced SA-biosynthesis and accumulation (Wildermuth et al., 2001; Strawn et al., 2007; Vlot et al., 2009). The *med4-2* mutant lacks in hydrolysing MeSA to SA (Vlot et al., 2008b). The JA-related JAR1 catalyses the formation of JA-Ile and can also be induced by Auxin. JAR1 is further described to play a role in SAR (Niu et al., 2016). ABI1 is important for ABA signal transduction and is also a negative regulator of ABA-induced stomatal closure (Allen et al., 1999). As mentioned in 1.6, AZI1 is involved in systemic immunity triggered by pathogens or azelaic acid as well as in the priming of SA induction (Jung et al., 2009; Yu et al., 2013). GLY1 is necessary for the biosynthesis of G3P and is involved in SAR (Lim et al., 2002; Mandal et al., 2011). *XTH25* encodes a predicted xyloglucan endotransglucosylase that can be induced by SA (Thibaud-Nissen et al., 2006), might physically interact with LEC1 and LLP1 (4.10, Table 12), and appears to affect SAR (Figure 19).

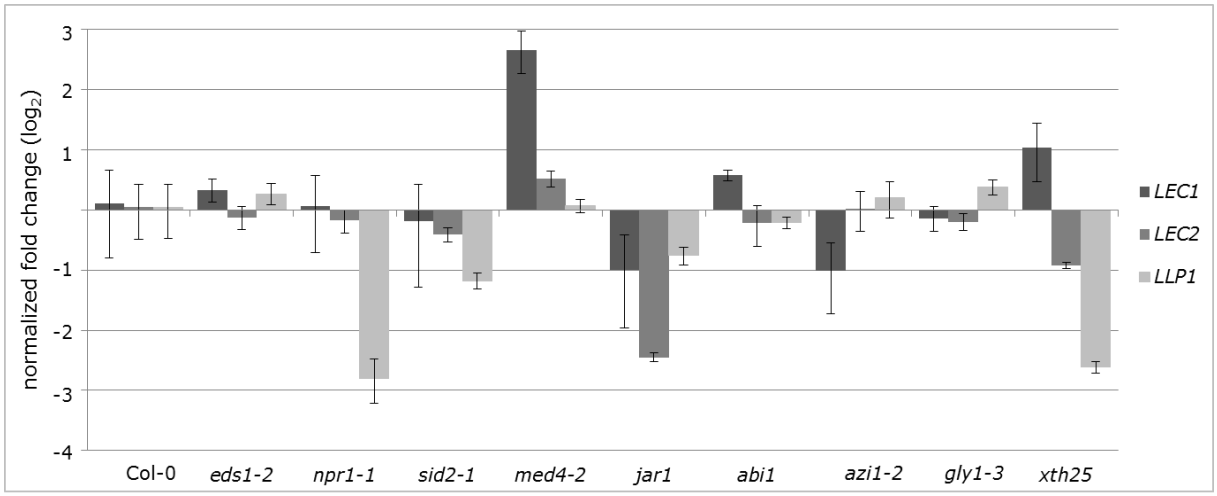


**Figure 20: Basal gene expression of the lectins in mutant lines related to SA-, ABA- or JA-pathway**

Leaves of 4-week-old plants were harvested and RNA was isolated of *eds1-2, npr1-1, sid2-1, med4-2, jar1, abi1* and *xth25*. qPCR on three individual plants for each genotype was performed to elucidate basal gene expression of *LEC1, LEC2* and *LLP1*. Transcript levels were normalized to that of *AtUBIQUITIN*. Bars represent the average ± standard deviation of three replicates. Asterisks indicate statistically significant differences to Col-0 (* $p<0.05$, ** $p<0.01$, student´s t-test).

There was no significant difference in *LEC1, LEC2* and *LLP1* transcript accumulation in *eds1-2* compared to Col-0 (Figure 20). The *npr1* mutant (Cao et al., 1997) showed a strong reduction of *LLP1* transcript accumulation, but no difference in *LEC1* or *LEC2* expression. In the *sid2* mutant (Wildermuth et al., 2001) only *LLP1* was slightly downregulated by ~2-fold. NPR1 acts downstream of SA, and SID2 is important for pathogen-induced SA-biosynthesis. Interestingly, the *LLP1* expression level in *med4-2*, which is defective in demethylation of MeSA to SA, was comparable to that in Col-0, while the transcript accumulation of *LEC1* was 6-fold up regulated in *med4-2* (Figure 20). This suggests that LEC1 could coincide with MeSA, as higher MeSA levels might be associated with *LEC1* gene expression. *LEC1* and *LLP1* were slightly downregulated in *jar1* (Staswick et al., 1998) by ~2- to ~1.8-fold respectively, as compared to Col-0 (Figure 20). Also, the gene expression of *LEC2* was 6-fold down-regulated in *jar1* compared to Col-0 plants. JAR1 is involved in the perception of JA signals which possibly associates LEC2 with JA-dependent defence signalling. No significant regulation of *LEC1, LEC2* and *LLP1* could be observed in *abi1*, which is defective in ABA signal transduction, as compared to Col-0. The *azi1-2* mutant showed a 2-fold reduction of *LEC1* but no transcriptional changes for *LEC2* and *LLP1.* Basal expression of the lectins seemed to be same in *gly1-3* and Col-0. Interestingly, in the *xth25* mutant upregulation of *LEC1* (2-fold) and downregulation of *LEC2* (2-fold) and *LLP1* (6-fold) could be observed. In conclusion, these results give a hint that the lectins may play a role in stress tolerance mediated by different phytohormones (4.1).

# 5. Discussion

Nowadays the focus of the agricultural politics is a higher yield by using less and more specific pesticides. Therefore information regarding the plant immune system is necessary to enable a better plant health care and support defence responses. Analysing the legume lectin-like lectins at the interface between systemic resistance and abiotic stress responses is one step forward in finding possible ways of balancing these antagonizing responses towards enhancing stress resilience and yield.

## 5.1 Regulation and localisation of LEC1, LEC2 and LLP1

Lectins are abundant and diverse proteins with binding capacity to carbohydrates. They contain at least one non-catalytic domain that in most cases binds reversibly to a specific mono- or oligosaccharide. In plants, there are 12 known families of lectins, with the legume lectin family, including legume-like lectins, representing the largest family (1.8). Genes of the legume lectin family can be induced by pathogens or elicitor treatments (Singh et al., 2012; Lannoo and Van Damme, 2014). A high number of them are secretory proteins which accumulate in the vacuoles, in the cell wall or the intercellular space (Chrispeels and Raikhel, 1991).

In previous proteomic research new potential SAR signals in *Arabidopsis thaliana* were identified (Breitenbach, 2012; Breitenbach et al., 2014). These were found by comparing the apoplast-enriched extracts from wild type and *eds1-2* mutant plants that were induced for SAR signal generation. Among others, LLP1 and its two closest homologues LEC1 and LEC2 were identified (Breitenbach et al., 2014). *LLP1* is induced by SA or BTH treatment but the accumulation of SA is not affected by mutations in *LLP1* (Armijo et al., 2013; Breitenbach et al., 2014). After exogenous SA application, *llp1* mutant plants support elevated *PR1* expression and resistance to various *Pseudomonas* strains. Together, LLP1 is hypothesized not to act downstream of SA but in parallel with SA (Breitenbach et al., 2014). The LLP1 homologue LEC1 accumulates in young plants after MeJA and ET treatment (Lyou et al., 2009). Whereas LLP1 is associated with the SA pathway, LEC1 is assumed to play a role in JA/ET-responsive defence reactions (Lyou et al., 2009; Armijo et al., 2013; Breitenbach et al., 2014). Application of chitin induces *LEC1* accumulation in Col-0 and in JA/ET defective mutants, indicating that *LEC1* expression by chitin is JA/ET-independent (Lyou et al., 2009). Similarly, *LEC2* is strongly induced by chitin in Col-0 and in mutants which are defective in the JA- and SA-dependent pathways (Ramonell et al., 2002; Zhang et al., 2002). Microarray data revealed *LEC2* as a jasmonate-responsive gene (Jung et al., 2007a).

In this thesis, the roles of LLP1, LEC1 and LEC2 during SAR were examined (1.10). To this end, the expression of the corresponding genes was measured in Col-0 and mutants

defective in *EDS1*, a main regulator of SAR (1.4). EDS1 acts as positive regulator of SA accumulation and negative regulator of JA/ET defence signalling. Hence, EDS1 is suggested to be a regulator of the antagonism between the SA- and ET/JA-mediated defence systems (Brodersen et al., 2006). To study putative interactions of LEC1, LEC2 and LLP1 with EDS1 in different defence pathways, Col-0 and *eds1-2* mutants were treated with different phytohormones and analysed for gene expression of *LEC1, LEC2* and *LLP1.* The data showed that *LEC1* was upregulated after MeJA treatment in Col-0 and *eds1-2* (4.1, Figure 8A). This verified the work of Lyou et al. (2009) and also showed that the induction of *LEC1* by MeJA was EDS1-independent. After challenging the plants with ABA, *LEC1* was downregulated in Col-0 which approved Anderson et al. (2004) that the JA/ET responsive defence gene is suppressed by ABA (Anderson et al., 2004). Same could be observed in the *eds1-2* plants, indicating that *LEC1* transcript regulation by ABA is EDS1-independent (4.1, Figure 8C). In Col-0 *LEC2* was upregulated by MeJA confirming the microarray data of Jung et al. (2007) and downregulated by ABA (Jung et al., 2007a). The regulation of *LEC2* by MeJA and ABA was independent of EDS1 (4.1, Figure 8A,C). Neither *LEC1* nor *LEC2* was induced by exogenous application of SA or BTH (4.1, Figure 8B,D). In contrast, *LLP1* was induced after SA and BTH treatment in Col-0 which approved results from Breitenbach et al. (4.1, Figure 8B,D)(Breitenbach et al., 2014). Same was observed in the *eds1-2* mutant, indicating that the regulation of *LLP1* by BTH is EDS1-independent. Furthermore, it was shown, that the induction of *LLP1* is fast responsive, since *LLP1* is induced after 2h and transcript level remained the same until 24hpi in Col-0 and *eds1-2*. In Col-0, *LLP1* was downregulated by ABA, but upregulated by the same treatment in *eds1-2* (4.1, Figure 8C), suggesting that *LLP1* is negatively influenced by EDS1 under ABA treatment.

Together, these results suggest that LEC1, LEC2 and LLP1 respond to biotic stress in an *EDS1*-independent manner. LEC1 and LEC2 play a preferential role in the jasmonate-mediated defence system. Although EDS1 is a negative regulator of the JA/ET defence system, the regulation of *LEC1* and *LEC2* was not affected by *EDS1* because their induction by MeJA was EDS1-independent (4.1, Figure 8). It is known, that *EDS1* is a positive regulator of SA and that the induction of *LLP1* is induced by SA (4.1, Figure 8). The induction of *LLP1* might not be dependent on *EDS1,* because LLP1 acts in parallel with SA (Breitenbach et al., 2014). It seems that LEC1, LEC2 and LLP1 might act on the interface between biotic and abiotic stress tolerance.

*LEC1, LEC2* and *LLP1* encode proteins with a legume lectin-like domain, which contains a cleavable N-terminal signal sequence, indicating that the proteins accumulate in extracellular regions. Armijo et al. (2013) located LLP1 primarily bound to the apoplastic side of the plasma membrane. These authors suggested that LLP1 plays a role in a membrane-associated mechanism induced by SA (Armijo et al., 2013). By measuring

protein extracts of soluble and microsomal fractions, they also found a minor amount of soluble LLP1, suggesting that two forms of LLP1 are produced. To verify the localization of LLP1 and to localize LEC1 and LEC2, promoter:protein-GFP constructs expressing *LEC1, LEC2* or *LLP1* from their respective native promoters were generated (3.11.2). To examine the functionality of these constructs, they were agro-infiltrated in tobacco plants. Transient transformation in *Nicotiana benthamiana* led to significant accumulation of GFP-tagged LEC1, LEC2, and LLP1. The GFP signal of LEC1:*LEC1-GFP* was mostly observed in parenchymal cells. It is not perceptible if LEC1 is located in the cytoplasm, the vacuole or other cellular regions like the chloroplasts. Chloroplasts are mostly found in the parenchymal layer and less in the epidermal cells (Stephens, 2006). LEC1 was mainly detected in the parenchymal layer and is probable located in extracellular regions due to its cleavable signal sequence. However, it could be also located near the chloroplast, which could not be perceived in this work. To determine the subcellular location of LEC1 it is necessary to further analyse stable transformed *Arabidopsis thaliana* containing LEC1:*LEC1-GFP.*

LEC2 and LLP1 were detected in the epidermal layer of transiently transformed tobacco leaves. It is not discernible if the GFP signal is connected to the plasma membrane or soluble. To ascertain the localization, it is necessary to include dyes in future experiments which stain the plasma membrane and the cell wall, respectively. The staining should go hand in hand with plasmolysis of the plant tissue to withdraw the plasma membrane from the cell wall. In this way, precise localisation and possible membrane binding of the lectins can be evidenced. Strikingly, only 20-30% of the agroinfiltrated leaf areas showed an evaluable GFP signal. Hence, the native gene promoters from *Arabidopsis* which were used in this work might not be equally efficient when ectopically expressed in *N. benthamiana.* Along the same lines, protein localization in *N. benthamiana* might also not exactly mirror that in *Arabidopsis.* Hence, it will be necessary to generate transgenic *Arabidopsis* to investigate the localization of LEC1, LEC2 and LLP1. This can be done by using the constructs described here, because the significant accumulation of GFP-tagged LEC1, LEC2, and LLP1 in *N. benthamiana* indicates that the native promoter regions that were chosen for *LEC1, LEC2* and *LLP1* were adequate for functional transcript accumulation.

## 5.2 Local defence response is not regulated by *LEC1*, *LEC2* and *LLP1*

Comparing the CDS of *LEC1* and *LEC2,* it could be seen, that they share a similarity of 89% (4.3, Table 10). Furthermore, *LEC1* and *LLP1* are 76% homologous and *LEC2* shares 77% homology of its CDS with that of *LLP1.* Due to their very close relationship, LEC1, LEC2, and LLP1 might fulfil similar functions in the plant. As described above, it is assumed that *LEC1, LEC2* and *LLP1* play a role in plant defence and might interact. To

ascertain, if LEC1, LEC2 and/or LLP1 compensate for each other (i.e. are functionally redundant) or interact during stress responses, RNAi-mediated silencing lines in the background of Col-0 or *eds1-2* were generated (4.3, Table 11). Homozygous lines of the third generation were tested by qPCR for basal transcript accumulation of *LEC1, LEC2* and *LLP1* (4.3, Figure 10). Two independent single insertion lines of the double silencing construct (C2 5-2, C2 6-2) and the triple silencing construct (C3 12-2, C3 13-1) in Col-0 as well as one single insertion line (e3 3-5) in the background of *eds1-2* were used for experiments in this work (4.3, Figure 10).

