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Genome-Wide Investigation into Roles of Arabidopsis Receptor-Like Proteins in Pathogen Defense

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Contents

Chapter 1

General Introduction

Plants directly or indirectly provide food for humans, animals and other heterotrophs. Because plant diseases result in crop losses and can cause famines, they have been a threat to mankind throughout history. Plants are continuously exposed to a wide range of pathogens including viroids, viruses, bacteria, mycoplasmas, fungi, oomycetes and nematodes as well as feeding insects (Agrios, 2007). Although plants are constantly exposed to various pathogens and insects with diverse attacking and feeding strategies, diseases occur relatively rarely. Nonhost resistance, which is defined as resistance of an entire plant species to all strains of a particular pathogen, is the most common form of disease resistance and is known to be highly effective and durable. Hence, most pathogen species cannot infect most plant species. In fact, the host range of a pathogen can be restricted to one single plant species. Resistance of plants at the host species level is called host resistance, and is often cultivar- or accession-specific. Plant defense can be subdivided in different levels of resistance responses that rank from preformed defense barriers to primary and secondary innate immune responses that, if successful, lead to nonhost and host resistance, respectively (Chisholm et al., 2006; Jones and Dangl, 2006).

Preformed Defenses

Components of nonhost resistance are the preformed or constitutive defenses consisting of plant structures functioning as barriers and anti-microbial compounds (Heath 2000, Nürnberger et al., 2004). Physical barriers are provided by a waxy cuticle, rigid plant cell walls and structures on the plant surface such as hairs and trichomes that can prevent the invasion of pathogens or feeding of insects. Chemical defenses include phytoanticipins, which act as antimicrobial compounds, such as phenolics, tannins and saponins (Heath, 2000; Nürnberger et al., 2004). A specific toxic secondary metabolite is often restricted to a narrow set of species within a phylogenetic group. For example, the saponin avenacin produced in roots of oat plants can be detoxified by the adapted oat root pathogen *Gaeumannomyces graminis* var. *avenae* but not by the closely related wheat pathogen *G. graminis* var. *tritici*, thus, providing evidence that avenacin is required for nonhost resistance (Papadopoulou et al., 1999). Furthermore, *Brassicaceae* species produce isothiocyanates upon tissue disruption, such as wounding. Isothiocyanates can be harmful to a wide range of predators, such as insects and bacteria, and are generated by cleaving of preformed nontoxic glucosinolates by the enzyme myrosinase. The localization of glucosinolates and myrosinase before tissue disruption is not fully clear but it is thought that they are stored in separate cell compartments, different cell types, or in the same cell compartment with the myrosinase in an inactive form (Halkier and Gershenzon, 2006).

Inducible Defenses

Inducible defenses are triggered by recognition of a pathogen. Basal defense, which can be a constituent of both nonhost and host resistance, provides basal level resistance that prevents infection by a wide range of microbes. Elicitors of basal defense can be plant cell wall-derived structures released by hydrolytic activity of enzymes secreted by invading microbes, but also common features of the pathogen, referred to as pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides, chitins, glucans and flagellins (Nürnberger et al., 2004; Schwessinger and Zipfel, 2008). These general elicitors are present or may be released during the invasion of both host and nonhost pathogens. PAMPs, which are usually indispensable in the lifestyle of microbes, are recognized by conserved pattern recognition receptors (PRRs) in order to induce basal defense, also called PAMP-triggered immunity (PTI) or primary innate immunity (Chisholm et al., 2006; Jones and Dangl, 2006). PTI is associated with MAP kinase signaling, transcriptional induction of pathogenesis related (PR) genes, production of reactive oxygen species, deposition of callose to reinforce the cell wall at sites of penetration, and phytoalexin production, all of which contribute to prevention of microbial proliferation (Nürnberger et al., 2004; Zhao et al., 2005; Chisholm et al., 2006). In Arabidopsis, several phytoalexin-deficient (*pad*) mutants have been identified that are involved in basal resistance (Glazebrook et al., 1997). For example, the *PAD3* gene, encoding a P450 monooxygenase, is required for biosynthesis of the Arabidopsis phytoalexin camalexin (Zhou et al., 1999) and a mutation in *PAD3* has been found to compromise resistance against *Alternaria brassicicola* (Thomma et al., 1998; 1999).

During evolution, some pathogens have acquired the ability to counteract PTI by developing and delivering specific effectors into plants. These effectors suppress or interfere with basal defense signaling and thus enhance pathogen growth and disease development (Espinosa and Alfano, 2004; Chisholm et al., 2006; Jones and Dangl, 2006; He et al., 2007; Schwessinger and Zipfel, 2008). In turn, resistant plant genotypes were found to have evolved a sophisticated effector-triggered immunity (ETI) during co-evolution, with disease resistance (R) proteins that specifically detect directly or indirectly certain pathogen effectors, which are now called avirulence (Avr) proteins (Chisholm et al., 2006; Jones and Dangl, 2006). ETI is race-cultivar specific, and is also called secondary innate immunity. This type of resistance was first described by Flor (1942) and formed the basis for his gene-for-gene hypothesis. Interestingly, as stated in the so-called guard hypothesis, R proteins monitor the status of the host effector target (Dangl and Jones, 2001; Mackey et al., 2002; Shao et al., 2003), rather than that they directly interact with the pathogen effector. Direct interaction has been demonstrated only in a few cases (Scofield et al., 1996; Tang et al., 1996;

Jia et al., 2000; Leister and Katagiri, 2000; Deslandes et al., 2003; Dodds et al., 2006; Burch-Smith et al., 2007). Recognition of an Avr protein by its cognate R protein initiates a rapid resistance response consisting of localized cell death, the so called hypersensitive response (HR), and associated defense responses (Chisholm et al., 2006; Jones and Dangl, 2006). When comparing compatible (adapted) and incompatible (non-adapted) host-pathogen interactions, general elicitors (PAMPs) and specific elicitors (Avrs) trigger partially overlapping defense signaling responses in plants, but the responses induced by Avrs were found to lead to stronger defense activation (Tao et al., 2003; Navarro et al., 2004; Zipfel et al., 2004; Schwessinger and Zipfel, 2008). Although one might predict that plant resistance responses mediated by the same type of R protein would induce similar defense responses, microarray analysis of tomato resistance to *C. fulvum* and *V. dahliae*, which is conveyed by the same type of R proteins, demonstrated significant differences in the induced gene set (van Esse et al., 2009).

Over the recent years, evidence accumulates for RNA silencing to play a role in defense responses against bacteria, apart from viral defense (Voinnet, 2008). The gene silencing was found to result from inhibition of gene transcription (transcriptional gene silencing, TGS) or from post-transcriptional degradation of RNA (post-transcriptional gene silencing, PTGS), and correlated with the accumulation of small double-stranded RNA fragments of 20 to 27 nucleotides, so-called small RNAs (sRNAs). These corresponded to the promoter of the silenced gene, or to the degraded RNA in TGS and PTGS, respectively (Hamilton and Baulcombe, 1999; Mette et al., 2000). By now, several small RNA species, such as micro RNAs (miRNAs) and small interfering RNAs (siRNAs), were found to regulate plant defense responses upon pathogen infections (Katiyar-Agarwal et al., 2006; 2007; Navarro et al., 2006; 2008). Furthermore, it was recently suggested that the transcriptional regulation of resistance gene loci may be under the control of RNA silencing, as was demonstrated for the *RPP5*-locus for recognition of the oomycete downy mildew pathogen *Peronospora parasitica* (Yi and Richards, 2007).

Elicitor Perception

Receptors functioning in pathogen surveillance are located on the plant cell surface or inside the cell (Chisholm et al., 2006; Jones and Dangl, 2006). In the past decade many plant immune receptors, providing resistance to bacteria, viruses, fungi, oomycetes, nematodes and insects, have been identified and were categorized into five protein classes (Fig.1; Dangl and Jones, 2001). While one class represents intracellular serine/threonine kinases such as the Pto kinase from tomato (Loh and Martin, 1995), another class only includes two Arabidopsis RPW8 membrane proteins with a putative coiled-coil domain (Xiao et al., 2001).

However, the largest class of plant resistance receptors encodes central nucleotide-binding site domain plus C-terminal leucine-rich repeat (NB-LRR) proteins that reside intracellularly, of which the LRR domain is generally thought to mediate ligand perception (Kobe and Kajava, 2001; Kinoshita et al., 2005). At the N-terminus, these NB-LRR proteins carry either a region with similarity to the N-terminus of the Toll and Interleukin 1 receptor (TIR-NB-LRR proteins), or a leucine-zipper (LZ) or a coiled-coil (CC) motif (CC-NB-LRR proteins; Pan et al., 2000; Meyers et al., 2003). In Arabidopsis, several NB-LRR proteins have been found to confer resistance to different races of the downy mildew *Hyaloperonospora parasitica* and the bacterium *Pseudomonas syringae* (Bent et al., 1994; Grant et al., 1995; Parker et al., 1997; McDowell et al., 1998; Gassmann et al., 1999; Bittner-Eddy et al., 2000; Cooley et al., 2000; van der Biezen et al., 2002; Sinapidou et al., 2004; Rehmany et al., 2005). Furthermore, tobacco N provides resistance against tobacco mosaic virus, while tomato Mi confers not only resistance against nematodes but also against aphids (Whitham et al., 1994, Rossi et al., 1998; Vos et al., 1998). The two remaining immune receptor classes harbor an extracellular LRR (eLRR) domain, of which each eLRR repeat is composed of 23 to 25 amino acids with the conserved consensus sequence LxxLxxLxLxxNxLt/sgxIpxxLG (Jones and Jones, 1997). In addition, both classes contain a single-pass transmembrane domain, but while the receptor-like kinases (RLK) contain a cytoplasmic serine/threonine kinase domain, the receptor-like proteins (RLP) only contain a short cytoplasmic tail without obvious signaling motifs except for the putative endocytosis motif found in some members (Joosten and de Wit, 1999; Fritz-Laylin et al., 2005; Kruijt et al., 2005; Wang et al., 2008). In Arabidopsis, the RLKs form the largest group of eLRR-containing cell-surface receptors with over 200 representatives in the Arabidopsis genome (Shiu and Bleecker, 2003). These include the PPRs FLAGELLIN SENSITIVE 2, FLS2, and the EF-Tu receptor, EFR, that mediate plant innate immunity upon perception of bacterial PAMPs flagellin (Gómez-Gómez and Boller, 2000; Chinchilla et al., 2006) and EF-Tu, respectively (Zipfel et al., 2006). In addition, the Arabidopsis RLK AtPep1 receptor PEPR1 was found to bind the endogenous peptide elicitor AtPep1 (Yamaguchi et al., 2006), which activates defense against pathogens (Huffaker et al., 2006). Furthermore, an RLK in rice, Xa21, has been found to confer resistance against the bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Song et al., 1995). In Arabidopsis, the second largest group of eLRR-containing cell surface receptors, containing 57 members, is formed by the RLPs (Joosten and de Wit, 1999; Fritz-Laylin et al., 2005; Kruijt et al., 2005; Wang et al., 2008). While functional analysis of Arabidopsis RLPs is limited, several RLPs in other plant species were found to confer resistance against pathogens.

Receptor-Like Proteins

The first RLP identified was the tomato Cf-9 protein that mediates resistance against strains of the biotrophic leaf mold fungus *Cladosporium fulvum* that secrete the corresponding effector molecule Avr9 (Jones et al., 1994). By now, several Cf resistance proteins have been discovered in tomato that all belong to the RLP family. Overall, the amino acid sequence of RLPs can be divided into 7 conserved domains (A to G) with a signal peptide (A), a cysteine-rich domain (B), the LRR domain (C), a spacer (D), an acidic domain (E), the transmembrane domain (F), and a short cytoplasmic region (G). The eLRR-containing C domain is subdivided into three domains with a non-LRR island domain (C2) interrupting two eLRR regions (C1 and C3) (Fig. 1B; Jones and Jones, 1997). Most of the *Cf* genes appear in gene clusters that were grouped into two large gene families, both containing members with currently unknown function and *Cf* resistance genes that recognize the presence or activity of specific *C. fulvum* avirulence molecules. These include Cf-2, Cf-4, Cf-4E, Cf-5, Cf-9 and 9DC that confer recognition of the *C. fulvum* Avr2, Avr4, Avr4E, Avr5 and Avr9 proteins (Jones et al., 1994; Dixon et al., 1996; 1998; Thomas et al., 1997; Takken et al., 1999; Kruijt et al., 2004). In fact, some of these secreted (a-)virulence effector molecules, Avr2 and Avr4, were demonstrated to contribute to virulence (Thomma et al., 2005; van Esse et al., 2007; 2008).

In addition to *Cf* genes, the tomato *RLP* gene family harbors two other *RLP* loci, the *Ve* and *LeEIX* gene clusters. The *Ve* locus, which consists of the two genes *Ve1* and *Ve2* (Kawchuk et al., 2001), provides resistance against soil-borne vascular wilt pathogens of the genus *Verticillium*, including *V. dahliae* and *V. albo-atrum* (Kawchuk et al., 1994; 1998; Diwan et al., 1999). By introducing the *Ve1* or *Ve2* gene in potato, both genes were shown to provide resistance against an aggressive race 1 isolate of *V. albo-atrum* (Kawchuk et al., 2001). However, when the *Ve1* and *Ve2* genes were separately expressed in susceptible tomato plants, only *Ve1* was demonstrated to confer resistance against different *Verticillium* species (Fradin et al., 2009).

Xylanase (EIX) produced by the biocontrol fungus *T. viride* is recognized by a single dominant locus in tomato and tobacco, where it elicits ethylene biosynthesis which results in induction of defense (Bailey et al., 1993; Ron et al., 2000). In tomato, this locus comprises three homologous *LeEIX* genes of which two, *LeEIX1* and *LeEIX2*, have been cloned and belong to the tomato RLP gene family (Ron and Avni, 2004). Both LeEIX1 and LeEIX2 were demonstrated to bind EIX, although only LeEIX2 was able to transmit the signal that induced an HR.

In addition to tomato, RLPs have been implicated in disease resistance in apple. The Apple *Vf* locus, derived from the crabapple species *Malus floribunda,* confers resistance to five races of the apple scab fungus *Venturia inaequalis* but not to the newly identified races 6 and 7 (Durel et al., 2003; Guerin et al., 2007). The *Vf* locus comprises a cluster of four RLP genes, *HcrVfa1* to *HcrVfa4* (for homologue of the *C. fulvum* resistance genes of the *Vf* region), of which *HcrVfa1*, *HcrVfa2* and *HcrVfa4* encode typical RLPs while *HcrVfa3* contains an insertion at the end of the LRR motif, resulting in truncated transcripts (Vinatzer et al., 2001; Xu and Korban, 2002). Expression of *HcrVfa1* or *HcrVfa2*, but not of *HcrVfa4*, in susceptible

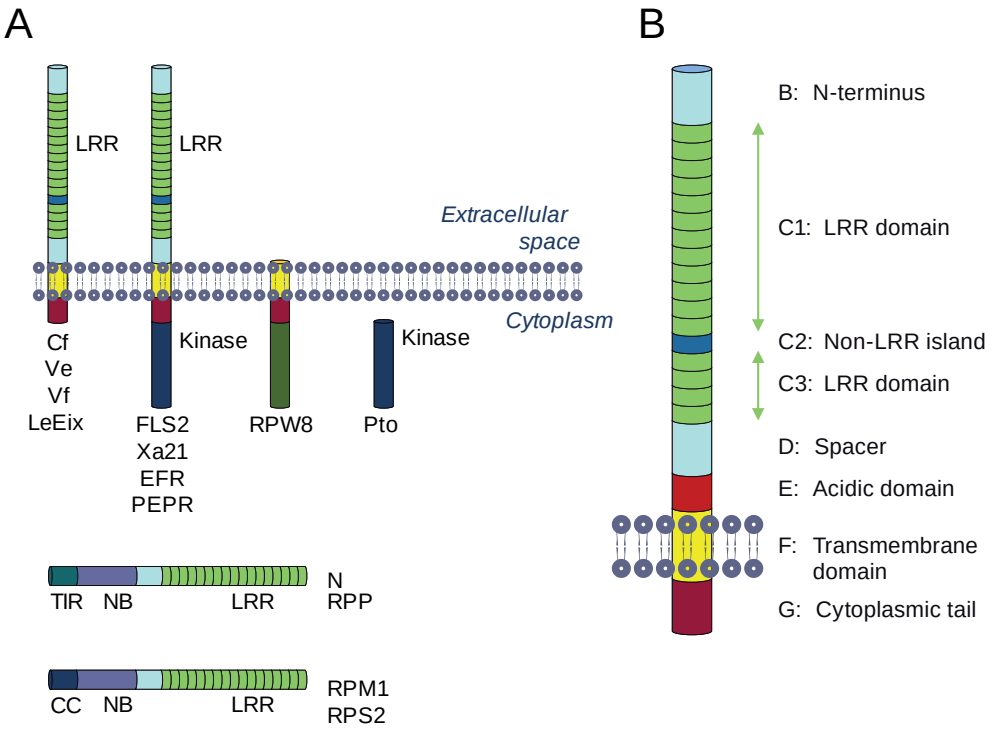

Figure 1. Schematic representation of the five major structural classes of plant R proteins and PRRs (A) and of the RLP domain structure (B).

A One class, the RLPs, is represented by the tomato Cf, Ve and apple Vf R proteins as well as the tomato LeEIX proteins mediating EIX perception. The RLK class includes Arabidopsis FLS2, EFR and PEPR proteins mediating perception of PAMPs or endogenous elicitors (PEPR) as well as the rice R protein Xa21. Arabidopsis RPW8 and tomato Pto represent other classes of R proteins. The largest class of R proteins is the NB LRR class that can be divided into two subclasses. While the TIR-NB-LRR subclass contains R proteins such as tobacco N and Arabidopsis RPP proteins, the CC-NB-LRR subclass is represented by Arabidopsis RPM1 and RPS2.

B Typical domain structure of a mature RLP. See text for details.

apple cultivars provided resistance against *V. inaequalis* strains that belong to races 1 to 5 (Belfanti et al., 2004; Malnoy et al., 2008).