Studies on *lec1* and *llp1-1* revealed that they might play an important role in systemic acquired resistance and are probably less important for local defence responses (Breitenbach, 2012; Breitenbach et al., 2014). Here, the RNAi-mediated silencing lines and the T-DNA insertion lines were first tested for local defence to a variety of *P. syringae* strains. To this end, 4-week-old plants were exposed to the different pathogens, whose growth was recorded as a measure for plant susceptibility. *Pst*, a virulent strain, induces an *EDS1*-dependent basal defence response (Aarts et al., 1998; Breitenbach et al., 2014). As a result, higher bacterial titres were observed in the *eds1-2* mutant compared to Col-0 plants (4.4, Figure 11A). Compared to Col-0, the T-DNA insertion lines *lec1, llp1-1* and *llp1-3* showed similar behaviour in defence response to *Pst*. The growth behaviour of *Pst* in the RNAi-mediated silencing lines C2 5-2, C2 6-2, C3 12-2 and C3 13-1 was comparable to that in Col-0. Same could be observed in e3 3-5 compared to its parental line *eds1-2*. Together, all genotypes behaved similarly as their respective parental lines when infected with *Pst* (4.4, Figure 11A), suggesting that LEC1, LEC2 and LLP1 are not essential for basal defence in *Arabidopsis* against *Pst.*The avirulent strains *Pst AvrRpm1* and *Pst AvrRpt2* induce defence responses which are *EDS1*-independent (Aarts et al., 1998; Breitenbach, 2012). Hence, bacterial growth of *Pst AvrRpm1* was comparable in Col-0 and *eds1-2* (4.4, Figure 11B). All transgenic lines showed similar bacterial titres and growth pattern compared to their parental lines. Therefore, the local immune response to *Pst AvrRpm1* is not affected by *LEC1, LEC2* and *LLP1*. Comparable to the immune response to *Pst AvrRpm1*, also local defence to *Pst AvrRpt2* was not altered in Col-0 and Col-0 transgenic lines. The immune response in *eds1-2* was comparable to that in Col-0, but the triple silencing line e3 3-5 mounted lower *Pst AvrRpt2* titres at 7dpi than Col-0 or *eds1-2* did. This suggests that the immune response to *Pst AvrRpt2* was accelerated in the absence of EDS1 and at least one or more lectins (4.4, Figure 11C). Because this effect occurs relatively late after the onset of the infection, it seems unlikely that the accelerated immune response at 7dpi significantly affects plant health.

The defence response induced by *AvrRps4* is dependent on *EDS1* (Aarts et al., 1998) which was supported by significantly higher *Pst AvrRps4* growth in *eds1-2* mutant as

compared to Col-0 plants in Figure 11D. A delayed immune response to *Pst AvrRps4* was observed in the *lec1* mutant and in the triple silencing line C3 13-1 whereby bacterial titres adapted to same level as in Col-0 at T4. This suggests that LEC1 might support a fully intact defence response. In contrast, the single T-DNA insertion lines *llp1-1* and *llp1-3* as well as the double silencing lines C2 5-2 and C2 6-2 did not show a delayed immune response. Thus, if LEC2 and LLP1 together are either present (*lec1*) or absent (C3), a delayed immune response occurs indicating a possible interference of LEC1, LEC2 and LLP1 during the defence response induced by *AvrRps4*. This interference might be associated with EDS1, because a delayed immune response was not observed in the absence of *EDS1* (*eds1-2*) or in the triple silencing line e3 3-5. Taken together, it seems that *LLP1* does not affect *EDS1*-dependent ETI downstream of *AvrRps4,* but there is a positive effect of *LEC1* on *EDS1*-dependent ETI.

In short, basal resistance and *EDS1*-independent ETI appeared to be unaffected by *LEC1, LEC2* and *LLP1* (4.4, Figure 11A-C). In contrast, a putative and moderate influence of LEC1 on *Pst AvrRps4*-triggered ETI was observed by delayed resistance to *Pst AvrRps4* growth in *lec1* and C3 13-1 (4.4, Figure 11D). Because the immune response to *Pst AvrRps4* is dependent on *EDS1*, a positive correlation in *EDS1*-dependent plant defence should be considered between *EDS1* and *LEC1* and/or *LEC2* and *LLP1*.

EDS1 is one of the main regulators of SA-dependent defence responses and SAR. To trigger systemic immune responses, plants were locally infected with *Pst AvrRpm1* (4.5). As described above, local bacterial titres and pathogen growth did not differ between Col-0, *eds1-2* and their transgenic lines (4.4, Figure 11B). To confirm that there are no differences in the immune response also at transcriptional level, transcript accumulation of the SA-responsive *PR1* gene was measured (4.5, Figure 12). *PR1* is induced in reaction to pathogen attack and is a common marker gene for SAR (1.5). The basal *PR1* expression was comparable in Col-0 wild type plants and the mutants and transgenic lines used in this study (4.5, Figure 12, T0). After pathogen challenge, Col-0 showed elevated transcript accumulation of *PR1* at 2dpi as expected. Same was observed in the T-DNA insertion lines and the RNAi-mediated silencing lines in the background of Col-0, indicating that LEC1, LEC2 and LLP1 do not play a crucial role in the local *PR1* response to *Pst AvrRpm1*. In *eds1-2* and e3 3-5 plants *PR1* transcript accumulation in response to *Pst AvrRpm1* was not observed at 2dpi. Interestingly, local defence response to *Pst AvrRpm1* is EDS1-independent (Aarts, Metz et al. 1998), therefore these results were unexpected. It is not yet evidenced, that the local defence response of *eds1-2* to *Pst AvrRpm1* is linked to the expression of *PR1*. It might be that the expression of the defence gene *PR1* is delayed, because *PR1* transcript levels were comparable in *Pst AvrRpm1*-infected Col-0 and *eds1-2* plants at 4dpi (8.1, Figure S3). Although defence

response to *Pst AvrRpm1* is EDS1-independent, the data lead to the suggestion that EDS1 is needed for a fast ETI response to *Pst AvrRpm1.*

### 5.3 LEC1, LEC2 and LLP1 work additively during systemic acquired resistance

SAR is a long-lasting mechanism of enhanced defence to subsequent infections against a broad spectrum of microorganisms (Conrath et al., 2015). It occurs in the systemic healthy tissue of local pre-infected plants (Vlot et al., 2017). SA accumulates during SAR leading to a local defence and a systemic SA-dependent immunity (Vlot et al., 2009). It is known that mutation in *LEC1* compromises SAR but not local resistance to *P. syringae* (Breitenbach, 2012). Same was observed for mutations in *LLP1* (Breitenbach et al., 2014). Furthermore, *LLP1* is an early responsive gene induced via a SA-mediated pathway (Armijo et al., 2013). In 2014, it was shown that LLP1 promotes systemic but not local resistance possibly in parallel with SA (Breitenbach et al., 2014).

Canonical SAR experiments showed that *Pst* growth in the systemic tissue after a local *Pst AvrRpm1* infection compared to mock was reduced in Col-0 wild type, but not in the T-DNA insertion lines *lec1, llp1-1* and *llp1-3* (4.6, Figure 13A, Figure 13B). This confirmed that the *lec1* and *llp1* mutants are SAR-defective. The double silencing lines in the background of Col-0 were also defective in SAR, indicating that RNAi-mediated silencing of *LEC1 and LEC2* compromised SAR. The triple silencing lines C3 12-2 and C3 13-1 mounted comparable *Pst* titres in MOCK-pretreated plants as compared to Col-0. Interestingly, C3 12-2 and C3 13-1 supported significantly more growth of *Pst* in the systemic tissue compared to MOCK-pretreated plants (4.6, Figure 13A, Figure 13B), suggesting that systemic defence was suppressed compared to MOCK. In other words, the plants became more susceptible in the systemic tissue after priming, suggesting that LEC1, LEC2 and LLP1 act additively during SAR and together fine-tune systemic resistance.

SAR is characterized among others, by the increased expression of a number of *PR* genes, encoding defence-related proteins such as the antimicrobial peptide PR1. To ascertain, if the systemic susceptibility is also taken place on transcriptional level, expression levels of *PR1* were measured. Systemic *PR1* expression was elevated in local *PstAvrRpm1*-pretreated compared to local MOCK-pretreated Col-0 plants (4.6, Figure 13C, Figure 13D). The T-DNA insertion lines *lec1, llp1-1, llp1-3* and the double silencing lines C2 5-2 and C2 6-2 also supported higher *PR1* transcript accumulation in SAR-induced compared to MOCK-pretreated plants. In contrast, *PR1* expression in the triple silencing lines C3 12-2 and C3 13-1 was downregulated in the systemic tissue after challenging the local leaves with *PstAvrRpm1* compared to MOCK. This supports the hypothesis that LEC1, LEC2 and LLP1 might act additively in SAR. As discussed above

(5.2), there might be a possible interaction of LEC1, LEC2, LLP1 and the SAR regulator EDS1. Therefore, SAR was investigated in *eds1-2* and the triple silencing line e3 3-5 (4.3). Compared to Col-0, bacterial titres in systemic tissue of locally MOCK-treated plants were higher in both *eds1-2* and e3 3-5 as compared to Col-0 (4.6, Figure 14A), supporting data that the defence response to *Pst* is *EDS1*-dependent (Aarts et al., 1998; Breitenbach et al., 2014)(4.4, Figure 11A). SAR was abolished in *eds1-2* and e3 3-5 confirming published data that *eds1-2* is SAR-deficient (4.6, Figure 14A) (Truman et al., 2007; Breitenbach et al., 2014). Hence, in contrast to Col-0, systemic *PR1* induction in *eds1-2* was marginal in SAR-induced compared to locally MOCK-treated plants (4.6, Figure 14B). Same was observed in the triple silencing line e3 3-5, indicating that EDS1 is required for an intact interplay of LEC1, LEC2 and LLP1 and that the systemic induced susceptibility (SIS) in the C3 lines similarly to SAR in Col-0 depends on *EDS1*.

Taken together, the data suggest that the reduced transcript accumulation of *LEC1*, *LEC2* and *LLP1* in the triple silencing lines is responsible for systemic defence signalling not only failing but leading to systemic susceptibility. It is known that virulent *P. syringae* is able to suppress plant defence in the systemic tissue which is known as systemic induced susceptibility (SIS) (Cui et al., 2005). The role of EDS1 during SIS is unknown, but SIS requires the bacterial toxin coronatine, a structural and functional mimic of the defence hormone JA (Cui et al., 2005; de Torres Zabala et al., 2009). Cui et al. (2005) suggest that SIS may be a consequence of the mutually antagonistic interaction between the SA and JA signalling pathways.

## 5.4 LEC1, LEC2 and LLP1 may form a synergistic node of crosstalk between SA and JA

Because SAR is mediated by SA, SA-induced resistance was investigated to gain further insight into a possible role of the lectins in SA signalling. Therefore the responses of the T-DNA insertion mutants *lec1, llp1-1, llp1-3, eds1-2* and the RNAi-mediated silencing lines C2 5-2, C2 6-2, C3 12-2, C3 13-1 and e3 3-5 to pathogen infection was surveyed after the application of exogenous SA. In Col-0, SA locally enhanced resistance leading to a reduced growth of *Pst* in treated plants (4.7, Figure 15A, Figure 15B). Same was observed in the T-DNA insertion lines and the RNAi-mediated silencing lines in the background of Col-0. SA-induced resistance was not altered in these lines (4.7, Figure 15A, Figure 15B). After SA treatment, also *eds1-2* responded with enhanced resistance to *Pst*, confirming published data, that EDS1 acts upstream of SA in SA-induced immunity (Falk et al., 1999; Feys et al., 2001). Notably, the *eds1-2* triple silencing line e3 3-5 was nonresponsive to SA and supported *Pst* growth to similar levels in the MOCK-treated and SA-treated plants. Hence, EDS1 might rely on *LEC1*, *LEC2* and/or *LLP1* for an adequate resistance response downstream of SA.

*PR1* transcript accumulation in SA-treated leaves was measured before *Pst* infection (4.7, Figure 15C, Figure 15D). In Col-0, SA-induced resistance in Col-0 was accompanied by enhanced *PR1* expression. Also in *lec1, llp1-1* and *llp1-3* SA-induced *PR1* expression was not altered, while the RNAi-mediated silencing lines C2 5-2, C2 6-2, C3 12-1 and C3 13-1 responded to SA treatment with normal *PR1* induction that was comparable to that in Col-0 (4.7, Figure 15C, Figure 15D). Similarly, *PR1* transcript accumulation was elevated to the same level in SA-treated *eds1-2* as in Col-0 plants. Thus, as expected, the local SA-related immune response was not compromised in *eds1-2* (Falk et al., 1999; Feys et al., 2001). Interestingly, also e3 3-5 showed comparable *PR1* transcript accumulation as its parental line *eds1-2* after SA treatment (4.7, Figure 15C), although resistance to *Pst* growth after SA treatment was not induced (4.7, Figure 15A). This suggests that *EDS1* requires *LEC1, LEC2* and *LLP1* during SA-dependent immune responses partially independently of *PR1* transcript accumulation. In *eds1-2*, basal transcript accumulation of *LEC1, LEC2* and *LLP1* was not different from that in Col-0 (4.12, Figure 20), indicating that the basal regulation of the lectins is independent of *EDS1*. Basal gene expression of *LEC1, LEC2* and *LLP1* in mutant lines connected to SA synthesis and signalling might give a hint where to order the lectins during SA-dependent plant defence (4.12, Figure 20). It is known that NPR1 acts downstream of SA (Cao et al., 1997) whereas LLP1 acts in parallel with SA (Breitenbach et al., 2014). Because *npr1* supported reduced transcript accumulation of *LLP1*, it is possible NPR1 might act upstream of LLP1. SID2 acts upstream of SA and is essential for SA-biosynthesis induced by pathogen attack (Wildermuth et al., 2001). In *sid2* mutant lines, transcript accumulation of *LLP1* was slightly downregulated, suggesting that pathogen-induced SA-biosynthesis is also correlated to LLP1. The mutant line *med4-2* is defective in the hydrolysis of MeSA to SA (4.12, Figure 20). *LLP1* gene expression was not affected by the accumulation of MeSA, although LLP1 is induced by SA. It seems that transcript accumulation of *LEC1* is supported by shifting the MeSA:SA equilibrium towards MeSA because *LEC1* accumulation was 6-fold up regulated in *med4-2* (4.12, Figure 20).