In Arabidopsis, only two *RLP* genes, *TOO MANY MOUTHS* (*TMM*) and *CLAVATA2* (*CLV2*), were characterized in detail. While *TMM* regulates stomatal distribution across the epidermis by initiation of stomatal precursor cells (Nadeau and Sack, 2002), *CLV2* is involved in maintenance of a balanced meristematic cell population (Jeong et al., 1999). CLV2 was proposed to stabilize the RLK CLV1 (Jeong et al., 1999), which acts as a receptor for the extracellular peptide ligand CLV3 (Ogawa et al., 2008). Recently, CLV2 was not only found to act in concert with CLV1 but also in parallel with the receptor kinase CORYNE (CRN) in order to perceive the CLV3 signal (Müller et al., 2008). The maize gene *FASCIATED EAR* (*FEA2*; Taguchi-Shiobara et al., 2001) is characterized as a CLV2 homolog, indicating that CLV2 function is conserved across species. However, only in 2005 the first Arabidopsis *RLP* with a role in pathogen defense was identified. This RLP was found to be induced upon treatment of Arabidopsis seedlings with the fungal PAMP chitin. T-DNA insertion mutants for this chito-oligomer-responsive *RLP* gene displayed enhanced susceptibility to the powdery mildew pathogen *Erysiphe cichoracearum* (Ramonell et al., 2005).

OUTLINE OF THE THESIS

While considerable advances have been made in our understanding of NB-LRR and RLK signaling in Arabidopsis plant innate immunity (DeYoung and Innes, 2006; McHale et al., 2006; Nürnberger and Kemmerling, 2006; Li and Jin, 2007; Tameling and Joosten, 2007; Afzal et al., 2008; Zipfel, 2008), relatively little is known about the role and function of Arabidopsis RLPs (Fritz-Laylin et al., 2005; Kruijt et al., 2005). This thesis represents a contribution to the identification of roles for Arabidopsis RLPs.

Chapter 2 describes the identification of the 57 (*At*)*RLP* genes in the *Arabidopsis thaliana* genome and the assembly of a genome-wide collection of T-DNA insertion lines. This collection was functionally analyzed with respect to alterations in plant growth and development and sensitivity to various stress responses, including susceptibility towards pathogens. A number of novel phenotypes were revealed for our *CLV2* (*AtRLP10*) and *TMM* (*AtRLP17*) mutants. In addition, the *AtRLP41* gene was identified to be involved plant hormone sensitivity, while another *AtRLP* gene, *AtRLP30* (and possibly also *AtRLP18*) was found to be required in plant defense. Most of the T-DNA insertion lines, however, displayed no altered phenotype in development and upon abiotic and biotic stress challenges.

Chapter 3 presents an RNA interference (RNAi) strategy to target the expression of multiple *AtRLP* genes simultaneously, followed by functional analysis of the resulting RNAi lines. RNAi lines for a construct predicted to target *AtRLP41* amongst other *AtRLP* genes displayed enhanced hormone sensitivity similar to the *AtRLP41* knock-out line. This observation confirmed that RNAi-mediated gene silencing can be used as a mechanism to investigate the function of RLP receptors. However, novel phenotypes were not discovered in this analysis.

RNA silencing is a conserved mechanism in eukaryotes that plays an important role in various biological processes including regulation of gene expression, genome stability and protection of plants against invading nucleic acids such as transgenes and viruses. Recently, RNA silencing has also been found to influence defense against bacterial plant pathogens in Arabidopsis. In chapter 4 we show that gene silencing plays a role in plant defense against vascular fungi belonging to the *Verticillium* genus. Several components of RNA silencing pathways were tested, of which many were found to affect *Verticillium* defense.

In chapter 5 all results obtained in this thesis are discussed and placed in a broader perspective including recent data from literature.

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Chapter 2

A Genome-Wide Functional Investigation into Roles of Receptor-Like Proteins in Arabidopsis

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Abstract

Receptor-like proteins (RLPs) are cell surface receptors that typically consist of an extracellular LRR-domain, a transmembrane domain and a short cytoplasmatic tail. In several plant species, RLPs have been found to play a role in disease resistance, such as the tomato Cf and Ve proteins, and the apple HcrVf2 protein that mediate resistance against the fungal pathogens *Cladosporium fulvum*, *Verticillium* spp. and *Venturia inaequalis*, respectively. In addition, RLPs play a role in plant development; Arabidopsis TOO MANY MOUTHS (TMM) regulates stomatal distribution, while Arabidopsis CLAVATA2 (CLV2) and its functional maize ortholog FASCIATED EAR2 regulate meristem maintenance. In total, 57 *RLP* genes have been identified in the Arabidopsis genome and a genome-wide collection of T-DNA insertion lines was assembled. This collection was functionally analyzed with respect to plant growth and development and sensitivity to various stress responses including susceptibility towards pathogens. A number of novel developmental phenotypes were revealed for our *CLV2* and *TMM* insertion mutants. In addition, one *AtRLP* gene was found to mediate abscisic acid sensitivity and another *AtRLP* gene was found to influence nonhost resistance towards *Pseudomonas syringae* pv. *phaseolicola*. This genome-wide collection of Arabidopsis *RLP* gene T-DNA insertion mutants provides a tool for future investigations into the biological roles of RLPs.

Introduction

For decades, it was thought that the communication between plant cells occurs through the cell wall-spanning cytoplasmic bridges called plasmodesmata. However, since the identification of the first plant cell-surface receptor (Walker and Zhang, 1990) it is known that, similar to other multicellular organisms, plants can perceive extracellular signals at the plasma membrane. Since then, many plant cell-surface receptors have been found to play key roles in very diverse processes ranging from growth and development in which they perceive endogenous self signals, to recognition of other organisms, in which they perceive exogenous non-self signals (Diévart and Clark, 2004).

A common structural element of many plant cell-surface receptors is the extracellular leucine-rich repeat (eLRR) domain that is generally thought to mediate ligand perception (Kobe and Kajava, 2001; Kinoshita et al., 2005). These eLRRs are composed of 23 to 25 amino acids with the conserved consensus sequence LxxLxxLxLxxNxLt/sgxIpxxLG (Jones

and Jones, 1997). The largest group of eLRR-containing cell-surface receptors is formed by the receptor-like kinases (RLKs) that are composed of an eLRR domain, a single-pass transmembrane domain, and a cytoplasmic kinase domain, with over 200 representatives in the Arabidopsis genome (Shiu and Bleecker, 2003). The second largest group of eLRR-containing cell surface receptors is formed by the receptor-like proteins (RLPs) that differ from RLKs in that they lack the cytoplasmic kinase domain and only have a short cytoplasmic tail that lacks obvious motifs for intracellular signaling except for the putative endocytosis motif found in some members (Joosten and de Wit, 1999; Kruijt et al., 2005). Typically, the amino acid sequence of RLPs has been divided into the conserved domains A through G with a putative signal peptide (A), a cysteine-rich domain (B), the LRR domain (C), a spacer (D), an acidic domain (E), the transmembrane domain (F), and a short cytoplasmic region (G). Furthermore, the LRR-containing C domain is subdivided into three domains in which the non-LRR island C2 domain interrupts the C1 and C3 LRR regions (Jones and Jones, 1997).

Recently, considerable advances have been made in our understanding of the role and function of RLKs and how they relay extracellular signals to initiate an intracellular response (Nürnberger and Kemmerling, 2006; Li and Jin, 2007). By contrast, very little is known about RLP signaling (Fritz-Laylin et al., 2005; Kruijt et al., 2005). The first *RLP* gene identified was tomato *Cf-9* that mediates resistance against strains of the leaf mold fungus *Cladosporium fulvum* that carry the avirulence gene *Avr9* (Jones et al., 1994). *C. fulvum* is a biotrophic pathogen that is characterized by strictly apoplastic growth (Thomma et al., 2005). To date, several *Cf* resistance genes have been cloned from tomato that all to belong to the *RLP* gene family (Dixon et al., 1996; 1998; Thomas et al., 1997; Takken et al., 1999). In addition to *Cf* genes, the *RLP* gene family in tomato comprises two *Ve* genes that have been reported to provide resistance against vascular wilt pathogens of the genus *Verticillium* (Kawchuk et al., 2001) that, like *C. fulvum*, grow extracellularly without penetrating plant cells (Fradin and Thomma, 2006). Finally, the tomato *RLP* family comprises two *LeEIX* genes that encode receptors for the ethylene-inducing xylanase produced by extracellularly growing *Trichoderma* biocontrol fungi (Ron and Avni, 2004).

In addition to tomato, RLPs have been implicated in disease resistance in other plant species (Kruijt et al., 2005). Apple *HcrVf-2* confers resistance to the apple scab fungus *Venturia inaequalis* (Belfanti et al., 2004). Furthermore, an Arabidopsis chitin-inducible *RLP* gene has been implicated in resistance against the powdery mildew pathogen *Erysiphe cichoracearum* (Ramonell et al., 2005).