One of the main negative regulators of SA-dependent immune responses is JA. Both pathways interact in a complex, mostly antagonistic crosstalk (Pieterse et al., 2001; Kunkel and Brooks, 2002; Glazebrook et al., 2003). *LEC1* and *LEC2* are induced in Col-0 and *eds1-2* by the JA-derivate MeJA (4.1, Figure 8A). To gather insight into the role of the lectins during JA-dependent defence, plants were challenged with *A.brassicicola* (4.8, Figure 16). It is known that *LEC1* expression is induced by *A.brassicicola* (Mukherjee et al., 2010). Also, the immune response to *A.brassicicola* is mediated by JA and independent of SA (Thomma et al., 1998). Col-0 was more resistant to *A.brassicicola* compared to the T-DNA insertion lines *lec1, llp1-1* and *llp1-3.* Same was observed for the silencing lines C2 5-2, C2 6-2 and C3 13-1. All mutants and transgenic lines supported

the occurrence of bigger lesions resulting from the fungal infection, suggesting that all three lectins, LEC1, LEC2 and LLP1, affect the *Arabidopsis* immune response against *A.brassicicola.* It also seems that defence against *A. brassicicola* is independent of *EDS1*, because *eds1-2* mounted similarly sized *A. brassicicola* lesions as Col-0 plants did. Interestingly, the same held true for the triple silencing line e3 3-5, suggesting that LEC1, LEC2 and LLP1 might be partially dependent on or regulated by *EDS1* for an intact immune response to *A.brassicicola.* Transcript accumulation of *LEC1, LEC2* and *LLP1* was measured in *jar1*, which is defective in JA-signalling perception (4.12, Figure 20)(Staswick et al., 1998). All lectins were down regulated in *jar1* compared to Col-0. Particularly *LEC2* transcript accumulation was strongly reduced, suggesting that LEC2 might be associated with JA-mediated immune responses.

Although in this thesis, SAR is induced by an avirulent *Pseudomonas* strain, it should be considered that the systemic immune response can be affected by both SA- and JA-dependent defence pathways (Vlot et al., 2017). It seems that the LEC1, LEC2 and LLP1 are required for EDS1-dependent SAR response downstream of SA independently of *PR1* gene expression. During JA-mediated defence response, *LEC1*, *LEC2* and *LLP1* might be partially dependent on or regulated by *EDS1.*

## 5.5 ABA-related stress response in *Arabidopsis thaliana* is impaired by LEC1, LEC2 and LLP1

Data in 4.1 showed that transcript accumulation of *LEC1* and *LEC2* is decreased under ABA treatment. Therefore, the ABA response in seedling development under salt stress was monitored (4.9).

The expression of the well-known ABA marker genes *ABA-INSENSITIVE1* (*ABI1*), *RESPONSIVE TO ABA18* (*RAB18*) and *RESPONSIVE TO DESSICATION29B* (*RD29B*) were chosen for examination (Guo et al., 2009). *ABI1* encodes a protein phosphatase 2C that is required for proper ABA responsiveness both in seeds and in vegetative tissues and is a negative regulator of ABA signal transduction (Lu et al., 2015). The expression of *RAB18* is under direct ABA regulation through ABA-responsive elements (ABRE) in the promoter (Yoshida et al., 2015), and *RD29B* is a dehydration-responsive gene, which is mediated mainly by ABA (Virlouvet et al., 2014). *RAB18* was induced in the T-DNA insertion lines *lec1, llp1-1, llp1-3* and the triple silencing lines C3 12-2, C3 13-1 in the background of Col-0 at 100mM NaCl (4.9, Figure 17) to a higher level compared to that in Col-0. Hence, enhanced tolerance of high salt conditions in the absence of one or more lectins was associated with elevated *RAB18* transcript accumulation and thus probably ABA signalling. Vice versa, ABA treatment reduced the transcript accumulation of *LEC1,*

*LEC2 and LLP1* in Col-0 (4.1, Figure 8C). Together, the data support an inverse correlation between the lectins and ABA-related immune responses in *Arabidopsis*.

In *eds1-2* and the triple silencing line e3 3-5, transcript accumulation of *RAB18* was significantly higher than that in Col-0 (4.9, Figure 17), with e3 3-5 supporting significantly higher transcript accumulation of *RAB18* than *eds1-2*. This suggests that the lectins respond to high salinity in an EDS1-independent manner. *RD29B* transcript accumulation was induced in Col-0 at 100mM NaCl. Only *lec1* and *llp1-1* showed a basal downregulation of *RD29B* in the absence of stress (4.9, Figure 17D, preliminary data). For all tested lines, the expression pattern for *RD29B* at 100mM NaCl was similar to that of *RAB18* at 100mM NaCl (4.9, Figure 17B, Figure 17A). Hence, it seems that the higher resistance in the transgenic lines to NaCl was linked to the ABA-pathway and not due to ionic stress.

Thus, in response to salt stress, the mutant lines l*ec1*, *llp1-1* and *llp1-3* and the RNAi lines showed similar patterns, suggesting a similar function of LEC1 and LLP1. A prediction for LEC2 is not speculated because there is no T-DNA insertion line available for *LEC2* and double silencing lines were not included in this experiment.

Taken together, the lectins might affect the *Arabidopsis* response to high salinity in an *EDS1*-independent manner. It is possible that in the lectin-deficient plant lines, high salinity leads to a higher accumulation of ABA which in turn induces *RAB18* transcript accumulation. The fact that ABA treatment reduces the transcript accumulation of *LEC1, LEC2 and LLP1* in Col-0 (4.1, Figure 8C) further supports an inverse correlation between the lectins and ABA-related immune responses in *Arabidopsis*.

### 5.6 LEC2 and LLP1 might bind to a xyloglucan transferase/hydrolase

Lectins have a putative carbohydrate-binding capacity to bind glycoproteins, glycolipids, or polysaccharides with high affinity (Lannoo and Van Damme, 2014; De Schutter and Van Damme, 2015). As it was discussed in 5.3, LEC1, LEC2 and LLP1 might bind to a yet unknown SAR signal, SAR regulator or membrane-associated SAR-relevant protein containing a carbohydrate domain. Therefore, to find possible interaction partners that might act as co-regulators of the lectins during SAR Yeast-2-Hybrid assays were performed (4.10). The results of these assays could not be verified and are therefore no evidence for real and physical interactions with LEC1, LEC2 and LLP1. This might be due to the chosen sequence used for cloning, where the first 63 base pairs of each lectin gene code for a signal peptide, which directs the proteins to the apoplast and is most often cleaved in plants. As yeast is not capable of recognizing and naturally cleaving plant proteins, the plant signal peptide can disturb protein binding. Only one very promising putative binding partner who showed presumptive interaction with two of the lectins was

included in this thesis and analysed (4.10, Table 12). The xyloglucan transferase/hydrolase XTH25 showed a putative interaction with LEC2 and LLP1 and is similarly to LEC2 and LLP1 predicted to localise to the extracellular region. In 2013, Gruner et al. found three *XTH* genes that are downregulated during SAR (Gruner et al., 2013). Two of them (XTH4 and XTH31) code for proteins that are constituents of the cell wall proteome in *Arabidopsis* hypocotyls (Irshad et al., 2008). It is also known, that in response to abiotic stress the accumulation of XTHs is elevated in cell wall metabolism (Le Gall et al., 2015). Taken together, it is possible that XTH25 could act as a binding partner of LEC2 and/or LLP1 (4.10). To investigate if XTH25 is also involved in the systemic defence response SAR assays were performed with *xth25* mutant plants (4.11, Figure 19). It seemed that SAR in *xth25* was compromised. SAR appeared to be abolished in only one out of three experiments (4.11, Figure 19A). However, in the other two experiments, SAR was significant in *xth25*, but weaker than in Col-0 and thus compromised (4.11, Figure 19B). Analyses of the transcript accumulation of *LEC1, LEC2* and *LLP1* in the *xth25* mutant revealed that *LEC1* was slightly upregulated, whereas *LEC2* and *LLP1* transcript accumulation was downregulated as compared to in Col-0 (4.11, Figure 19). Therefore, compromised SAR in *xth25* might be due either to the absence of XTH25 or to reduced *LLP1* and *LEC2* transcript accumulation (4.11, Figure 19). In future experiments, *XTH25* should be considered as a possible functional LEC1, LEC2 and LLP1 interactor in SAR.

## 5.7 Conclusion

The lectins LEC1, LEC2 and LLP1 belong to the legume lectin like family which are transcriptionally induced by pathogens or elicitor treatments (Lannoo and Van Damme, 2014). As described in 1.3, crosstalk between hormones and other regulatory components takes place when biotic and abiotic stresses occur concurrently (1.3, Figure 3). Figure 21 shows a simplified depiction of Figure 3 including putative roles of LLP1, LEC1 and LEC2 during hormonal crosstalk.

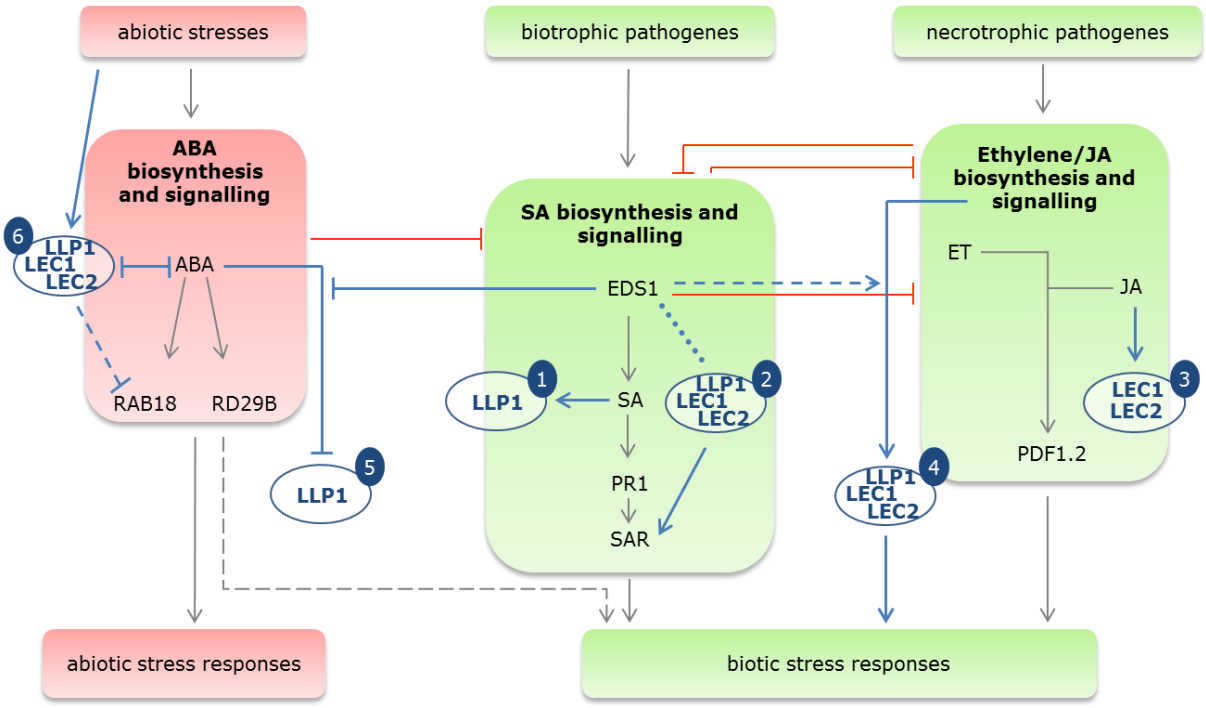


**Figure 21: LLP1, LEC1 and LEC2 inhibit abiotic and support biotic stress responses**

Putative roles of LLP1, LEC1 and LEC2 in abiotic stress responses (in red) mediated by ABA and in biotic stress responses (in green) mediated by SA (left) and JA (right). Arrows (→) signify positive regulation or gene induction, while repression or an inhibitory interaction is presented by the ⊣ symbol and inverse correlation is shown in double blocks (⊢⊣). Grey and red lines indicate well-known and established relationships. Blue lines and blue numbers (1), (2), (3), (4), (5), (6) illustrate the proposed impact of *LLP1, LEC1* and *LEC2* on plant defence as hypothesized from the experimental results presented in this thesis. Dashed lines indicate indirect impact or partial regulation, whereas the dotted line represents assumed coherence. LLP1: legume-lectin like protein1, LEC1: lectin1, LEC2: lectin2, ABA: abscisic acid, RAB18: responsive to abscisic acid18, RD29B: responsive to desiccation 29B, EDS1: enhanced disease susceptibility 1, SA: salicylic acid, PR1: pathogenesis-related protein1, SAR: systemic acquired resistance, ET: ethylene, JA: jasmonic acid, PDF1.2: plant defensin1.2.

When challenged with biotrophic pathogens, *Arabidopsis* plants trigger SA biosynthesis and signalling to initiate a biotic stress response. (**1**) Treatment of Col-0 with SA and BTH induced *LLP1* transcript accumulation which confirmed published data (4.1, Figure 8B,D)(Armijo et al., 2013; Breitenbach et al., 2014). The blue dotted line in (**2**) illustrates, that for a full defence response, all three lectins are required in coherence with EDS1, but independently of *PR1* gene expression (4.4, Figure 11, 4.5, Figure 12). However, stress response after SA challenge in various transgenic and mutant lines showed, that LLP1, LEC1 and LEC2 are not necessary for immune signalling downstream of SA in Col-0 (4.7, Figure 15). Nevertheless, LEC1, LEC2 and LLP1 act additively during SAR and are essential for the fine-tuning of systemic resistance in collaboration with EDS1 (4.6, Figure 13, Figure 14). EDS1 might need *LEC1*, *LEC2* and/or *LLP1* to support an adequate SAR response in parallel with SA. It seems that pathogen-induced SA-

biosynthesis is associated with LLP1 accompanied by EDS1 and that EDS1 is required for an intact interplay of LLP1 with LEC1 and LEC2 (4.7, Figure 15). This is supported by the fact that the triple silencing line e3 3-5 shows deviating results in SAR and systemic *PR1* induction as compared to the triple silencing lines in the background of Col-0 (4.6, Figure 13A-D, Figure 14). In addition, *PR1* expression data imply that LEC1, LEC2 and LLP1 play a role in SAR signal perception and less in SAR signal generation.

Beside the SA-pathway, also the JA-pathway plays a crucial role in biotic stress responses (Figure 21, green). Hence, roles of LEC1, LEC2 and LLP1 were examined in JA-mediated defence. (**3**) The exogenous application of the JA-derivate MeJA induced *LEC1* and *LEC2*, but not *LLP1* (4.1, Figure 8A). The dashed arrow pointing to (**4**) in Figure 21 displays that *LEC1*, *LEC2* and *LLP1* might be partially dependent on or regulated by *EDS1* during JA-mediated defence responses (4.8, Figure 16). In addition to *LEC1* and *LEC2*, *LLP1* is included in arrow 4, because mutants lacking *LLP1* showed enhanced susceptibility to the necrotrophic pathogen *A.brassicicola* (4.8, Figure 16) leading to the conclusion, that *LLP1* might be involved in JA-dependent stress responses. Similarly, experiments with *A.brassicicola* revealed, that LEC1 and/or LEC2 play a role in defence against necrotrophic fungal pathogens. Hence, all three lectins LEC1, LEC2 and LLP1 might be necessary for an intact immune response against necrotrophic fungi (4.8, Figure 16).

Taken together, LEC1, LEC2 and LLP1 are important for biotic stress responses in *Arabidopsis.* Apparently, LLP1 is stronger involved in the defence response against biotrophic pathogens as it is induced by SA and is assumed to act in parallel with SA (Figure 21(**1**)). In contrast, LEC1 and LEC2 are regulated by the JA-derivate MeJA and are therefore assumed to play a stronger role during necrotrophic pathogen attack (Figure 21(**3**)). Nevertheless, all three lectins are essential for full biotic stress responses, including SAR and JA-mediated immunity against *A. Brassicicola* (Figure 21(**2**),(**4**)).