RLPs also play significant roles in plant development. For example, Arabidopsis CLAVATA2 (CLV2) was found to be crucial for maintaining a balanced meristematic stem cell population and is required for the accumulation and stability of CLV1, which is an RLK (Jeong et al., 1999). It has been proposed that CLV1 and CLV2 undergo a physical interaction to form a heterodimer to act as receptor for the predicted extracellular peptide ligand CLV3 (Trotochaud et al., 1999; Rojo et al., 2002; Ogawa et al., 2008). Upon ligand perception by the ectodomain (Ogawa et al., 2008), the kinase domain of CLV1 is thought to be activated to initiate the downstream signaling that is required to maintain the stem cell population (Rojo et al., 2002; Diévart and Clark, 2004). In maize, an ortholog of the *CLV2* gene has been identified as *FASCIATED EAR2* (*FEA2*; Taguchi-Shiobara et al., 2001). Furthermore, the RLK thick tassel dwarf1 has been identified as a CLV1 ortholog, suggesting that the CLAVATA signaling pathway is conserved between monocots and dicots (Bommert et al., 2005). Another *RLP* gene, *TOO MANY MOUTHS* (*TMM*), is involved in plant development in Arabidopsis, and regulates stomatal distribution across the epidermis (Nadeau and Sack, 2002). Although a physical interaction between TMM and any other RLP or RLK has not been established, TMM was found to negatively regulate three RLKs of the *ERECTA* family (Shpak et al., 2005).

Previously, in the Arabidopsis genome 56 putative *RLP* genes (*AtRLPs*) have been identified that are assembled at 33 loci (Fritz-Laylin et al., 2005). So far, a function has only been assigned to the three *AtRLP* genes described above (Jeong et al., 1999; Nadeau and Sack, 2002; Ramonell et al., 2005), implicating that the other RLPs are orphan proteins. In the complete genome sequence of the monocot plant rice, 90 *RLP* genes have been identified (Fritz-Laylin et al., 2005). Genes involved in plant development are presumably under evolutionary pressure to maintain a specific function which reduces sequence drift across orthologs, while disease resistance genes are under strong diversifying selection to produce highly divergent sequences with distinct recognition capacities (Fritz-Laylin et al., 2005). Based on the sequence comparison between Arabidopsis and rice *RLP* genes, and building on the hypothesis that developmental genes are less likely to be duplicated and undergo diversifying selection than are disease resistance genes (Leister, 2004), nine *AtRLP* genes were proposed as putative developmental orthologous genes, while the remaining *AtRLP* genes were proposed to be candidate disease resistance genes (Fritz-Laylin et al., 2005). In this manuscript, we report on the assembly and functional analysis of a genome-wide collection of *AtRLP* family T-DNA knock-out lines. This collection has been screened for altered phenotypes in growth and development, but also alterations in response to pathogen challenge. Our analysis has revealed novel phenotypes linked with mutations in the well-studied AtRLPs TMM and CLV2. Furthermore, one *AtRLP* gene is found to play a role in ABA signaling, a process in

which RLP-activity has not been implicated previously. Remarkably, despite an extensive list of pathogens tested including adapted and non-adapted pathogens of Arabidopsis, we have only been able to identify one *AtRLP* gene with a role in basal nonhost resistance against the non-adapted bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola*. The described *AtRLP* T-DNA collection is a valuable source for future investigations into the biological roles of RLPs.

RESULTS

AtRLP Gene Structure and AtRLP Protein Analysis

At the onset of this project, a bioinformatic analysis to investigate the structure of all the *AtRLP* genes was undertaken. To this end, BLAST searches were performed on the Arabidopsis genome sequence using the predicted protein sequences of the previously characterized RLPs CLV2, TMM and Cf-9 as queries. The set of Arabidopsis genes obtained in this way was further analyzed for presence of a signal peptide, eLRRs, a transmembrane domain and a short cytoplasmic tail lacking kinase motifs in the predicted protein. Although a previously published study has identified in total 56 *AtRLP* genes (Fritz-Laylin et al., 2005), our analysis revealed a set of 57 putative *AtRLP* genes (Table 1). All 57 *AtRLP* genes are assigned AtRLP numbers in consecutive order according to their gene numbers along the Arabidopsis genome (Table 1). The additional *AtRLP* gene identified here, denoted as *AtRLP5*, corresponds to At1g34290, and, although it carries only two eLRRs, the predicted protein complies with the canonical RLP domain composition.

Pairwise amino acid sequence comparison revealed that AtRLPs display low overall sequence identity, with only 10 pairwise combinations that share over 70% identity (Supplemental Table S1). Of these, the proteins encoded by the neighboring genes *AtRLP41* and *AtRLP42* share the highest level of identity (86%). Furthermore, both proteins are highly similar to AtRLP39 (85% and 82% identity, respectively), and the corresponding genes reside in close proximity to each other, suggesting recent gene multiplication. Two other AtRLP proteins, AtRLP44 and AtRLP57, are found to be similar in length and domain composition, sharing 80% identity (Fig. 1; Supplemental Table S1), although the genes that encode these proteins are located on different chromosomes. To further assess the structures of *AtRLP* genes, the exon boundaries and corresponding flanking intron sequences were determined. While only 21% of the genes in the Arabidopsis genome are composed of a single exon (The Arabidopsis Genome Initiative, 2000), 37 of the 57 (65%) AtRLP-encoding genes were found to contain a

Table 1. List of the Arabidopsis RLP (AtRLP) genes and corresponding T-DNA insertion lines used in this study.

Gene name [a]	AGI code	T-DNA line ordered	Mutant name
AtRLP1	at1g07390	SALK_059920 [g]	*Atrlp1-1*
		SALK_116923	*Atrlp1-2*
AtRLP2	at1g17240	SALK_049366 [f]	*Atrlp2-1*
AtRLP3	at1g17250	SALK_051677	*Atrlp3-1*
		SAIL_204_D01 [b]	*Atrlp3-2*
AtRLP4	at1g28340	SALK_039264 [h]	*Atrlp4-1*
AtRLP5	at1g34290	SALK_112291	*Atrlp5-1*
AtRLP6	at1g45616	SALK_080898	*Atrlp6-1*
		SAIL_84_E01 [b]	*Atrlp6-2*
		SALK_020071 [i]	
AtRLP7	at1g47890	SALK_030269	*Atrlp7-1*
AtRLP8	at1g54480	SM_3_38632	*Atrlp8-1*
		SM_3_20200	*Atrlp8-2*
AtRLP9	at1g58190	SALK_061979	*Atrlp9-1*
		SALK_023419	*Atrlp9-2*
AtRLP10 (CLV2)	at1g65380	GABI_686A09	*Atrlp10-1*
		clv2-3 (EMS) [c]	*clv2-3*
AtRLP11	at1g71390	SALK_013218	*Atrlp11-1*
AtRLP12	at1g71400	SALK_151456	*Atrlp12-1*
AtRLP13	at1g74170	SALK_020984	*Atrlp13-1*
AtRLP14	at1g74180	SAIL_513_A08 [b]	*Atrlp14-1*
AtRLP15	at1g74190	SALK_041143	*Atrlp15-1*
		GABI_077G01 [i]	
AtRLP16	at1g74200	SALK_032150	*Atrlp16-1*
AtRLP17 (TMM)	at1g80080	FLAG_014F03 [b]	*Atrlp17-1*
		tmm-1 (EMS) [d]	*tmm-1*
		SAIL_165_F02 [b, i]	
AtRLP18	at2g15040	SAIL_400_H02 [b]	*Atrlp18-1*
AtRLP19	at2g15080	FLAG_524A03 [b, e, j]	*Atrlp19-1*
AtRLP20	at2g25440	SALK_130147[f]	*Atrlp20-1*
AtRLP21	at2g25470	SAIL_693_F05	*Atrlp21-1*
		SALK_133403 [i]	
AtRLP22	at2g32660	SALK_125231	*Atrlp22-1*
AtRLP23	at2g32680	SALK_034225	*Atrlp23-1*
AtRLP24	at2g33020	SALK_046236	*Atrlp24-1*
AtRLP25	at2g33030	SALK_048434 [e]	*Atrlp25-1*
AtRLP26	at2g33050	SALK_104127 [f]	*Atrlp26-1*
		SALK_026997 [i]	
AtRLP27	at2g33060	SALK_029443	*Atrlp27-1*
AtRLP28	at2g33080	SM_3_1740	*Atrlp28-1*
AtRLP29	at2g42800	SALK_022220	*Atrlp29-1*
AtRLP30	at3g05360	SALK_122528	*Atrlp30-1*
		SALK_008911	*Atrlp30-2*
		SALK_122536	*Atrlp30-3*
		SALK_145342	*Atrlp30-4*
AtRLP31	at3g05370	SALK_058586	*Atrlp31-1*
		SALK_094160	*Atrlp31-2*
AtRLP32	at3g05650	FLAG_588C11 [b]	*Atrlp32-1*
AtRLP33	at3g05660	FLAG_048F06 [b]	*Atrlp33-1*
		SALK_087631	*Atrlp33-2*
		SALK_085252	*Atrlp33-3*
AtRLP34	at3g11010	SALK_067155	*Atrlp34-1*
		SALK_085506 [i]	
AtRLP35	at3g11080	SALK_096171	*Atrlp35-1*
		SALK_016143	*Atrlp35-2*
AtRLP36	at3g23010	SALK_086147	*Atrlp36-1*
AtRLP37	at3g23110	SALK_041785	*Atrlp37-1*
		SALK_012745 [j]	*Atrlp37-2*
AtRLP38	at3g23120	SALK_017819	*Atrlp38-1*
		GT_5_105490 [b, i]	
AtRLP39	at3g24900	SALK_126505	*Atrlp39-1*
		SALK_126504 [i]	
AtRLP40	at3g24982	GABI_564D03	*Atrlp40-1*
AtRLP41	at3g25010	SALK_024020	*Atrlp41-1*
		SM_3_20242	*Atrlp41-2*
		SM_3_38956	*Atrlp41-3*
AtRLP42	at3g25020	SALK_080324 [g]	*Atrlp42-1*
		SALK_094190 [g]	*Atrlp42-2*
AtRLP43	at3g28890	SALK_041685	*Atrlp43-1*
AtRLP44	at3g49750	SALK_097350 [e]	*Atrlp44-1*
		SALK_045246 [f]	*Atrlp44-2*
AtRLP45	at3g53240	GABI_620G05	*Atrlp45-1*
		FLAG_339H12 [b, f]	*Atrlp45-2*
AtRLP46	at4g04220	SALK_048207 [e]	*Atrlp46-1*
		SAIL_15_A02 [b, i]	
AtRLP47	at4g13810	SALK_105921	*Atrlp47-1*
AtRLP48	at4g13880	SALK_036842	*Atrlp48-1*
AtRLP49	at4g13900	SALK_067372	*Atrlp49-1*
		SALK_116910	*Atrlp49-2*
AtRLP50	at4g13920	SALK_070876 [e]	*Atrlp50-1*
AtRLP51	at4g18760	SALK_143038	*Atrlp51-1*
		SAIL_740_C06 [e]	*Atrlp51-2*
AtRLP52	at5g25910	SALK_107922	*Atrlp52-1*
		SALK_054976 [i]	
AtRLP53	at5g27060	SALK_124008	*Atrlp53-1*
AtRLP54	at5g40170	SAIL_306_E09 [b]	*Atrlp54-1*
AtRLP55	at5g45770	SALK_139161 [g]	*Atrlp55-1*
		SALK_076590	*Atrlp55-2*
AtRLP56	at5g49290	SALK_129306	*Atrlp56-1*
		SALK_010565	*Atrlp56-2*
AtRLP57	at5g65830	SALK_077716	*Atrlp57-1*

Legend of Table 1.

a In chronological order along the five Arabidopsis chromosomes.

b SAIL-lines are in CS8846, FLAG-lines in WS-2 and GT-line is in L*er* background.

c EMS mutant *clv2-3* (Jeong et al., 1999).

d EMS mutant *tmm-1* (Nadeau and Sack., 2002).

e T-DNA insertion site within 300 nucleotides upstream of the open reading frame.

f T-DNA insertion site between 300 and 1000 nucleotides upstream of the open reading frame.

g T-DNA insertion site within 300 nucleotides downstream of the open reading frame.

h T-DNA insertion site within an intron.

i T-DNA insertion site could not be confirmed by PCR, no homozygous T-DNA insertion line was obtained.

j No homozygous line for the T-DNA insertion was be obtained.

single exon (Fig. 1). Interestingly, within the group of genes that contain multiple exons, *AtRLP9*, *AtRLP14*, *AtRLP15*, *AtRLP21* and *AtRLP56* have introns at similar positions in the genes (Fig. 1). Similarly, the introns of *AtRLP19*, *AtRLP33* and *AtRLP34* are localized at comparable positions (Fig. 1). Furthermore, all the *AtRLP* genes that contain multiple exons cluster in a phylogenetic tree (Fig. 1).

Next, the domain composition was analyzed for all predicted AtRLP proteins. As has been noted previously (Fritz-Laylin et al., 2005), the AtRLPs exhibit great variation at the sequence level and also in the numbers of eLRRs (Fig. 1). The predicted sizes of the AtRLPs range from 218 amino acids (AtRLP25) to 1784 amino acids (AtRLP9), whereas the eLRRs vary in number from 2 (AtRLP5) to 49 (AtRLP9; Fig. 1). Of the 57 AtRLPs, 18 are predicted to contain two transmembrane domains, one at the N-terminus and one at the C-terminus, although it is presently unclear whether the N-terminal transmembrane domain indeed functions as such. Furthermore, it has previously been noted that not all RLPs contain an island domain (C2) within the eLRR region, with TMM as an example (Nadeau and Sack, 2002). Of the 57 AtRLPs, 45 are predicted to contain a C2 island domain nested in between two eLRR blocks (C1 and C3). Remarkably, in 42 of those RLPs the island domain is followed by a C3 domain that contains exactly four eLRRs (Fig. 1). This distinct domain organization has not only been observed for some functionally characterized RLPs, but also for some RLKs (Jones et al., 1994; Song et al., 1995; Clark et al., 1997; Li and Chory, 1997; Jeong et al., 1999; Gómez-Gómez and Boller, 2000; Taguchi-Shiobaraet al., 2001). For all *AtRLP* genes, corresponding cDNA sequences, EST sequences, Massively Parallel Signature Sequencing data and/or micro-array data are deposited in public databases, demonstrating that all 57 *AtRLP* genes are actively transcribed (Supplemental Figs. S1 and S2).

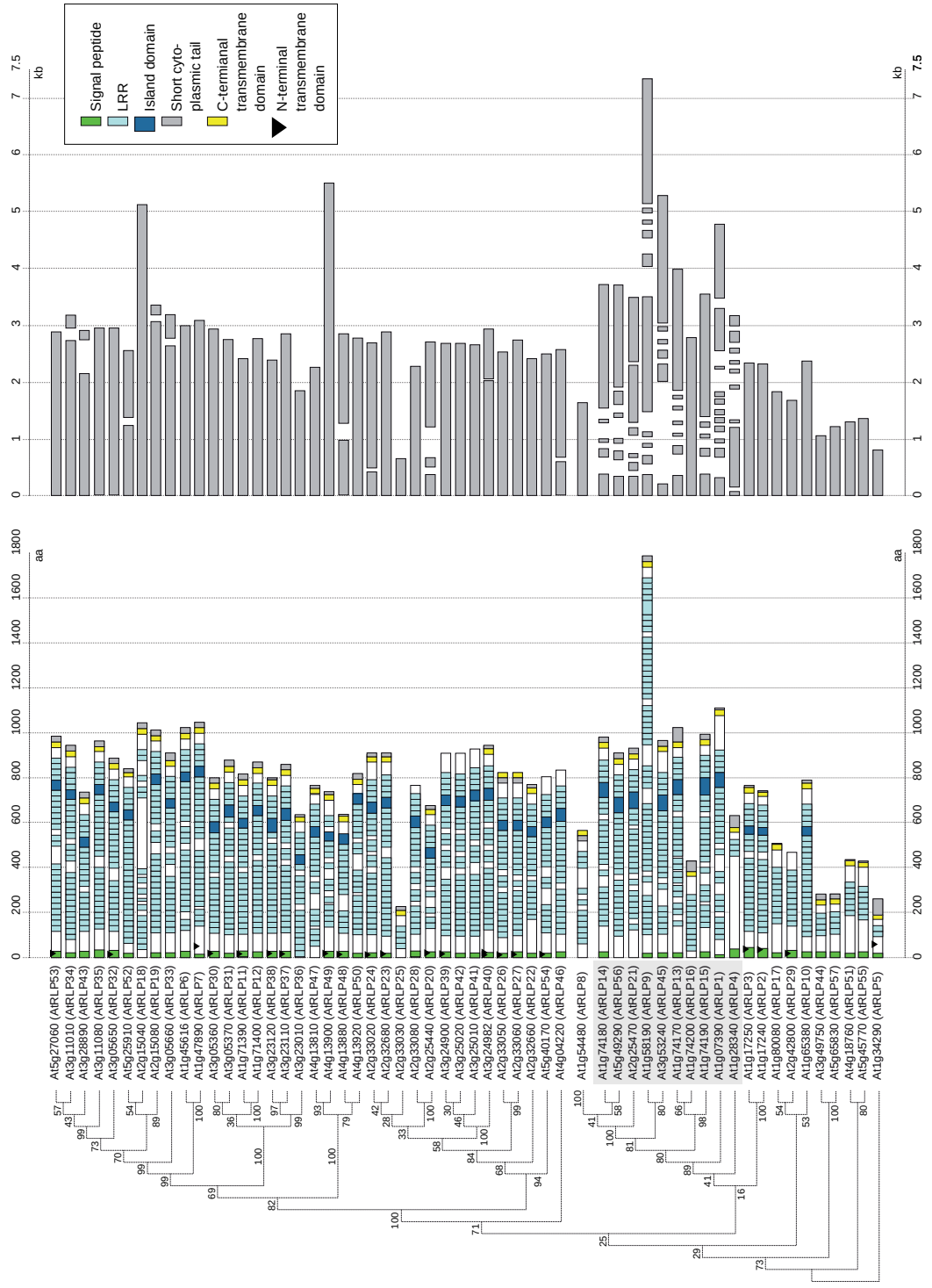
Signal peptide
LRR
Island domain
Short cyto-plasmic tail
C-termianal transmembrane domain
N-terminal transmembrane domain
kb
aa
At5g27060 (AtRLP53)
At3g11010 (AtRLP24)
At3g28890 (AtRLP43)
At3g11080 (AtRLP35)
At3g05650 (AtRLP32)
At5g25910 (AtRLP52)
At2g15040 (AtRLP18)
At2g15080 (AtRLP19)
At3g05660 (AtRLP33)
At1g45616 (AtRLP6)
At1g47890 (AtRLP7)
At3g05360 (AtRLP30)
At3g05370 (AtRLP31)
At1g71390 (AtRLP11)
At1g71400 (AtRLP12)
At3g23120 (AtRLP38)
At3g23110 (AtRLP37)
At3g23010 (AtRLP36)
At4g13810 (AtRLP47)
At4g13900 (AtRLP49)
At4g13880 (AtRLP48)
At4g13920 (AtRLP50)
At2g33020 (AtRLP24)
At2g32680 (AtRLP23)
At2g33030 (AtRLP25)
At2g33080 (AtRLP28)
At2g25440 (AtRLP20)
At3g24900 (AtRLP39)
At3g25020 (AtRLP42)
At3g25010 (AtRLP41)
At3g24982 (AtRLP40)
At2g33050 (AtRLP26)
At2g33060 (AtRLP27)
At2g32660 (AtRLP22)
At5g40170 (AtRLP54)
At4g04220 (AtRLP46)
At1g54480 (AtRLP8)
At1g74180 (AtRLP14)
At5g49290 (AtRLP56)
At2g25470 (AtRLP21)
At1g58190 (AtRLP9)
At3g53240 (AtRLP45)
At1g74170 (AtRLP13)
At1g74200 (AtRLP16)
At1g74190 (AtRLP15)
At1g07390 (AtRLP1)
At1g28340 (AtRLP4)
At1g17250 (AtRLP3)
At1g17240 (AtRLP2)
At1g80080 (AtRLP17)
At2g42800 (AtRLP29)
At1g65380 (AtRLP10)
At3g49750 (AtRLP44)
At5g65830 (AtRLP57)
At4g18760 (AtRLP51)
At5g45770 (AtRLP55)
At1g34290 (AtRLP5)