In addition to biotic stressors, plants are also confronted with abiotic stresses which initiate ABA biosynthesis and signalling (Figure 21, red). (**5**) Phytohormone treatment revealed that *LLP1* is negatively influenced by EDS1 under ABA treatment (4.1, Figure 8C). The double block in (**6**) illustrates an inverse correlation between the lectins and the defence response of *A.thaliana* to salt stress. That inverse correlation to ABA might be dependent on the interplay of *LEC1*, *LLP1* and maybe *LEC2*. Furthermore, LEC1, LEC2 and LLP1 might respond to high salinity in an ABA-related, but *EDS1*-independent manner (4.9, Figure 17D,E). That in turn might impair the transcript accumulation of ABA-related defence genes as displayed by the dashed block symbol (Figure 21(**6**)).

That leads to the conclusion that LEC1, LEC2 and LLP1 might act on the interface between biotic and abiotic stress tolerance. Considering that the triple silencing line shows an inverse SAR response and SA signalling is antagonized by JA and ABA, all three phytohormones must be considered. In Cui et al. (2005) it was shown, that systemic induced susceptibility was caused by the production of the pathogen-derived functional and structural mimic of JA, coronatin (Cui et al., 2005). It was recently shown that coronatin action in antagonizing SAR likely depends on ABA signalling components that are activated by coronatin (Mine et al., 2017). In this thesis it was revealed that the lectins LEC1, LEC2 and LLP1 play a positive role in JA dependent defence response. Therefore, it is likely that JA biosynthesis and/or signalling are reduced in the absence of the lectins which in turn leads to an attenuated JA-mediated immune activation (as observed in the interaction between the lectin mutants and RNAi lines with *A. brassicicola*, 4.8, Figure 16). Local immune response against biotrophic pathogens is not impaired by the lectins and MOCK treatment is comparative in wild-type and transgenic plants. During SAR, primary infection significantly enhanced secondary growth of *Pst* in uninfected leaves as compared with mock-treated plants in the absence of all three lectins. One explanation might be that this is a consequence of the mutually antagonistic interaction between the SA and JA/ABA signalling pathways. Considering a possible synergistic effect of the lectins on SA and JA, we should probably focus here on ABA signalling, components of which are responsible for coronatin-induced systemic induced susceptibility (Cui et al., 2005; Mine et al., 2017). Also, SAR signalling is suppressed by ABA (Yasuda et al., 2008): ABA treatment suppresses the induction of SAR both upstream and downstream of SA (Tsuda and Somssich, 2015). On the contrary, SAR abates ABA signalling in response to salt stress. Both suppressions occurred from the complex crosstalk between the ABA and SAR signalling networks. Thus, SAR and ABA signalling are mutually antagonistic (Hofmann, 2008). It was shown in this thesis that the lectins are downregulated by ABA in Col-0 (4.1, Figure 8) leading to the suggestion of an inverse correlation between the lectins and ABA. This was further supported by an elevated transcriptional response to ABA in the absence of one or more of the lectins (4.9, Figure 17). Therefore, during SAR the lack of the lectins might be responsible for an exaggerated ABA response. This in turn can impair SAR signalling, in particular in the absence of all three lectins leading to a higher susceptibility to secondary growth of *Pst* in local pre-infected plants compared to local MOCK-treated plants. Hence, the comprehensive interaction of the lectins in phytohormone cross-talk is most notable during systemic defence response.

## 6. Outlook

In this thesis, the roles of LLP1, LEC1 and LEC2 during SAR were examined. Also, the SA regulator EDS1 was proved to play a major role in this study. Interactions of LEC1, LEC2 and LLP1 with EDS1 in different defence pathways were examined. It seems that after exogenous application of SA and MeJA transcripts of *LEC1*, *LEC2* and *LLP1* accumulate in an *EDS1*-independent manner. Notably, treatment of SA leads to transcript accumulation of *LLP1* whereas MeJA induces *LEC1* and *LEC2*. Therefore, experiments regarding SA- and JA-dependent immune response were conducted. Local defence response to *Pseudomonas* is not regulated by *LEC1*, *LEC2* and *LLP1.* However, a delayed immune response to *Pst AvrRps4* was observed in the *lec1* mutant and in the triple silencing line C3 13-1 indicating that *LLP1* does not affect *EDS1*-dependent ETI downstream of *AvrRps4,* but there is a positive effect of *LEC1* on *EDS1*-dependent ETI. It would be very interesting to study if SAR in *lec1* and the triple silencing line is also delayed when plants were first primed by *Pst AvrRps4.*

It was shown here for the first time that LEC1, LEC2 and LLP1 act additively during SAR and together fine-tune systemic resistance. To reveal more insight, experiments using petiole exudates from Col-0, *lec1* and *llp1-1* would elucidate if LEC1 and LLP1 are involved in SAR signal generation and/or perception. Including the mutant line *eds1-2*, relationship of EDS1 and LEC1, LEC2 and LLP1 will throw light on their interaction during SAR. For detailed insight into the role of the lectins in JA-mediated defence response it will be necessary to look for transcript accumulation of JA-responsive genes during JA-related defence in transgenic lectin lines as well as *eds1-2*. It would be also of interest to test various JA-dependent stresses. Hence transgenic lectin lines can be exposed to further necrotrophic fungi, to see if the stress response in this thesis is a class effect or narrowed to special fungi. Also herbivory attack or mechanical wounding lead to a JA-dependent immune response and can be taken into account.

Previously, LEC1 and LLP1 were found by comparing the apoplast-enriched extracts from wild type and *eds1-2* mutant plants. To gain first insights into the role of the lectins during plant defence responses, experiments were performed by using single T-DNA insertion lines (*lec1, llp1-1, llp1-3*). These mutant lines were obtained from the Nottingham Arabidopsis Stock Centre. By BLAST sequence search, a close homologue of LEC1, LECTIN2 (LEC2) was found and included in this study. Later on, multiple silencing lines were generated to gain insight into the putative interplay of LEC1, LEC2 and LLP1. To reveal if the experimental outcomes of the *LEC1/LEC2* double silencing line is due to the reduced transcript level of *LEC1* or *LEC2* or maybe of both, it is necessary to generate transgenic *Arabidopsis* silencing only *LEC2*, for which T-DNA insertion lines are not available. This can be done by using artificial micro RNAs (amiRNAs) (Schwab et al.,

2006) to ensure specific gene silencing of *LEC2*, which shares 89% nucleotide identity with *LEC1.* Subsequently, SAR experiments should be performed with this *LEC2* silencing line to verify that LEC2 is essential for SAR. Furthermore, SA-dependent defence responses and responses to other biotic and abiotic stresses need to be verified to reveal a clear position of LEC2 in plant defence.

Exogenous application of ABA leads to a downregulation of *LEC1* and *LEC2* independently of *EDS1*. In contrast, ABA regulates *LLP1* in the absence of EDS1 suggesting an inverse correlation during abiotic stress. Growth on MS plates with different concentrations of ABA will evaluate the effects of ABA-dependent plant development and confirm if the salt stress experiments are ABA-related or an osmotic effect. In addition, to exclude toxic effects of NaCl, non-toxic osmotic substances like mannitol should be included. The stress experiment needs to be repeated including repetition of the gene expression of *RD29B.* Furthermore in 4.9 (Figure 17B, Figure 17C) it seems that *llp1-1* developed bigger seedlings under osmotic stress conditions compared to *lec1* (4.9, Figure 17B). Regarding the fact that bigger leaves mostly correlate with more stomata, it would be interesting to have a closer look at ABA-induced stomatal closure. Higher stomata density leads to a greater balance in behavioural control over water loss rate and $CO_2$ uptake. This might lead to higher resistance of seedlings with high stomata density to salt stress compared to seedlings with less stomata density.

*LEC1, LEC2* and *LLP1* encode proteins with a legume lectin-like domain, which contains a cleavable N-terminal signal sequence, indicating that the proteins accumulate in extracellular regions. To investigate the localization of LEC1, LEC2 and LLP1, microscopy experiments need to be repeated including staining and plasmolysis as recommended in 5.1. Furthermore, stable transformed *Arabidopsis* need to be generated. This can be done by using the constructs described in 4.2, because the significant accumulation of GFP-tagged LEC1, LEC2, and LLP1 indicate that the native promoter regions that were chosen for *LEC1, LEC2* and *LLP1* were adequate for functional transcript accumulation. In vivo experiments during various stress responses would further reveal the dynamics and function of the lectins in response to infection. Also the expression pattern of each lectin can be ascertained, which would be important to exhibit the interplay of the lectins, especially during SAR. Hence, the lectin protein localization can be revealed to give further insight into the function of the lectins. Regarding the N-terminal signal peptide mentioned above, it is likely that the lectins are located somewhere else than where they are expressed. Hence, it might also be of interest, where the gene expression of the lectins occurs. Therefore, the native promoter region mentioned above can be used to drive only GFP. It is possible that the different lectins are expressed at different organs of the plant like the stem and the leaves. Also different parts of the leaves might express different lectins. Thus it might be that cells nearby the vascular tissue express one or

more of the lectins in a higher value than cells which are at the edge of the leaves. Furthermore it might be of interest if the subcellular expression pattern changes during stress response and pathogen attack. It is likely that cells nearby the infection site change the gene expression of one or more lectins. The expression pattern in vivo will also reveal insight in the behavioural gene expression of *LEC1, LEC2* and *LLP1* during SAR in the local and systemic tissue.

To identify potential co-signalling partners of LEC1, LEC2 and LLP1, Y2H data have to be repeated. In this thesis, primary screening and secondary phenotyping were accomplished as described in 4.10. However, it was not possible to verify the Y2H data by the last verification step. The chosen sequence for the cloning procedure of the vectors for the Y2H assay contained a gene code for a signal peptide. In plants, this signal peptide is most likely to be cleaved before or after the protein reaches its point of destination. Because of that, it is likely that the direct binding of the putative proteins were disturbed by the signal peptide and no clear interaction was detected. Therefore, it might be necessary to generate new DB-X constructs without the according signal peptide as referred in 5.6. It is expected, that XTH25 will be evidenced as putative binding partner for LEC2 and LLP1.

In parallel to Y2H it might be of interest to perform oligosaccharide microarray assays to find putative binding partners. Lectins are putative carbohydrate binding proteins which recognize specific sugar structures and are involved in a variety of biological processes such as cell–cell and host–pathogen interactions as well as innate immune responses (Chandra et al., 2006). To clarify the carbohydrate-binding capacity, recombinant proteins need to be generated and purified from e.g. insect cells in cooperation with the Protein Expression and Purification Facility (PEPF, Helmholtz-Zentrum München). They can then be analysed for their predicted glycan-binding or enzymatic activities, respectively. Therefore, available plant oligosaccharide microarrays (Pedersen et al., 2012) can be used to elucidate if these lectins bind oligosaccharides. The results will provide hints towards the possible substrates of LEC1, LEC2 and LLP1. The lectins may promote SAR by direct or indirect binding to glycan structures accumulating in the cell wall. In local infected tissue it might be possible that they bind glycolipids connected to the apoplastic side of the plasma membrane, which leads to a modification of the membrane permeability or activates signal transporters to release systemic signals. In the systemic uninfected tissue it might be that they are directly or indirectly capable of recognizing the systemic signal which in turn leads to a possible signal cascade that in the end is responsible for the induction of defence genes. By identifying the binding oligosaccharide, the role of *LEC1*, *LEC2* and *LLP1* during SAR might be clearer. One question to be answered can be if the lectins have an active function when bound, or if their purpose during SAR is to release bound oligosaccharides. Such a question might be

answered by this microarray, especially if the identified oligosaccharides are part of already known SAR signals or SAR interactors. Apart from that it is also possible that the identified oligosaccharides are yet unknown to play a role in SAR. Therefore, more detailed research needs to be done which might lead to the identification of new SAR signals or SAR interactors.

In research, crops became more important over the last years as the focus turns also on sustainable agriculture. It would be very interesting to monitor legume lectin like proteins in crops like barley. In barley it is known that systemic resistance is not associated with SA but rather with MeJA and ABA (Dey et al., 2014). In this thesis it was shown that in *A.thaliana* LEC1, LEC2 and LLP1 may act on the interface between SA, JA and ABA networks notably during SAR. Therefore it is likely that also in barley they play a role in systemic resistance also at the interface of the different phytohormones. By silencing *LEC1, LEC2* and *LLP1* in barley or other crop species, it can be clarified if the lectins also play a role in crops and therefore are important for agricultural research.

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# 8. Supplemental Data

## 8.1 Replicate experiments

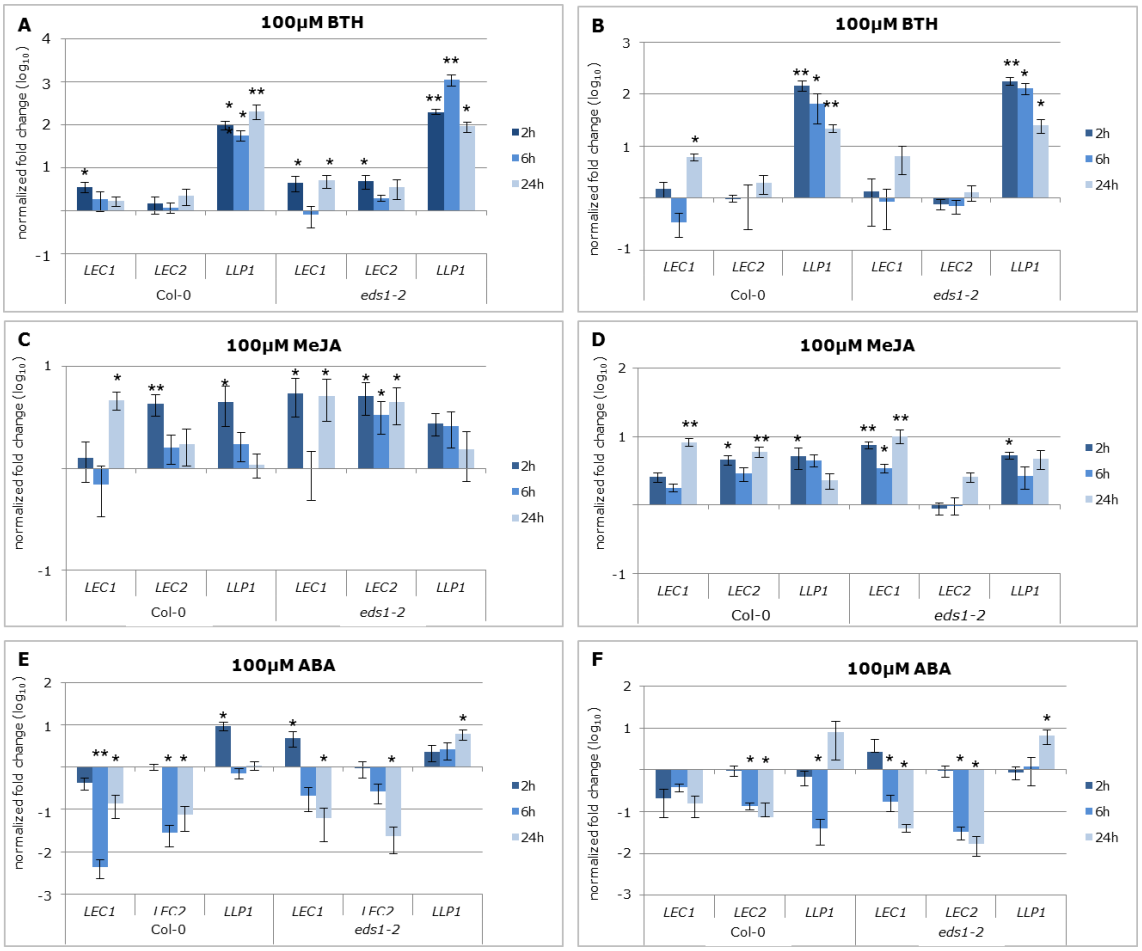


**Figure S1: Replicate experiments of Figure 8; Transcript accumulation of LEC1, LEC2 and LLP1 in Col-0 and eds1-2 after different phytohormone treatments**

16-day-old plants were sprayed with 100µM MeJA, 100µM BTH or 100µM ABA. Aerial parts were harvested 2hpi, 6hpi and 24hpi to monitor the gene expression of *LEC1*, *LEC2* and *LLP1* compared to MOCK treatment and relative to that of *AtTUBULIN*. Asterisks indicate significant differences to MOCK-treated plants.