Figure 1. A phylogenetic view of AtRLP protein domain configurations and the corresponding *RLP* gene structures as shown by exon/intron boundaries.

Left Phylogenetic tree of the AtRLP family that also includes CLV2 (AtRLP10) and TMM (AtRLP17). The tree was generated from the alignment of C3-F domains of all AtRLPs with 100 bootstrap replicates as indicated on the branch of the tree. The AGI code and *AtRLP* gene number is indicated on the left. Genes are organized according to the order along the chromosomes.

Middle Domain organizations as predicted by SMART/Pfam. Each colored box represents a domain as indicated. The arrowhead shows the putative N-terminal transmembrane domain. The open box means an amino acid fragment not showing any significant motif or domain.

Right *RLP* gene structure presented by gray boxes for exons and spaces for the introns.

Assembly of a Genome-Wide Collection of *AtRLP* Gene T-DNA Insertion Mutants

To identify putative T-DNA insertion lines for all the *AtRLP* genes we queried the T-DNA Express database of the SALK Institute Genome Analysis Laboratory (SIGnAL; http://signal.salk.edu). Since often several different insertion lines could be identified for each *AtRLP* gene, insertion lines were selected based on the position of the T-DNA insertion within the coding sequence, to enhance the likelihood of successful disruption of gene function. Preferably, T-DNA insertion lines of the Columbia (Col-0) ecotype were selected with exon insertions (Table 1). However, if not available, lines with predicted intron (one line), promoter (11 lines) or terminator (four lines) insertions were chosen. For the 57 *AtRLP* genes, in total 89 T-DNA insertion lines were selected (Table 1) that were evaluated for presence of the predicted T-DNA insertion using PCR (Supplemental Table S2). Ten lines did not have the predicted insertion, whereas 79 were confirmed to carry a T-DNA insertion in the gene of interest and for which homozygosity of the T-DNA insert was pursued. For two T-DNA insertion lines, FLAG_524A03 and SALK_012745 with an insertion in *AtRLP19* and *AtRLP37*, respectively, only heterozygous insertion lines were obtained suggesting that homozygosity of these T-DNA mutations caused embryonic lethality. However, subsequent segregation and complementation analysis could not confirm embryo lethality caused by T-DNA homozygosity in these lines and they were not used for further analysis. Although we were able to identify another T-DNA insertion line for *AtRLP37* that was carried to homozygosity (Table 1), unfortunately, no alternative T-DNA insertion line was available for *AtRLP19*. Overall, in the complete collection of 77 homozygous *AtRLP* T-DNA insertion lines, at least one line was obtained for 56 of the 57 *AtRLP* genes, while for 19 *AtRLP* genes multiple mutants were identified (Table 1).

Phenotypic Alterations in Growth and Development of *AtRLP* Gene T-DNA Insertion Mutants

We examined the phenotypes of the complete collection of homozygous T-DNA insertion lines with respect to various different characteristics related to plant growth and development. The T-DNA lines were examined for root development, rosette growth, inflorescence emergence, and the development and appearance of flowers and seeds. In addition, stomatal patterning across the cotyledons and leaves, formation of the leaf cuticle, and the leaf vascular patterns were analyzed. Two *AtRLP* genes, *CLV2* (*AtRLP10*) and *TMM* (*AtRLP17*) have previously been implicated in plant development (Jeong et al., 1999; Nadeau and Sack, 2002). Our analysis showed that the T-DNA insertion lines *Atrlp10-1* and *Atrlp17-1* for the *CLV2* and *TMM* gene, respectively, displayed phenotypes that have previously been reported for a number of mutants in these genes (Yang and Sack, 1995; Kayes and Clark, 1998; Jeong et al., 1999; Nadeau and Sack, 2002). Similar to the ethyl methanesulfonate (EMS) mutant *tmm-1*, the stomata of the knock-out allele *Atrlp17-1* that carries a T-DNA in the ATG start codon of the coding sequence were found to cluster across the leaf epidermis (Fig. 2, A-D and J). Complementation of *Atrlp17-1* with the wild-type *TMM* allele resulted in disappearance of the stomatal clustering phenotype (Fig. 2E), showing that *Atrlp17-1* is a true *TMM* mutant allele. In addition, as expected, the *Atrlp10-1* mutant with a knock-out in the *CLV2* gene displayed enlarged shoot meristem (Fig. 3, D and E), and alterations in the development of the gynoecia, flowers, carpels, pedicels, and stamens (data not shown). Like other *CLV2* mutants, the *Atrlp10-1* mutant fails to respond to *in vitro* treatment with a synthetic peptide that corresponds to the conserved CLE motif that is present in the CLV3-like peptide ligands (Fig. 3H; Fiers et al., 2005). However, while the previously described *CLV2* mutants (*clv2-1* to *clv2-5*) generally have four carpels (Kayes and Clark, 1998), *Atrlp10-1* shows only a mild carpel phenotype with 2.6 carpels on average (Fig. 3N).

Interestingly, despite the relatively weak carpel phenotype, *Atrlp10-1* exhibits a number of phenotypes that have not previously been reported for any of the *CLV2* mutants (Fig. 3). Plants from the *Atrlp10-1* T-DNA insertion line grow slower, develop more rosette leaves and shorter stems, and flower at a later stage than wild-type plants and the *clv2-3* mutant (Fig. 3, I-M). During flowering, the meristem of the main inflorescence stops producing flowers for a short period, upon which flowering is resumed (Fig. 3, A, B and G). However, side stems do not show this temporary termination of the flower meristem. Linkage analysis in a segregating population has demonstrated that the temporary termination of flowering phenotype is linked to a homozygous T-DNA knockout in *Atrlp10-1*. Moreover, complementation of *Atrlp10-1* with the wild-type *CLV2* allele restored all *clv2* mutant phenotypes (Fig. 3, C and L-N).

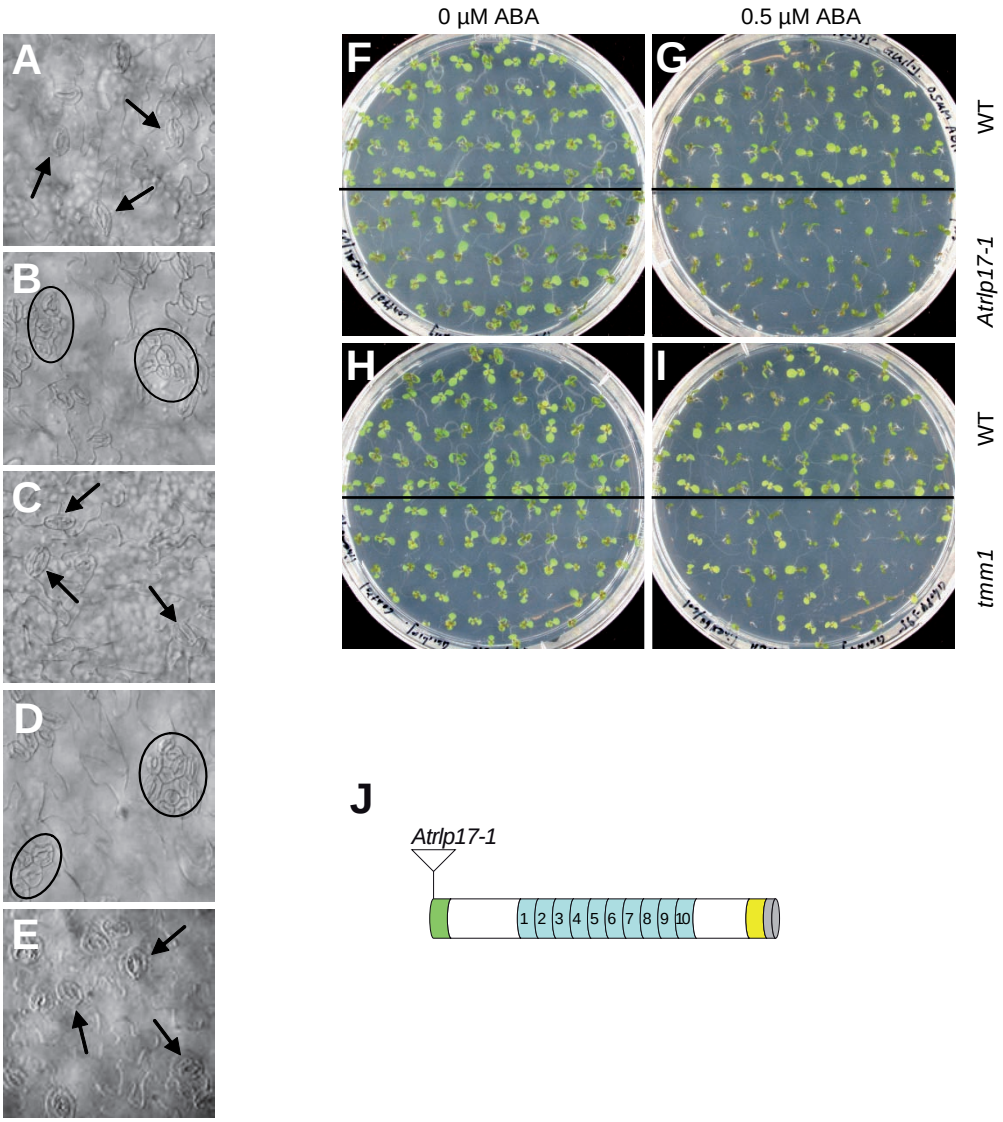

Figure 2. Characterization of the *AtRLP17-1* mutant allele.

A-E Comparison of stomata distribution of wild-type (A and C) with *AtRLP17-1* (B), *tmm-1* mutant (D), and *Atrlp17-1* mutant after complementation with a wild-type *TMM* allele (E). The arrows indicate single stomata, while the circles indicate stomatal clusters.

F-I Comparison of ABA response of wild-type (top half of the plate) with *Atrlp10-1* (F and G, bottom half of the plate) or *tmm-1* (H and I; bottom half of the plate) in the absence (F and H) and presence (G and I) of ABA.

J Location of T-DNA insertion in *Atrlp17-1*.

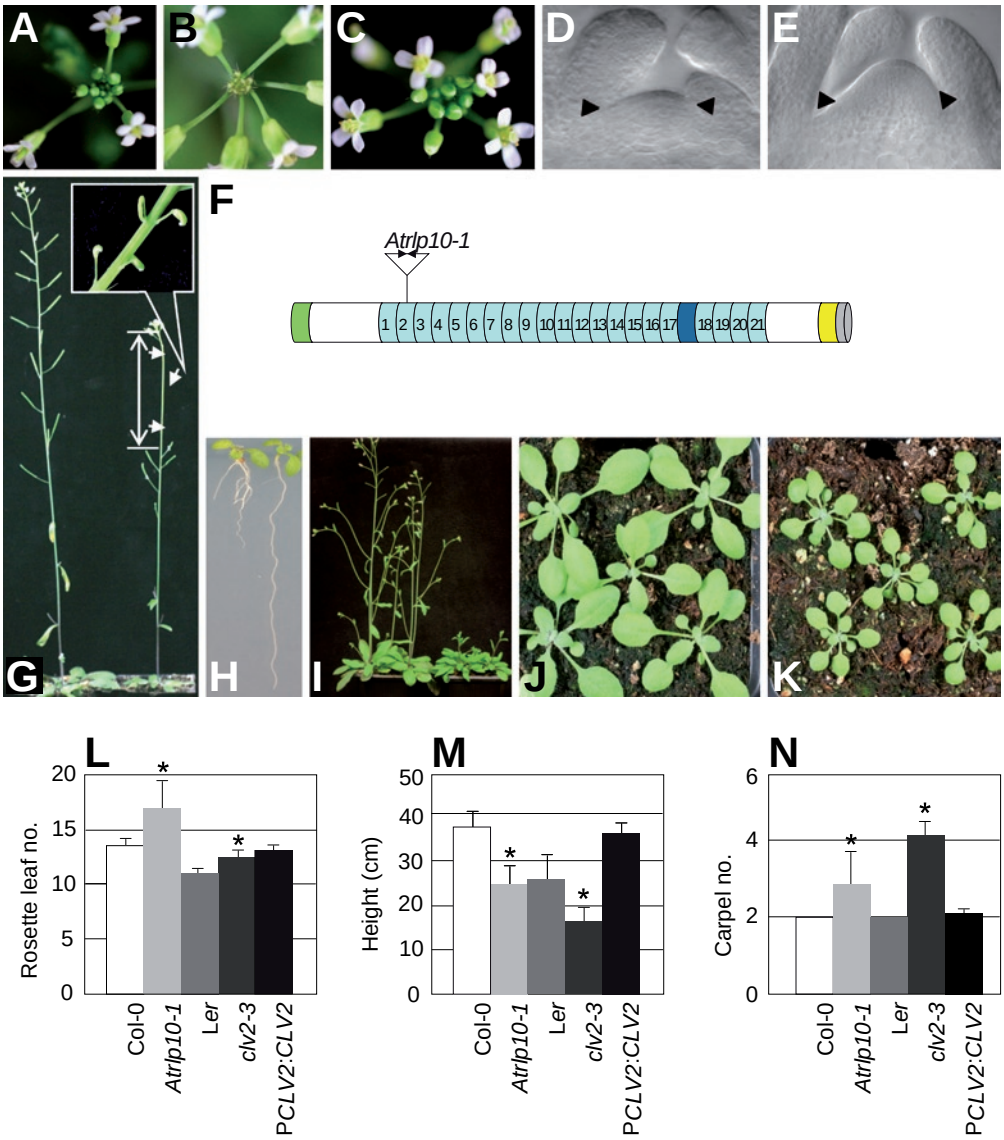

Figure 3. Characterization of the *AtRLP10-1* mutant allele.

A Wild-type inflorescence meristem. **B** *Atrlp10-1* inflorescence meristem. **D, E** Cleared shoot meristem of wild-type (D) and *Atrlp10-1* (E). Arrowheads indicate meristem borders. **F** Location of T-DNA insertion in *AtRLP10* (*CLV2*). **G** Comparison of inflorescence development of wild-type (left) with *Atrlp10-1* mutant (right). The zoom-in picture indicated no siliques were developed because of the temporary termination of inflorescence meristem of *Atrlp10-1* mutant. **H** The eight day-old wild-type seedling (left) showed a short root phenotype while *Atrlp10-1* (right) shows no effect with 10 µM CLV3p treatment. **I** Comparison of four-week-old plants of wild-type (left) with *Atrlp10-1* mutant (right). **J, K** Comparison of two-week-old plants of wild-type (J) with *Atrlp10-1* mutant (K).

L-N The mean of the rosette leaf number (N), height of the primary stem (M), and carpel number (N) of wild-types, *clv2-3*, *Atrlp10-1* and *Atrlp10-1* upon complementation with the wild-type *CLV2* allele. Asterisks indicate significant differences ($P < 0.01$) compared to the respective wild-types.

Conditional Phenotypic Alterations of *AtRLP* Gene T-DNA Insertion Mutants

We tested the collection of T-DNA lines for altered conditional developmental phenotypes including gravitropism, response to darkness or treatment with different hormones and a CLV3-like peptide ligand (Supplemental Table S3). For most of the treatments, no consistent differential responsiveness within the collection of *AtRLP* gene knock-out lines was observed (data not shown). The only treatment that resulted in a reliable phenotype was a treatment with the plant hormone abscisic acid (ABA). In addition to the previously described stomatal clustering phenotype, *tmm-1* and *Atrlp17-1* that both carry a mutation in the *AtRLP* gene *TMM* displayed decreased sensitivity to ABA. Although seedlings of non-treated *Atrlp17-1* and *tmm-1* mutants were phenotypically indistinguishable from control plants (Fig. 2, F and H), exogenous application of ABA induced chlorosis in control plants but not in mutants, and reduced the growth of *Atrlp17-1* and *tmm-1* mutants (Fig. 2, G and I) in comparison to the respective control plants. These results indicate that TMM plays a role in ABA-induced chlorosis and growth reduction in Arabidopsis.

Assessment of the Roles of *AtRLP* Genes in Plant Defense

To determine whether *AtRLP* genes play a role in the perception and signaling of abiotic stress signals, we have tested the sensitivity of the collection of T-DNA insertion lines for several abiotic stress inducers. These included inducers of salt stress, osmotic stress, drought stress, reactive oxygen stress and heavy metal stress (Supplemental Table S3). No consistent phenotypic alterations were observed for any of these abiotic stress stimuli within the collection of T-DNA mutant lines in comparison to wild-type plants.

We have also investigated the possible roles of *AtRLP* genes in the recognition of plant pathogens. The collection of T-DNA insertion lines was assessed for altered phenotypic responses upon pathogen challenge with a diverse range of host-adapted and non-adapted necrotrophic or biotrophic pathogens (Thomma et al., 2001). Non-adapted pathogens are pathogenic on other hosts but normally unable to colonize Arabidopsis. The bacterial pathogens *Pectobacterium atrosepticum, Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), and *Xanthomonas campestris* pv. *campestris*; the fungal pathogens *Alternaria brassicicola, Botrytis cinerea, Cladosporium cucumerinum, C. fulvum, Colletotrichum destructivum, Oidium neolycopersici, Plectosphaerella cucumerina, Sclerotinia sclerotiorum*, and *Verticillium dahliae*, and the oomycetes *Phytophthora infestans* and *Hyaloperonospora*

parasitica were among the pathogens tested (Supplemental Tables S3 and S4). Remarkably, none of the T-DNA insertion lines showed a significant phenotypic alteration in their sensitivity towards these pathogens.