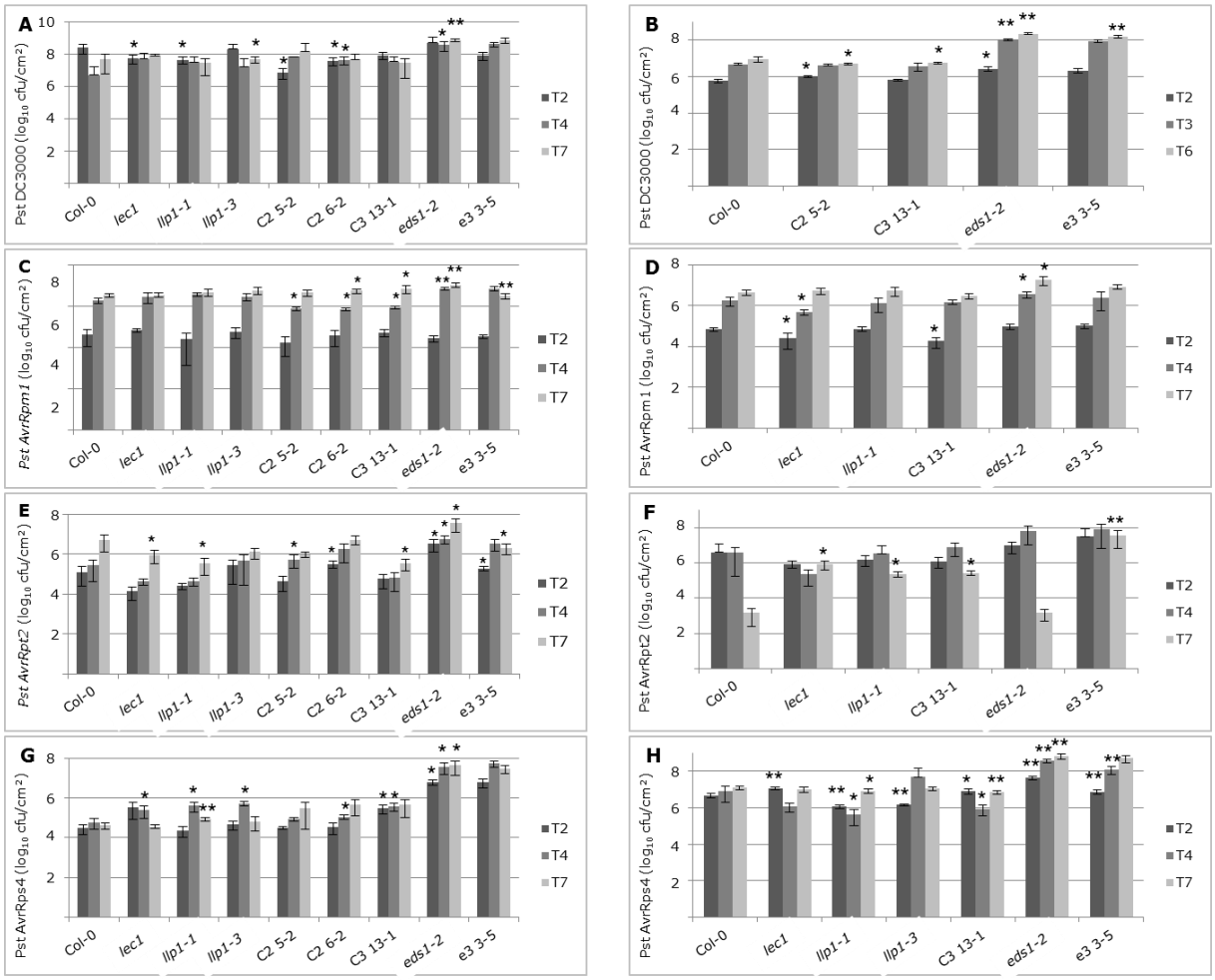


**Figure S2: Replicate experiments of Figure 11; Growth behaviour of different Pseudomonas strains in various *Arabidopsis* mutants and silenced plants**

4-week-old *Arabidopsis* plants were infected with (A,B) *Pst*, (C,D) *Pst AvrRpm1*, (E,F) *Pst AvrRpt2* and (G,H) *Pst AvrRps4.* Bacterial growth was measured at 2, 3 and 7 days after infection (T2, T4, and T7). Asterisks indicate significant differences compared to Col-0 plants for *lec1, llp1-1, llp1-3,* C2 5-2, C2 6-2, C3 13-1 and *eds1-2* and to *eds1-2* for e3 3-5 (*p<0,05 **p<0,01, Student's t test).

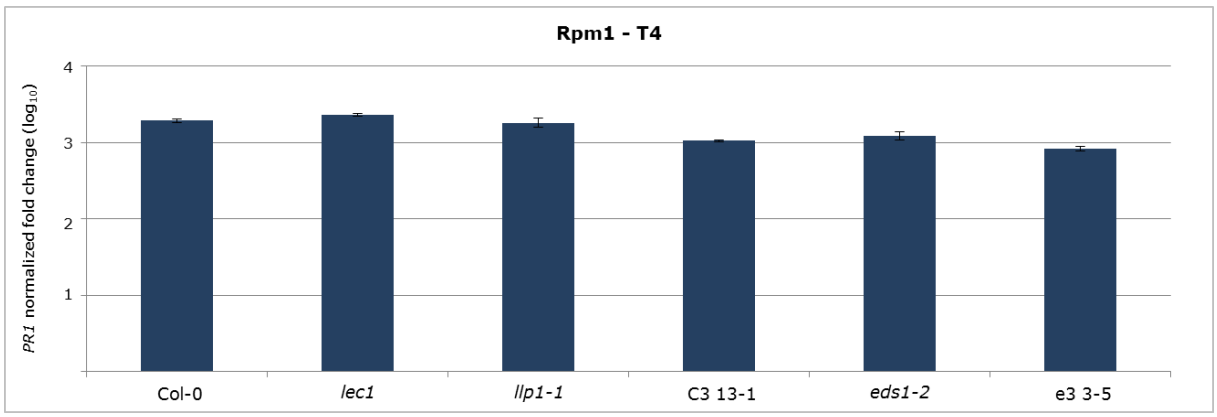


**Figure S3: local *PR1* gene expression 4 days after challenging different *Arabidopsis* mutants and silenced plants with *Pst* AvrRpm1**

*PR1* expression level was measured four days after infection of true leaves with *PstAvrRpm1* (T4) in the infected tissue. Transcript level of *PR1* was normalized to that of *AtUBIQUITIN* and is shown relative to that in untreated Col-0 plants. Experiment was performed once.

## 8.2 List of primers used in this thesis

**Table S1: Primer for localization assay**

| Acronym | Name | 5' 3' | Size (nt) | Annealing Temp (°C) |
|---|---|---|---|---|
| C1 | *At*LEC1 Prom for | CACCAGCAATCATCAAAAGACACAAGGAA | 29 | 60 |
| C2 | *At*LEC1 for | CACCTCCACACTTGAATCACA | 21 | 58 |
| C3 | *At*LEC1 Prom rev | TGTGTTTCTTTTGTTGTTGTTTGTGATTCAAGTGTGG | 38 | 60-70 |
| C4 | *At*LEC1 rev | GTTTTCAAACGACCAGCTC | 19 | 58 |
| C5 | *At*LEC1 STOP rev | TTAGTTTTCAAACGACCAGCTCCA | 24 | 60 |
| C6 | *At*LEC2 Prom for | CACCAAGTCAAAATTTCCGAGGTT | 24 | 60 |
| C7 | *At*LEC2 for | CACCTCAATCTTTCAATCAC | 20 | 52 |
| C8 | *At*LEC2 Prom rev | TGTGTGCGTTTTTTTTTTGGTGTTG | 25 | 61 |
| C9 | *At*LEC2 rev | GTTGTTTTTGGCGGCGTTT | 19 | 52 |
| C10 | *At*LEC2 STOP rev | TTAGTTGTTTTTGGCGGCGTT | 21 | 60 |
| C11 | *At*LLP1 Prom for | CACCGTAGCGTTCAAATCGGCAGT | 20 | 62 |
| C12 | *At*LLP1 for | CACCATGAAGATTCATAAACTCTGTTTTCTT | 31 | 52,3 |
| C13 | *At*LLP1 Prom rev | TGCGTTTTCGTAACTGCTGTTTA | 23 | 60 |
| C14 | *At*LLP1 rev | GATTCTCTTGGCACTGTTCTGGA | 23 | 52,3 |
| C15 | *At*LLP1 STOP rev | TTAGATTCTCTTGGCACTGTTCTGG | 25 | 64 |

**Table S2: Primer for T-DNA insertion knock out mutants**

| Acronym | Primer | 5' 3' | Size (nt) | Annealing Temp (°C) |
|---|---|---|---|---|
| T1 | LBb1(pBIN-pROK2) | GCGTGGACCGCTTGCTGCAACT | 22 | 60 |
| T2 | SALK_032673_RP | AATTCGACAGCAAAGATGTGG | 21 | 63 |
| HB-P2 | SALK_030762 LP | TCCGTGAAGAAAACAAACAAAG | 22 | 64 |
| HB-P3 | SALK_030762 RP | GAGACGAAACCCCATTCTCTC | 21 | 64 |
| HB-P4 | SALK_036814 LP | TTGGGATGCAAAGCAAATTAC | 21 | 60 |
| HB-P5 | SALK_036814 RP | CTTTCTCAGCAACAACGGAAG | 21 | 60 |
| T3 (xth) | SALK_204573_LP | AACCAACACATCATCAATGGC | 21 | 60 |
| T4 (xth) | SALK_204573_RP | TCATCTCTGGTTCGATCCAAC | 21 | 60 |

**Table S3: Primer for sequencing**

| Acronym | Primer | 5' 3' | Size (nt) | Annealing Temp (°C) |
|---|---|---|---|---|
| S1 | LEC1 S1rev | GTCTGCAACTTATTTTGTCTTGTGAG | 26 | 60 |
| S2 | LEC1 S2rev | CTTCGTCTCGCCCTCTTGTT | 20 | 60 |
| S3 | LEC1 S3rev | CAGCGACAAGAGAAGTCATCG | 21 | 60 |
| S4 | LEC2 S1rev | ACACTCCAAGGAAGAAGGTTGA | 22 | 60 |
| S5 | LEC2 S2rev | CACAAAGGGCCTAGCAGTGA | 20 | 60 |
| S6 | LEC2 S3rev | TGTGGTTCTCTGGCTTTCCA | 20 | 60 |
| S7 | LLP1 S1rev | CTCTTAGGCACGATCCGCAA | 20 | 60 |
| S8 | LLP1 S2rev | ACCCACCACATAGACAACAC | 20 | 58 |
| S9 | LLP1 S3rev | TGAAAGGGACTGGAGTGGAC | 20 | 58 |
| | M13 uni (-21) | TGT AAA ACG ACG GCC AGT | 18 | |
| | M13 rev (-29) | CAG GAA ACA GCT ATG ACC | 18 | |

**Table S4: Primer for qPCR**

| Acronym | Primer | 5' 3' | Size (nt) | Annealing Temp (°C) |
|---|---|---|---|---|
| Q1 | qP LEC1 for | TTTGGAGCTGGTCGTTTGAA | 20 | 60 |
| Q2 | qP LEC1 rev | ATTCACTCTACAACAATTAC | 20 | 60 |
| Q3 | qP LEC2 for | TTTGGAGCTGGTCGTTTGAA | 20 | 60 |
| Q4 | qP LEC2 rev | AGTTACCACTGAGTAGTATG | 20 | 60 |
| Q5 | qP LLP1 for | TGAGTAAACAGCAGTTACGA | 20 | 60 |
| Q6 | qP LLP1 rev | TGACGCCATCAGAAGCAGGA | 20 | 60 |
| Q7 | qP Tubulin for | GTACCTTGAAGCTTGCTAATCCTA | 24 | 60 |
| Q8 | qP Tubulin rev | GTCAAAGGTGCAAAACCAAC | 20 | 60 |
| Q9 | qP PR1 for | CTACGCAGAACAACTAAGAGGCAAC | 25 | 60 |
| Q10 | qP PR1 rev | TTGGCACATCCGAGTCTCACTG | 22 | 60 |
| Q11 | qP ABI1 for | AAGCGGATTCTCTTGTGGCA | 20 | 60 |
| Q12 | qP ABI1 rev | AAATACTCAGCCGCGGACAT | 20 | 60 |
| Q13 | qP PDF1.2. for | CCAAGTGGGACATGGTCAG | 19 | 60 |
| Q14 | qP PDF1.2. rev | ACTTGTGTGCTGGGAAGACA | 20 | 60 |
| Q15 | qP RAB18 for | TTCGGTCGTTGTATTGTGCTTT | 22 | 60 |
| Q16 | qP RAB18 rev | CCAGATGCTCATTACACACTCATG | 24 | 60 |

**Table S5: Primer for multiple silencing constructs**

| Acronym | Primer | 5′ 3′ | Size (nt) | Annealing Temp (°C) |
|---|---|---|---|---|
| HB-P18 | RNAi_2er_ *At*AED9-1 F | CACCCTCGAGGGATCCACTTCGATTCCTTCGATGGC | | 59.5 |
| HB-P19 | RNAi_2er_ *At*AED9-1 R | GATATCCGCCATCTGAAGCACCGGGCTGGGACAATGACGAAG | 279 | 59.5 |
| HB-P20 | RNAi_2er_ *At*AED9-2 F | CTTCGTCATTGTCCCAGCCCGGTGCTTCAGATGGCGGATATC | | 59.5 |
| HB-P21 | RNAi_2er_ *At*AED9-2 R | GGTACCAAGCTTTCAGGCGCGAGTGTAACC | 319 | 59.5 |
| HB-P22 | RNAi_3er_ *At*AED9-1 F | CACCCTCGAGGGATCCACTTCGATTCCTTCGATGGC | | 59.5 |
| HB-P23 | RNAi_3er_ *At*AED9-1 R | CCGCCATCTGAAGCACCGGGGCTGGGACAATGACGAAG | 279 | 59.5 |
| HB-P24 | RNAi_3er_ *At*AED9-2 F | CTTCGTCATTGTCCCAGCCCCGGTGCTTCAGATGGCGG | | 59.5 |
| HB-P25 | RNAi_3er_ *At*AED9-2 R | CGGCGGAAGTGGTGTGAGCTTCAGGCGCGAGTGTAACCG | 319 | 59.5 |
| HB-P26 | RNAi_3er_ *At*AED9-3 F | CGGTTACACTCGCGCCTGAAGCTCACACCACTTCCGCCG | | 59.5 |
| HB-P27 | RNAi_3er_ *At*AED9-3 R | GGTACCAAGCTTAAGCAAGGCCGTGACCAGGG | 284 | 59.5 |
| HB-P18 | RNAi_2er_ *At*AED9-1 F | CACCCTCGAGGGATCCACTTCGATTCCTTCGATGGC | | 59.5 |
| HB-P19 | RNAi_2er_ *At*AED9-1 R | GATATCCGCCATCTGAAGCACCGGGCTGGGACAATGACGAAG | 279 | 59.5 |
| HB-P20 | RNAi_2er_ *At*AED9-2 F | CTTCGTCATTGTCCCAGCCCGGTGCTTCAGATGGCGGATATC | | 59.5 |
| HB-P21 | RNAi_2er_ *At*AED9-2 R | GGTACCAAGCTTTCAGGCGCGAGTGTAACC | 319 | 59.5 |
| HB-P22 | RNAi_3er_ *At*AED9-1 F | CACCCTCGAGGGATCCACTTCGATTCCTTCGATGGC | | 59.5 |
| HB-P23 | RNAi_3er_ *At*AED9-1 R | CCGCCATCTGAAGCACCGGGGCTGGGACAATGACGAAG | 279 | 59.5 |

## 8.3 Additional information to the native promoter lines

**Table S6: Overview of transgenic plants; the vector constructs and their parental lines**

| | LEC1::*LEC1* | LEC1::*LEC1-GFP* | LEC1::*GFP* | LEC2::*LEC2-GFP* | LEC2::*GFP* | LLP1::*LLP1* | LLP1::*LLP1-GFP* | LLP1::*GFP* |
|---|---|---|---|---|---|---|---|---|
| Col-0 | | **X** | **X** | | **X** | | | **X** |
| *lec1* | **X** | **X** | | **X** | | | | |
| *llp1-1* | | | | | | **X** | **X** | |

**Table S7: DNA sequence used for cloning**

Promoter region is shaded in grey. Attached sequences are shown in small letters. The linker sequence is in grey font colour and GFP sequence is shaded in green.

| | |
|---|---|
| LEC1::*LEC1* | AGCAATCATCAAAAGACACAAGGAAAGTTTTGTGATTTTAATTAATATTAAGGATAATGACTTT<br>TCCCATCATACAAGAGAATCAATGAACATATCCTTTTTGTGACAAACTAACATTGTTCTACTTC<br>TTCTACTTAACTTAAGGTTTGTCCTCTAAAGCCACCAACGATCTGATAACGCCAGGAGAAATA<br>CCATCAGCAAGAGCCCCTAGTTCCGATATAAACAGAGCTTTTTCCTTCACTAAGCTCTGTAAC<br>TTCTTCGATTCAAACTCCATTTTCTCTTCATCTTCACATAGTAACAAGTAGGCCGTTTTCTCAT<br>GAGGAAACTGCACTAGTCTCTAAATGAGAGTAGCAAATAAATGTAAACAAAGATGATACCTTT<br>CTCTAGCATTGTATGCACAGCATGAAACGGAATTGCAGCAAAAAGATCAGCTCCTGGGAGCA<br>TCATCCAGGTGGGTTCACTAGAGTCATGGGATCCACATGTGGAGCAAACTTCTTGGACCAAA<br>GGTTTCGTTGAGCTCCCATGGATATCCTTCATCATAGAATCAAATGGAGACATAACACTAGTC<br>TTTGTTGTCCGAGCTCTCTTCCTTAGTGCTGTAAGAGCCTCTCGGTTTGCATTCCTCTCCTTGT<br>CATTCTCCACCATCTATTACACATATTACAAGCTCCATTTTGGTTAAAAAAACTTATTGACCTA<br>ACAACAATACATATCATATAATATCTGTTTTCTCACAAGACAAAATAAGTTGCAGACAACAATA<br>GATTGAAAAATCTTGTGCTTTCCTCAATCTGTCAATCACATATAAATTACGAAGTAGTGAATAT<br>TTCCAACTAGATACTACCAAAGCAGATTCCCGAGTACCTGATTTCGAGCTAATAGAAACTCAT<br>CAGCTTCAGCTTCAATTTTGTCCAATAGTGCAGCAAACTTCTTTGGATCCAAATCCATCTCTAG<br>TCTAACAGATTCCAACAAACAAATAGGCTCAGAATCAAATAAAGCATTATTGGTTGAAGGGGA<br>AAAAGGAAAAGATTTGATAAGTGGTATAGTCAATCTTCCTAATCCTGTACATCTCCATGTTAAC<br>CTACATCTTCATGCACAAATCAAACCACAACGAAGCCAGAAAAAAAGTGAAGCTATGGATACT<br>TTGTTTGCTTAAGCTCAATTTCAAAGACTCTAATTAGTACCATAAGCCACTCCATGTTTTCGTT<br>GTATGTATGTACCAACATCATACTAAACCTTTCACCATCGTTTCAGCTTCTATTCTATAGAGTG<br>ATTATTGAATTCCCAAATTTTTCAATATTGATACGATTAATGATTTCATCCAATTTCAGAGAAAC<br>GATTATAGAAATTAACAATTCTGATAAACCCTAAATGGTTAAAACCCTGAATTCCGAAACCCTA<br>AAACCTAATTCACAAAATCTCTCGTTCCTACGATGCAATAACGAAGGCAACCACAACACCAAC |

| | |
|---|---|
| | AAAAGTGTAAAACGATTCTTTAATTCATTTTTGCAGCATCTTAACAAGAGGGCGAGACGAAGT<br>AACTCAGAAACAAAATAATAACCTTTAAATGGATTTACAGACGAAGATTTAATAAGCAGAGGT<br>CCGGTGAGAATCTCGGTGACGCAATGAGAGGGACGAAGTTTATTAACAATATAAATTACATT<br>AAACCCCCGAGTTGAGTTATGGGCTAGGTATTTGGGCTATAAAAGGCCCGGCCCACTAATCA<br>TACCATTGAAAGGTGCGATCGTTCCCGCCGTCGTATCGTAGCACAAGAAAATGTAAATTTTAT<br>TTATTTGGTATTTAAAAATATACCTATTTAATAATTTGAGATGATAAAGTCATAAAACAGAGTA<br>ACTTTTTTGCATCCAAAAACGAGTTCGTGATATCAACTAAGCTTGTCAATGTGTTTTTTTGTAC<br>ACGAGACATTAAAATTCACAATGTTTCGTAGAGAAGGGGACAAGAAACACATAATATAATCAA<br>CCAATTATTATTACTCGAATCCATTATAATATTTTTTCAAAAGGATAAGATTTTGTTCATTGAAA<br>GACATCTGGGTGGGTCCATTTCACTGCTAAGCCCCTTGTGGTGGCGTCTCCAACGCATCCAC<br>TTGGCATTGTTTATTTGACCACTTTTTCGAATCATAGATTTTGAATATTCAAGCTCGATGGCTT<br>ATGTTAACTCTTATATAAACATATTATCAACCTCCAAATATTTTCATCATCAATCTTCTCCACAC<br>TTGAATCACAAACAACAACAAAAGAAACACAATGCAGATTCACAAACTCTGTTTTCTTGCTCT<br>GTTCTTAGCTAATGCAGCTTTTGCCGTCAAATTCAACTTCGATTCCTTCGATGGCAGCAACTT<br>GTTATTCCTTGGAGACGCTGAGCTTGGTCCTTCCTCTGATGGCGTAAGCCGATCCGGAGCTC<br>TTTCCATGACCCGAGACGAAACCCCATTCTCTCATGGTCAGGGTCTTTACATCAATCCAATCC<br>AATTCAAATCTTCAAACACTTCTTCTCCTTTTGATTTTAAAACTTCTTTCACTTTCTCCATCACT<br>CCTCGCACCAAACCTAATTCTGGTCAAGGCCTTGCCTTCGTCATTGTCCCAGCCGCTGATAAC<br>TCCGGCGCTTCCGGCGGAGGATATCTCGGAATCCTCAACAAAACCAACGATGGAAAGTCAGA<br>AAACAACCTCATATTTATCGAATTCGACACGTTTAAGAACAATGAGTCTAATGACATTAGTGG<br>TAACCATGTCGGAATCAACATCAACTCGATGACTTCTCTTGTCGCTGAGAAAGCTGGTTATTG<br>GGTTCAGACATTAGTCGGAAAAAGAAAAGTTTGGTCGTTTAAAGATGTGAATCTTAGCAGTG<br>GAGAGAGGTTCAAGGCTTGGATCGAGTTCAGAAGCAAAGACTCTAGGAATACCATTACCATC<br>GCCCCTGAAAACGTTAAGAAACCGAAGAGGCCTTTGATCCAAGGTTCGAGAGTACTCAATGA<br>TGTTCTTCTTCAAAACATGTACGCTGGTTTTGCTGGTTCCATGGGACGTGCCGGTGAGCGTC<br>ACGATGTTTGGAGCTGGTCGTTTGAAAACTAA |
| LEC1::*LEC1-GFP* | AGCAATCATCAAAAGACACAAGGAAAGTTTTGTGATTTTAATTAATATTAAGGATAATGACTTT<br>TCCCATCATACAAGAGAATCAATGAACATATCCTTTTTGTGACAAACTAACATTGTTCTACTTC<br>TTCTACTTAACTTAAGGTTTGTCCTCTAAAGCCACCAACGATCTGATAACGCCAGGAGAAATA<br>CCATCAGCAAGAGCCCCTAGTTCCGATATAAACAGAGCTTTTTCCTTCACTAAGCTCTGTAAC<br>TTCTTCGATTCAAACTCCATTTTCTCTTCATCTTCACATAGTAACAAGTAGGCCGTTTTCTCAT<br>GAGGAAACTGCACTAGTCTCTAAATGAGAGTAGCAAATAAATGTAAACAAAGATGATACCTTT<br>CTCTAGCATTGTATGCACAGCATGAAACGGAATTGCAGCAAAAAGATCAGCTCCTGGGAGCA<br>TCATCCAGGTGGGTTCACTAGAGTCATGGGATCCACATGTGGAGCAAACTTCTTGGACCAAA<br>GGTTTCGTTGAGCTCCCATGGATATCCTTCATCATAGAATCAAATGGAGACATAACACTAGTC<br>TTTGTTGTCCGAGCTCTCTTCCTTAGTGCTGTAAGAGCCTCTCGGTTTGCATTCCTCTCCTTGT<br>CATTCTCCACCATCTATTACACATATTACAAGCTCCATTTTGGTTAAAAAAACTTATTGACCTA<br>ACAACAATACATATCATATAATATCTGTTTTCTCACAAGACAAAATAAGTTGCAGACAACAATA<br>GATTGAAAAATCTTGTGCTTTCCTCAATCTGTCAATCACATATAAATTACGAAGTAGTGAATAT<br>TTCCAACTAGATACTACCAAAGCAGATTCCCGAGTACCTGATTTCGAGCTAATAGAAACTCAT<br>CAGCTTCAGCTTCAATTTTGTCCAATAGTGCAGCAAACTTCTTTGGATCCAAATCCATCTCTAG<br>TCTAACAGATTCCAACAAACAAATAGGCTCAGAATCAAATAAAGCATTATTGGTTGAAGGGGA |

AAAAGGAAAAGATTTGATAAGTGGTATAGTCAATCTTCCTAATCCTGTACATCTCCATGTTAAC
CTACATCTTCATGCACAAATCAAACCACAACGAAGCCAGAAAAAAAGTGAAGCTATGGATACT
TTGTTTGCTTAAGCTCAATTTCAAAGACTCTAATTAGTACCATAAGCCACTCCATGTTTTCGTT
GTATGTATGTACCAACATCATACTAAACCTTTCACCATCGTTTCAGCTTCTATTCTATAGAGTG
ATTATTGAATTCCCAAATTTTTCAATATTGATACGATTAATGATTTCATCCAATTTCAGAGAAAC
GATTATAGAAATTAACAATTCTGATAAACCCTAAATGGTTAAAACCCTGAATTCCGAAACCCTA
AAACCTAATTCACAAAATCTCTCGTTCCTACGATGCAATAACGAAGGCAACCACAACACCAAC
AAAAGTGTAAAACGATTCTTTAATTCATTTTTGCAGCATCTTAACAAGAGGGCGAGACGAAGT
AACTCAGAAACAAAATAATAACCTTTAAATGGATTTACAGACGAAGATTTAATAAGCAGAGGT
CCGGTGAGAATCTCGGTGACGCAATGAGAGGGACGAAGTTTATTAACAATATAAATTACATT
AAACCCCCGAGTTGAGTTATGGGCTAGGTATTTGGGCTATAAAAGGCCCGGCCCACTAATCA
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ACGAGACATTAAAATTCACAATGTTTCGTAGAGAAGGGGACAAGAAACACATAATATAATCAA
CCAATTATTATTACTCGAATCCATTATAATATTTTTTCAAAAGGATAAGATTTTGTTCATTGAAA
GACATCTGGGTGGGTCCATTTCACTGCTAAGCCCCTTGTGGTGGCGTCTCCAACGCATCCAC
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GTTCTTAGCTAATGCAGCTTTTGCCGTCAAATTCAACTTCGATTCCTTCGATGGCAGCAACTT
GTTATTCCTTGGAGACGCTGAGCTTGGTCCTTCCTCTGATGGCGTAAGCCGATCCGGAGCTC
TTTCCATGACCCGAGACGAAACCCCATTCTCTCATGGTCAGGGTCTTTACATCAATCCAATCC
AATTCAAATCTTCAAACACTTCTTCTCCTTTTGATTTTAAAACTTCTTTCACTTTCTCCATCACT
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TCCGGCGCTTCCGGCGGAGGATATCTCGGAATCCTCAACAAAACCAACGATGGAAAGTCAGA
AAACAACCTCATATTTATCGAATTCGACACGTTTAAGAACAATGAGTCTAATGACATTAGTGG
TAACCATGTCGGAATCAACATCAACTCGATGACTTCTCTTGTCGCTGAGAAAGCTGGTTATTG
GGTTCAGACATTAGTCGGAAAAAGAAAAGTTTGGTCGTTTAAAGATGTGAATCTTAGCAGTG
GAGAGAGGTTCAAGGCTTGGATCGAGTTCAGAAGCAAAGACTCTAGGAATACCATTACCATC
GCCCCTGAAAACGTTAAGAAACCGAAGAGGCCTTTGATCCAAGGTTCGAGAGTACTCAATGA
TGTTCTTCTTCAAAACATGTACGCTGGTTTTGCTGGTTCCATGGGACGTGCCGGTGAGCGTC
ACGATGTTTGGAGCTGGTCGTTTGAAAACcttgtacaaagtggtgatatcccgcggatggtgagcaagggc
gaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgtccg
gcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgccct
ggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgacttc
ttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgcg
ccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaac
atcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatc
aaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactaccagcagaacaccccc
atcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtccgccctgagcaaagaccccaacgag
aagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaag

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|---|---|
| LEC1::*GFP* | AGCAATCATCAAAAGACACAAGGAAAGTTTTGTGATTTTAATTAATATTAAGGATAATGACTTT<br>TCCCATCATACAAGAGAATCAATGAACATATCCTTTTTGTGACAAACTAACATTGTTCTACTTC<br>TTCTACTTAACTTAAGGTTTGTCCTCTAAAGCCACCAACGATCTGATAACGCCAGGAGAAATA<br>CCATCAGCAAGAGCCCCTAGTTCCGATATAAACAGAGCTTTTTCCTTCACTAAGCTCTGTAAC<br>TTCTTCGATTCAAACTCCATTTTCTCTTCATCTTCACATAGTAACAAGTAGGCCGTTTTCTCAT<br>GAGGAAACTGCACTAGTCTCTAAATGAGAGTAGCAAATAAATGTAAACAAAGATGATACCTTT<br>CTCTAGCATTGTATGCACAGCATGAAACGGAATTGCAGCAAAAAGATCAGCTCCTGGGAGCA<br>TCATCCAGGTGGGTTCACTAGAGTCATGGGATCCACATGTGGAGCAAACTTCTTGGACCAAA<br>GGTTTCGTTGAGCTCCCATGGATATCCTTCATCATAGAATCAAATGGAGACATAACACTAGTC<br>TTTGTTGTCCGAGCTCTCTTCCTTAGTGCTGTAAGAGCCTCTCGGTTTGCATTCCTCTCCTTGT<br>CATTCTCCACCATCTATTACACATATTACAAGCTCCATTTTGGTTAAAAAAACTTATTGACCTA<br>ACAACAATACATATCATATAATATCTGTTTTCTCACAAGACAAAATAAGTTGCAGACAACAATA<br>GATTGAAAAATCTTGTGCTTTCCTCAATCTGTCAATCACATATAAATTACGAAGTAGTGAATAT<br>TTCCAACTAGATACTACCAAAGCAGATTCCCGAGTACCTGATTTCGAGCTAATAGAAACTCAT<br>CAGCTTCAGCTTCAATTTTGTCCAATAGTGCAGCAAACTTCTTTGGATCCAAATCCATCTCTAG<br>TCTAACAGATTCCAACAAACAAATAGGCTCAGAATCAAATAAAGCATTATTGGTTGAAGGGGA<br>AAAAGGAAAAGATTTGATAAGTGGTATAGTCAATCTTCCTAATCCTGTACATCTCCATGTTAAC<br>CTACATCTTCATGCACAAATCAAACCACAACGAAGCCAGAAAAAAAGTGAAGCTATGGATACT<br>TTGTTTGCTTAAGCTCAATTTCAAAGACTCTAATTAGTACCATAAGCCACTCCATGTTTTCGTT<br>GTATGTATGTACCAACATCATACTAAACCTTTCACCATCGTTTCAGCTTCTATTCTATAGAGTG<br>ATTATTGAATTCCCAAATTTTTCAATATTGATACGATTAATGATTTCATCCAATTTCAGAGAAAC<br>GATTATAGAAATTAACAATTCTGATAAACCCTAAATGGTTAAAACCCTGAATTCCGAAACCCTA<br>AAACCTAATTCACAAAATCTCTCGTTCCTACGATGCAATAACGAAGGCAACCACAACACCAAC<br>AAAAGTGTAAAACGATTCTTTAATTCATTTTTGCAGCATCTTAACAAGAGGGCGAGACGAAGT<br>AACTCAGAAACAAAATAATAACCTTTAAATGGATTTACAGACGAAGATTTAATAAGCAGAGGT<br>CCGGTGAGAATCTCGGTGACGCAATGAGAGGGACGAAGTTTATTAACAATATAAATTACATT<br>AAACCCCCGAGTTGAGTTATGGGCTAGGTATTTGGGCTATAAAAGGCCCGGCCCACTAATCA<br>TACCATTGAAAGGTGCGATCGTTCCCGCCGTCGTATCGTAGCACAAGAAAATGTAAATTTTAT<br>TTATTTGGTATTTAAAAATATACCTATTTAATAATTTGAGATGATAAAGTCATAAAACAGAGTA<br>ACTTTTTTGCATCCAAAAACGAGTTCGTGATATCAACTAAGCTTGTCAATGTGTTTTTTTGTAC<br>ACGAGACATTAAAATTCACAATGTTTCGTAGAGAAGGGGACAAGAAACACATAATATAATCAA<br>CCAATTATTATTACTCGAATCCATTATAATATTTTTTCAAAAGGATAAGATTTTGTTCATTGAAA<br>GACATCTGGGTGGGTCCATTTCACTGCTAAGCCCCTTGTGGTGGCGTCTCCAACGCATCCAC<br>TTGGCATTGTTTATTTGACCACTTTTTCGAATCATAGATTTTGAATATTCAAGCTCGATGGCTT<br>ATGTTAACTCTTATATAAACATATTATCAACCTCCAAATATTTTCATCATCAATCTTCTCCACAC<br>TTGAATCACAAACAACAACAAAAGAAACACActtgtacaaagtggtgatatcccgcggatggtgagcaagg<br>gcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtgtc<br>cggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtgcc<br>ctggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctaccccgaccacatgaagcagcacgact<br>tcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagacccgc<br>gccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgacttcaaggaggacggcaa<br>catcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcat |

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|---|---|
| | caaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactaccagcagaacacccc<br>catcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtccgccctgagcaaagaccccaacga<br>gaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcggcatggacgagctgtacaag |
| LEC2::*LEC2-GFP* | CACCAAGTCAAAATTTCCGAGGTTTGCATCTTTCTTTGTCCACGATACTATTTGTTTTTTTTTC<br>TCTTTAATTCAATCATGTTTAATTTGAATCTGAGTTTGGTAGAAAGAATGAGGATATGTAGAGC<br>TACTAAAGATGCTATTTTTCTTAACAATAAAAGCATGTTTAGTTTAAAAATATGTCTTCTTTTCA<br>ATATAACATTTAGGCATTGCACTTAGAGAGAAATTGTTTCTTGGTGTAGAGATTAGCAGATGA<br>CATCAACGAAGGATACCAAAGTTTATATGGTGCTCAGGTAATTTATTTATTACTTGTTCTTGAG<br>AGTTTCTTGGTGAAAGCTAACTGAAACGCCATTAGTTTGTCTCAACTTTGAATCTAACGATAA<br>AAACGATAGAAAGATAAACATGGTTCCAAATTTTGTGCAATTATTATGTCTAGGAAAAGATAT<br>TAATAGTTGTGGTGGGAACAGGTGGAGAAAGTGAACGGAGTGGAAGTAAAGAATCTTAAGC<br>ATCTATGCGAGCTAATAGAGGAATGTAGCACCGAAGATTTGAGGCTGGAGTTTAAAAATCAT<br>AAGGTTTTGGTCCTCAACTATGAATCTGCTAAAAAGGCAACGTTACAAATCTTGGAACGTCAC<br>AAAATAAAGTCGGTCATATCAAAGGACATTTGTCTTCCTATGTTGCTTGATGATCCGTTCAAA<br>GACAATAAGATCAACCTTCTTCCTTGGAGTGTTCTTCCTTTGATGTTTGATTTTTCCTAATAAA<br>CAAGGATTGATTTCAAAAGGAGTTACATGACTAGCTATCACATGGAGAATATAATGAAAGAAT<br>GAAGACCATTCATACGTAGAAGAAGACATCTTCCAAGTTAAACTAAATAAACATATGTGTGTT<br>CTTATTTTTCTCAACGGATATGTGTTTTAATTGAGGTTTGTAATTGGTTTAAGAGAAAGTGTTT<br>TCTTATCAATGGTTTTTTTTTTTTTTATGTTTGTTGAATCAAAGAGATTTCACGTAAAAGTTAA<br>AACGTGTTATCTTAATTATTAGCATGATTACTTTTATCTTTCTTTTTTATCAAAAAACTTTTTAAT<br>GAGATTGCCAAACGTTTTTTTGCATCCACGTAAACTCCTCTGAACGTAAACTTTTCCAGAGTG<br>TATTTATGTGGACGTCAGAAAAACGTATTTTGCATCCACGTAACGAAAATAGTTCATATAAACT<br>AAAAGTGGCTATGTCTTACAGATATGTACACGACGCATGAAATTCGTAATGTTTCGTAGAGAA<br>AGGGGACAAGAAAATATAATTAATCAATTATTATTCAAAACAATTTATAATCGATTTTTTTTTTT<br>TCTTATAATTAATTATTACTCGAATCAATTATATTTTATCAAAAGAATAAAATTAATTTGTTCAT<br>GACATCTGATGGGTCCATTTTCACTGCTAGGCCCTTTGTGGTGGCGTCCGATCTGAATCGAA<br>TCCACTTGGCTTAATTTATTTGACCATTTTTCGAAGTATAAATTTGAATATTCAAACTCGATGG<br>CTTACGTTATCCCTTATATAAACACATTATCAACCTCCAAATATTTTCTCTTGGCTATCGAAATC<br>ATCAATCTTTCAATCACAAAAAACAACAACACCAAAAAAAAAACGCACACAATGCAGATTCAC<br>AAACTCTGTTTTCTTGTTCTGTTCTTAGCTAACGCAGCTTTTGCCGTCAAATTCAACTTCGATT<br>CCTTCGATGGCAGCAACTTGTTATTCCTAGGAGACGCAGAGCTTGGTCCTTCCTCTGATGGT<br>GTAAGCCGATCCGGAGCTTTATCCATGACCCGAGACGAGAACCCATTCTCTCATGGTCAAGG<br>TCTTTACATCAATCAAATCCCATTCAAACCTTCAAACACTTCTTCTCCTTTTTCATTTGAAACTT<br>CTTTCACTTTCTCCATCACTCCTCGCACCAAACCTAACTCCGGTCAAGGTTTCGCCTTCATCAT<br>AACCCCGGAAGCTGATAACTCCGGTGCTTCAGATGGCGGATATCTCGGAATCCTCAACAAAA<br>CCAACGATGGAAAGCCAGAGAACCACATCTTGGCTATCGAATTCGATACTTTTCAGAACAAA<br>GAGTTTCTAGACATTAGTGGTAACCATGTTGGAGTTAACATCAACTCAATGACTTCTCTTGTC<br>GCTGAGAAAGCTGGTTACTGGGTTCAGACAAGAGTCGGGAAAAGGAAAGTTTGGTCGTTTAA<br>AGATGTGAATCTTAGCAGTGGAGAGAGGTTCAAGGCTTGGGTTGAGTTCAGAAACAAAGACT<br>CTACGATTACGGTTACACTCGCGCCTGAAAACGTTAAGAAACCTAAGCGGGCTTTGATCGAA |

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|---|---|
| | GCTCCCAGAGTGCTCAATGAAGTTCTTCTTCAAAACATGTACGCCGGTTTTGCTGGTTCCATG<br>GGACGTGCCGTTGAGCGTCACGATATTTGGAGCTGGTCGTTTGAAAACGCCGCCAAAAACAA<br>Ccttgtacaaagtggtgatatcccgcggatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtc<br>gagctggacggcgacgtaaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagct<br>gaccctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcgtgc<br>agtgcttcagccgctaccccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggag<br>cgcaccatcttcttcaaggacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaac<br>cgcatcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacag<br>ccacaacgtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgagga<br>cggcagcgtgcagctcgccgaccactaccagcagaacaccccccatcggcgacggccccgtgctgctgcccgacaacca<br>ctacctgagcacccagtccgccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgacc<br>gccgccgggatcactctcggcatggacgagctgtacaag |
| LEC2::*GFP* | CACCAAGTCAAAATTTCCGAGGTTTGCATCTTTCTTTGTCCACGATACTATTTGTTTTTTTTTTC<br>TCTTTAATTCAATCATGTTTAATTTGAATCTGAGTTTGGTAGAAAGAATGAGGATATGTAGAGC<br>TACTAAAGATGCTATTTTTCTTAACAATAAAAGCATGTTTAGTTTAAAAATATGTCTTCTTTTCA<br>ATATAACATTTAGGCATTGCACTTAGAGAGAAATTGTTTCTTGGTGTAGAGATTAGCAGATGA<br>CATCAACGAAGGATACCAAAGTTTATATGGTGCTCAGGTAATTTATTTATTACTTGTTCTTGAG<br>AGTTTCTTGGTGAAAGCTAACTGAAACGCCATTAGTTTGTCTCAACTTTGAATCTAACGATAA<br>AAACGATAGAAAGATAAACATGGTTCCAAATTTTGTGCAATTATTATGTCTAGGAAAAGATAT<br>TAATAGTTGTGGTGGGAACAGGTGGAGAAAGTGAACGGAGTGGAAGTAAAGAATCTTAAGC<br>ATCTATGCGAGCTAATAGAGGAATGTAGCACCGAAGATTTGAGGCTGGAGTTTAAAAATCAT<br>AAGGTTTTGGTCCTCAACTATGAATCTGCTAAAAAGGCAACGTTACAAATCTTGGAACGTCAC<br>AAAATAAAGTCGGTCATATCAAAGGACATTTGTCTTCCTATGTTGCTTGATGATCCGTTCAAA<br>GACAATAAGATCAACCTTCTTCCTTGGAGTGTTCTTCCTTTGATGTTTGATTTTTCCTAATAAA<br>CAAGGATTGATTTCAAAAGGAGTTACATGACTAGCTATCACATGGAGAATATAATGAAAGAAT<br>GAAGACCATTCATACGTAGAAGAAGACATCTTCCAAGTTAAACTAAATAAACATATGTGTGTT<br>CTTATTTTTCTCAACGGATATGTGTTTTAATTGAGGTTTGTAATTGGTTTAAGAGAAAGTGTTT<br>TCTTATCAATGGTTTTTTTTTTTTTTTATGTTTGTTGAATCAAAGAGATTTCACGTAAAAGTTAA<br>AACGTGTTATCTTAATTATTAGCATGATTACTTTTATCTTTCTTTTTTATCAAAAAACTTTTTAAT<br>GAGATTGCCAAACGTTTTTTTGCATCCACGTAAACTCCTCTGAACGTAAACTTTTCCAGAGTG<br>TATTTATGTGGACGTCAGAAAAACGTATTTTGCATCCACGTAACGAAAATAGTTCATATAAACT<br>AAAAGTGGCTATGTCTTACAGATATGTACACGACGCATGAAATTCGTAATGTTTCGTAGAGAA<br>AGGGGACAAGAAAATATAATTAATCAATTATTATTCAAAACAATTTATAATCGATTTTTTTTTTT<br>TCTTATAATTAATTATTACTCGAATCAATTATATTTTATCAAAAGAATAAAATTAATTTGTTCAT<br>GACATCTGATGGGTCCATTTTCACTGCTAGGCCCTTTGTGGTGGCGTCCGATCTGAATCGAA<br>TCCACTTGGCTTAATTTATTTGACCATTTTTCGAAGTATAAATTTGAATATTCAAACTCGATGG<br>CTTACGTTATCCCTTATATAAACACATTATCAACCTCCAAATATTTTCTCTTGGCTATCGAAATC<br>ATCAATCTTTCAATCACAAAAAACAACAACACCAAAAAAAAAACGCACACActtgtacaaagtggtg<br>atatcccgcggatggtgagcaagggcgaggagctgttcaccggggtggtgcccatcctggtcgagctggacggcgacg<br>taaacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctg<br>caccaccggcaagctgcccgtgccctggcccaccctcgtgaccaccctgacctacggcgtgcagtgcttcagccgctacc<br>ccgaccacatgaagcagcacgacttcttcaagtccgccatgcccgaaggctacgtccaggagcgcaccatcttcttcaag |

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|---|---|
| | gacgacggcaactacaagacccgcgccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaaggg<br>catcgacttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatg<br>gccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgc<br>cgaccactaccagcagaacaccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagcacccagtcc<br>gccctgagcaaagaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgccgccgggatcactctcg<br>gcatggacgagctgtacaag |
| LLP1::*LLP1* | GTAGCGTTCAAATCGGCAGTCCCGCTTTTTATTGCTGCACCGCTGGACATTTCCAGTCACCTG<br>AAATTTTCCGTCGTGAAAGCTTACTTGTACTTGTGGGCATAAGGAAATAAGCTTCTCCCACTC<br>TGGATCCTTAGATTTGACGAGGTAGTAAATTTGGTGGAGGAGTGATATGAGCTCCGGCTCCG<br>GCTCGAGTTCCCTTATTTTAAAAAAGAGCAACTTGAGCTTCTGATCCGTAGCAGAATCTATAG<br>AGTTGACGAGAGGGATGTTTTCAGATACTAATGATATGAACTTCCTTAGCGGCTTCGGCTGG<br>GATTTCAAATCCATAGTGTTGAAGAGAGCTATGGTTTGAGTAGTGAGTGACATGAGTTTGGTC<br>TCCGGTTTTGGTAGCGAGTCCAAATCCATAGAGCTGAGGAGATCGATTATGCGACTAGTGAC<br>TAATACGACATAACTCGTTGCTTCATCCGAAATCCAAAAGAGAGGATCGACGAAAGACACTA<br>GTTGACTAATGCGTAATAAGAGCTCCGTCTGCTGCTCCGAATCCATAGCGTTCGCCGATAGA<br>GAGTTTATCTGAGTAATTAAGCGAGAGATTTGAATGATTTTGGTATGTTTGCTGCTCTCTCTAT<br>ATTGGTATATGTTTATCTAACCAAACAATGTATAATCATGTATACAGGCTATCCACTCTCCATC<br>CCATATTACAGGCTGTTCGAATACAAATTCAACGGTCAACTTTTAGAGATTAAAAAAATAAAG<br>TGTGACAAGACAATCTCAAGCTGTCCGTAGCTTGCGGATCGTGCCTAAGAGTTTTTCTTAGAC<br>TAATAATGAAGTTAATGAGGGTTACATTGGCGTTGGCCTATGCAACTTGCACCTTTTTCTATAT<br>TAAGTTGTGGGCCACAACGACTCATGGGCCATATTTAAAGCCTCAACTACACTGCTCAATCCG<br>TTTTTGCTCCTTTTTGCAAAAGAACCCTCCATTTTCTATTTTTTATTGTAAGTTCACAATATTTA<br>TTTGTTGAGAAAATATGAACCTTTGATCTCTCTAAAAAATTTGAACTTTTTAACCAATTGTGCA<br>AGTATTTTTGGGTTAAACTACAATATTACGATTACTATTTAAAAATCGTTCAAACGAAAAATCA<br>GAGATCGATTTACTAAGAAATCAATGGTAAGATTGTTGAACATTTCATATGTGTGATAAACTG<br>ATGAGAATTTGGGATGCAAAGCAAATTACTTTTTCCTGAGTGAATTTTCTTTGAAAATATCTCT<br>CGTAGAAAAATAAACAGCAATCGATCTTTGCATTGCATTGGTCCTTTTCTAGACCAATTACAC<br>CGAAGAAACACAAGGTTGTCTATGTTCCTCTTCAATACTCTCTAAGTAAATTTCTATACGACGT<br>AGTGTTGTCTATGTGGTGGGTAAAGAATTAAAGAACCCACTAGATTTTCCTTATCCTTTTGAC<br>GTAGTGTTTTCTATTCATTCATCCACATAATGATACCGTATACTATGCTTTCACATCGCAAAAG<br>ACAAGTACAATCTAATATACGAGATATGCGTACACTGTACACATACTCTGCTGAATGAGTGTA<br>TTCACAACAGTGTCTTCACGCGTTTACTTGACAAAATGTCATGTGCTGATGTGACCATTCGAT<br>GATTTTGTTTTAATTTTTTTTTCTTTCCATTATTAACAATCACGTTTTTTATTGGTCGAGTTCAAT<br>TTATGGAAAGGATAAGGAAATCTTGTAGGTGATGCGTTTACTTGACATCATACAATATGACGT<br>CATGTACATGGGCTTATGTTTTACTCTTTATAAATCACCACGTTAATAATTTCACTCTTTCAAAC<br>TCTCACTTGAGTAAACAGCAGTTACGAAAACGCAATGAAGATTCATAAACTCTGTTTTCTTGC<br>TCTGCTCTTAGCTCACACCACTTCCGCCGTCAACCTCAACCTCAACCTCAAAACCTCAGAGTT<br>GGTCTTCCTTGGAGACGCTGAGCTCGGTCCTGCTTCTGATGGCGTCAGCCGCTCCGGAGCTC<br>TCTCCATGACACGAGACGAGAACCCATTCTCTCACGGCCAAAGTCTTTGGTCCACTCCAGTCC<br>CTTTCAAGCCTTCTTCAAACTCTTCTTCTCCTTATCCATTTGAAACTTCTTTCACTTTCTCCATC |

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| LLP1::*LLP1-GFP* | GTAGCGTTCAAATCGGCAGTCCCGCTTTTTATTGCTGCACCGCTGGACATTTCCAGTCACCTG<br>AAATTTTCCGTCGTGAAAGCTTACTTGTACTTGTGGGCATAAGGAAATAAGCTTCTCCCACTC<br>TGGATCCTTAGATTTGACGAGGTAGTAAATTTGGTGGAGGAGTGATATGAGCTCCGGCTCCG<br>GCTCGAGTTCCCTTATTTTAAAAAAGAGCAACTTGAGCTTCTGATCCGTAGCAGAATCTATAG<br>AGTTGACGAGAGGGATGTTTTCAGATACTAATGATATGAACTTCCTTAGCGGCTTCGGCTGG<br>GATTTCAAATCCATAGTGTTGAAGAGAGCTATGGTTTGAGTAGTGAGTGACATGAGTTTGGTC<br>TCCGGTTTTGGTAGCGAGTCCAAATCCATAGAGCTGAGGAGATCGATTATGCGACTAGTGAC<br>TAATACGACATAACTCGTTGCTTCATCCGAAATCCAAAAGAGAGGATCGACGAAAGACACTA<br>GTTGACTAATGCGTAATAAGAGCTCCGTCTGCTGCTCCGAATCCATAGCGTTCGCCGATAGA<br>GAGTTTATCTGAGTAATTAAGCGAGAGATTTGAATGATTTTGGTATGTTTGCTGCTCTCTCTAT<br>ATTGGTATATGTTTATCTAACCAAACAATGTATAATCATGTATACAGGCTATCCACTCTCCATC<br>CCATATTACAGGCTGTTCGAATACAAATTCAACGGTCAACTTTTAGAGATTAAAAAAATAAAG<br>TGTGACAAGACAATCTCAAGCTGTCCGTAGCTTGCGGATCGTGCCTAAGAGTTTTTCTTAGAC<br>TAATAATGAAGTTAATGAGGGTTACATTGGCGTTGGCCTATGCAACTTGCACCTTTTTCTATAT<br>TAAGTTGTGGGCCACAACGACTCATGGGCCATATTTAAAGCCTCAACTACACTGCTCAATCCG<br>TTTTTGCTCCTTTTTGCAAAAGAACCCTCCATTTTCTATTTTTTATTGTAAGTTCACAATATTTA<br>TTTGTTGAGAAAATATGAACCTTTGATCTCTCTAAAAAATTTGAACTTTTTAACCAATTGTGCA<br>AGTATTTTTGGGTTAAACTACAATATTACGATTACTATTTAAAAATCGTTCAAACGAAAAATCA<br>GAGATCGATTTACTAAGAAATCAATGGTAAGATTGTTGAACATTTCATATGTGTGATAAACTG<br>ATGAGAATTTGGGATGCAAAGCAAATTACTTTTTCCTGAGTGAATTTTCTTTGAAAATATCTCT<br>CGTAGAAAAATAAACAGCAATCGATCTTTGCATTGCATTGGTCCTTTTCTAGACCAATTACAC<br>CGAAGAAACACAAGGTTGTCTATGTTCCTCTTCAATACTCTCTAAGTAAATTTCTATACGACGT<br>AGTGTTGTCTATGTGGTGGGTAAAGAATTAAAGAACCCACTAGATTTTCCTTATCCTTTTGAC<br>GTAGTGTTTTCTATTCATTCATCCACATAATGATACCGTATACTATGCTTTCACATCGCAAAAG<br>ACAAGTACAATCTAATATACGAGATATGCGTACACTGTACACATACTCTGCTGAATGAGTGTA<br>TTCACAACAGTGTCTTCACGCGTTTACTTGACAAAATGTCATGTGCTGATGTGACCATTCGAT<br>GATTTTGTTTTAATTTTTTTTTCTTTCCATTATTAACAATCACGTTTTTTATTGGTCGAGTTCAAT<br>TTATGGAAAGGATAAGGAAATCTTGTAGGTGATGCGTTTACTTGACATCATACAATATGACGT<br>CATGTACATGGGCTTATGTTTTACTCTTTATAAATCACCACGTTAATAATTTCACTCTTTCAAAC<br>TCTCACTTGAGTAAACAGCAGTTACGAAAACGCAATGAAGATTCATAAACTCTGTTTTCTTGC<br>TCTGCTCTTAGCTCACACCACTTCCGCCGTCAACCTCAACCTCAACCTCAAAACCTCAGAGTT<br>GGTCTTCCTTGGAGACGCTGAGCTCGGTCCTGCTTCTGATGGCGTCAGCCGCTCCGGAGCTC<br>TCTCCATGACACGAGACGAGAACCCATTCTCTCACGGCCAAAGTCTTTGGTCCACTCCAGTCC |

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| LLP1::*GFP* | GTAGCGTTCAAATCGGCAGTCCCGCTTTTTATTGCTGCACCGCTGGACATTTCCAGTCACCTG<br>AAATTTTCCGTCGTGAAAGCTTACTTGTACTTGTGGGCATAAGGAAATAAGCTTCTCCCACTC<br>TGGATCCTTAGATTTGACGAGGTAGTAAATTTGGTGGAGGAGTGATATGAGCTCCGGCTCCG<br>GCTCGAGTTCCCTTATTTTAAAAAAGAGCAACTTGAGCTTCTGATCCGTAGCAGAATCTATAG<br>AGTTGACGAGAGGGATGTTTTCAGATACTAATGATATGAACTTCCTTAGCGGCTTCGGCTGG<br>GATTTCAAATCCATAGTGTTGAAGAGAGCTATGGTTTGAGTAGTGAGTGACATGAGTTTGGTC<br>TCCGGTTTTGGTAGCGAGTCCAAATCCATAGAGCTGAGGAGATCGATTATGCGACTAGTGAC<br>TAATACGACATAACTCGTTGCTTCATCCGAAATCCAAAAGAGAGGATCGACGAAAGACACTA<br>GTTGACTAATGCGTAATAAGAGCTCCGTCTGCTGCTCCGAATCCATAGCGTTCGCCGATAGA<br>GAGTTTATCTGAGTAATTAAGCGAGAGATTTGAATGATTTTGGTATGTTTGCTGCTCTCTCTAT<br>ATTGGTATATGTTTATCTAACCAAACAATGTATAATCATGTATACAGGCTATCCACTCTCCATC<br>CCATATTACAGGCTGTTCGAATACAAATTCAACGGTCAACTTTTAGAGATTAAAAAAATAAAG<br>TGTGACAAGACAATCTCAAGCTGTCCGTAGCTTGCGGATCGTGCCTAAGAGTTTTTCTTAGAC<br>TAATAATGAAGTTAATGAGGGTTACATTGGCGTTGGCCTATGCAACTTGCACCTTTTTCTATAT<br>TAAGTTGTGGGCCACAACGACTCATGGGCCATATTTAAAGCCTCAACTACACTGCTCAATCCG<br>TTTTTGCTCCTTTTTGCAAAAGAACCCTCCATTTTCTATTTTTTATTGTAAGTTCACAATATTTA<br>TTTGTTGAGAAAATATGAACCTTTGATCTCTCTAAAAAATTTGAACTTTTTAACCAATTGTGCA<br>AGTATTTTTGGGTTAAACTACAATATTACGATTACTATTTAAAAATCGTTCAAACGAAAAATCA<br>GAGATCGATTTACTAAGAAATCAATGGTAAGATTGTTGAACATTTCATATGTGTGATAAACTG<br>ATGAGAATTTGGGATGCAAAGCAAATTACTTTTTCCTGAGTGAATTTTCTTTGAAAATATCTCT<br>CGTAGAAAAATAAACAGCAATCGATCTTTGCATTGCATTGGTCCTTTTCTAGACCAATTACAC<br>CGAAGAAACACAAGGTTGTCTATGTTCCTCTTCAATACTCTCTAAGTAAATTTCTATACGACGT |

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| Geburtsort | Gräfelfing |
| Familienstand | ledig |

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**Elisabeth Pabst, Heiko H. Breitenbach, Marion Wenig and A. Corina Vlot**

"The role of the SAR regulators LEC1, LEC2 and LLP1 during biotic and abiotic stress responses"
Poster, Congress on PR Proteins and Induced Resistance against Pathogens and Insects, Aachen, Germany, 2015