Examination of nonhost interactions was extended using the non-pathogenic bean pathogen *P. syringae* pv. *phaseolicola* strain 1448A (*Psp* 1448A) that is unable to colonize wild-type Col-0 due to changes to the challenged plant cell wall rather than a hypersensitive response (Soylu et al., 2005; de Torres et al., 2006). Colonization by *Psp* 1448A is known to be enhanced in Col-0 *fls2* mutants that lack the ability to perceive flagellin, irrespective whether inocula are applied to the leaf surface or infiltrated directly into the mesophyll (Zipfel et al., 2004; de Torres et al., 2006). The response of *AtRLP* T-DNA insertion lines to infiltration with bacterial suspensions was examined, and symptom development compared with both wild-type and *fls2* mutant plants in each set of experiments. We initially recorded the development of yellowing and patchy collapse of infiltrated tissues using an incremental seven point scoring system. Lines revealing differences in reaction compared with the wild-type Col-0 in the first experiment were further assessed by repeated tests including measurement of bacterial multiplication. The mutant *Atrlp30-1* recorded consistently enhanced symptom development and more bacterial multiplication with *Psp* 1448A (Fig. 4, A-C). Subsequently, additional insertion mutants in *AtRLP30* recovered from SALK stocks were likewise examined for their reaction to *Psp* 1448A (Fig. 4, D and H; Table 2). In all cases, enhanced symptom development was recorded (Table 2) that was associated with the recovery of higher mean numbers of bacteria from infiltrated tissue (Fig. 4D). Student's t-tests indicated that all of the mutants allowed significantly higher multiplication than Col-0 ($P = 0.05$, 0.01 and 0.08 for *Atrlp30-1*, *Atrlp30-2*, and *Atrlp30-3*, respectively). In all cases, the enhanced symptom development in *AtRLP30* T-DNA mutant lines was lower than observed in the Col-0 *fls2* mutant (Fig. 4, A-C; Table 2). Similar as for *Atrlp30* mutants, enhanced susceptibility towards *Psp* 1448A was recorded for *Atrlp18-1* mutants. However, we were unable to further confirm the phenotype due to absence of additional lines with T-DNA insertions in *At2g15040*.

Examination of the enhanced susceptibility phenotype of *Atrlp30* mutants was extended by examining *Pst* strains that carry the avirulence genes *AvrRpm1*, *AvrRpt2*, *AvrRps4*, *AvrPto* and *AvrPtoB*, and furthermore an *hrpA* and *hrcC* mutant of *Pst*, a coronatine-deficient *Pst* mutant and the non-adapted strain *P. syringae* pv. *tabaci*. However, *Atrlp30* mutants did not display enhanced susceptibility to any of these bacterial strains.

Because of its potential role in basal defense we examined the subcellular localization of the AtRLP30 protein in Arabidopsis. Transgenic plants expressing C-terminal YFP-tagged AtRLP30 were generated and examined by confocal microscopy.

A clear localization of YFP-tagged AtRLP30 to the plasma membrane was, as predicted, observed in the leaf epidermis (Fig. 4F) and petiole tissue (Fig. 4E), which could also be confirmed by western analysis using an antibody directed against the HA tag (Fig. 4G).

The enhanced susceptibility of the *Atrlp30* and *Atrlp18-1* T-DNA insertion mutants to *Psp* 1448A could be explained by an altered responsiveness to the pathogen-associated molecular pattern (PAMP) flagellin. Examination of expression data showed that *AtRLP30* is induced by various PAMPs, including flg22 (Supplemental Fig. S3). We therefore compared the effect of the flg22 flagellin peptide derived from *Psp* 1448A on the seedling growth of Col-0 and the *Atrlp30-1* T-DNA insertion mutant, but no differences were observed (Supplemental Fig. S3). The reduced basal defense observed in the *AtRLP30* mutant was therefore through a route other than flagellin perception. The analysis of response to flg22 was extended to the whole collection of *AtRLP* T-DNA insertion mutants. In no case was any significant alteration in the inhibition of seedling growth observed (Supplemental Table S5). Similarly, none of the *Atrlp* mutant lines had a significant alteration in its response to the necrosis-inducing elicitor protein from *Botrytis cinerea*, BcNEP1 (Schouten et al., 2008), compared to the controls.

Table 2. Symptom development in leaves of Col-0 and mutant lines after syringe inoculation with *Pseudomonas syringae* pv. *phaseolicola* strain 1448A.

Plant	DPI	Frequency of lesion type [a]								Mean score (SD) [b]
		0	1	2	3	4	5	6	7	
Col-0	4	4	5	15						1.46 (0.8)
	6	3	2	9	8	2				2.29 (1.0)
Atrlp30-1	4	1	2	9	2	10				2.75 (1.2)*
	6		5	5	6	7	1			3.75 (1.2)*
Atrlp30-3	4	1	3	11	3	6				2.42 (1.1)*
	6		2	2	7	7	6			3.54 (1.2)*
Atrlp30-4	4	2	2	7	6	7				2.58 (1.2)*
	6		1	3	4	7	9			3.83 (1.2)*
Col-*fls2*	4			2	4	9	7	2		4.13 (1.0)*
	6				3	3	8	6	4	5.21 (1.3)*

a Three half leaves on eight plants were infiltrated with bacteria at OD_{600} 0.25 (approximately 2×10^8 cells mL^{-1}). Symptom development was scored after four and six days and sites assigned to each progressive category; 0, no symptoms; 1, very pale yellowing; 2, pale yellowing; 3, yellowing over most of the area infiltrated; 4, pale yellowing with patchy collapse; 5, yellow with patchy collapse; 6, collapse of more than 50% of infiltration site; 7, collapse of all the infiltrated area. Lack of a number means no sites in the category.

b Asterisks indicate significant differences ($P < 0.1$) compared to Col-0 at the respective time points.

Mining of *AtRLP* Expression Data to Uncover Additional *AtRLP*-Regulated Biological Processes

In our unbiased screenings, few novel biological roles have been uncovered for *AtRLP* genes. To gain additional insight into the possible biological processes in which *AtRLP* genes are involved, the Genevestigator online search tool Meta-Analyzer (Zimmermann et al., 2004) was used (Supplemental Fig. S2). This analysis revealed that the expression of the *AtRLP* genes in the context of different organs, growth stages, and stress responses is very diverse. Most *AtRLP* genes are expressed in many organs and developmental stages. *AtRLP4*, which was predicted as putative developmental orthologs (Fritz-Laylin et al., 2005), is ubiquitously and highly expressed across almost all the developmental stages and organs, confirming a potential basic function in plant development (Supplemental Figs. S1 and S2). However, the development of the *Atrlp4-1* mutant is indistinguishable from that of wild-type plants. Some *AtRLP* genes are specifically expressed in only one or a few organs, such as *AtRLP5*, *AtRLP8*, *AtRLP11*, *AtRLP45*, and *AtRLP48* that are mainly expressed in pollen (Supplemental Fig. S2), suggesting they may play a role at the reproductive stage. However, no defective pollen phenotypes were observed for mutants in those respective genes. The stress response expression data upon challenge with pests and pathogens, hormones and abiotic stress factors (Supplemental Fig. S2) show differential expression patterns for all *AtRLP* genes. Strikingly, *AtRLP48* is highly induced only upon hormone treatment, and for two hormone treatments (ABA and zeatin), *AtRLP48* is the only *AtRLP* gene induced. Nevertheless, *Atrlp48-1* showed no phenotype upon treatment with these hormones (data not shown).

As many as 25 *AtRLP* genes (*AtRLP2-4*, *7*, *13*, *19*, *20*, *22*, *23*, *26*, *28*, *34-38*, *40-43*, *46*, *47*, *50*, *52* and *54*) are predominantly expressed in senescent leaves (Supplemental Fig. S2). Of these, five AtRLP-encoding genes (*AtRLP7*, *20*, *28*, *36*, and *42*) are almost exclusively induced in senescent leaves (Supplemental Fig. S2), suggesting a possible function in senescence-related processes. Therefore, we tested whether the 25 *AtRLP* genes are involved in senescence-related processes by subjecting leaves of the corresponding mutants to submergence in ABA. Most of the mutants did not show any altered phenotypes. However, three independent T-DNA insertion lines (Salk_024020, SM_3_20242, SM_3_38956) of *AtRLP41* displayed enhanced-sensitivity upon exogenous application of 100 µM ABA, since the mutant leaves were bleached while wild-type leaves remained green (Fig. 5A). Therefore, our results indicate that *AtRLP41* plays a role in ABA responses.

Previously, *AtRLP51* was reported to be locally induced in roots by the non-pathogenic, root-colonizing rhizobacterium *Pseudomonas fluorescens* WCS417r (Verhagen et al., 2004). This bacterium activates induced systemic resistance (ISR) against a broad range of pathogens (Pieterse et al., 1996).

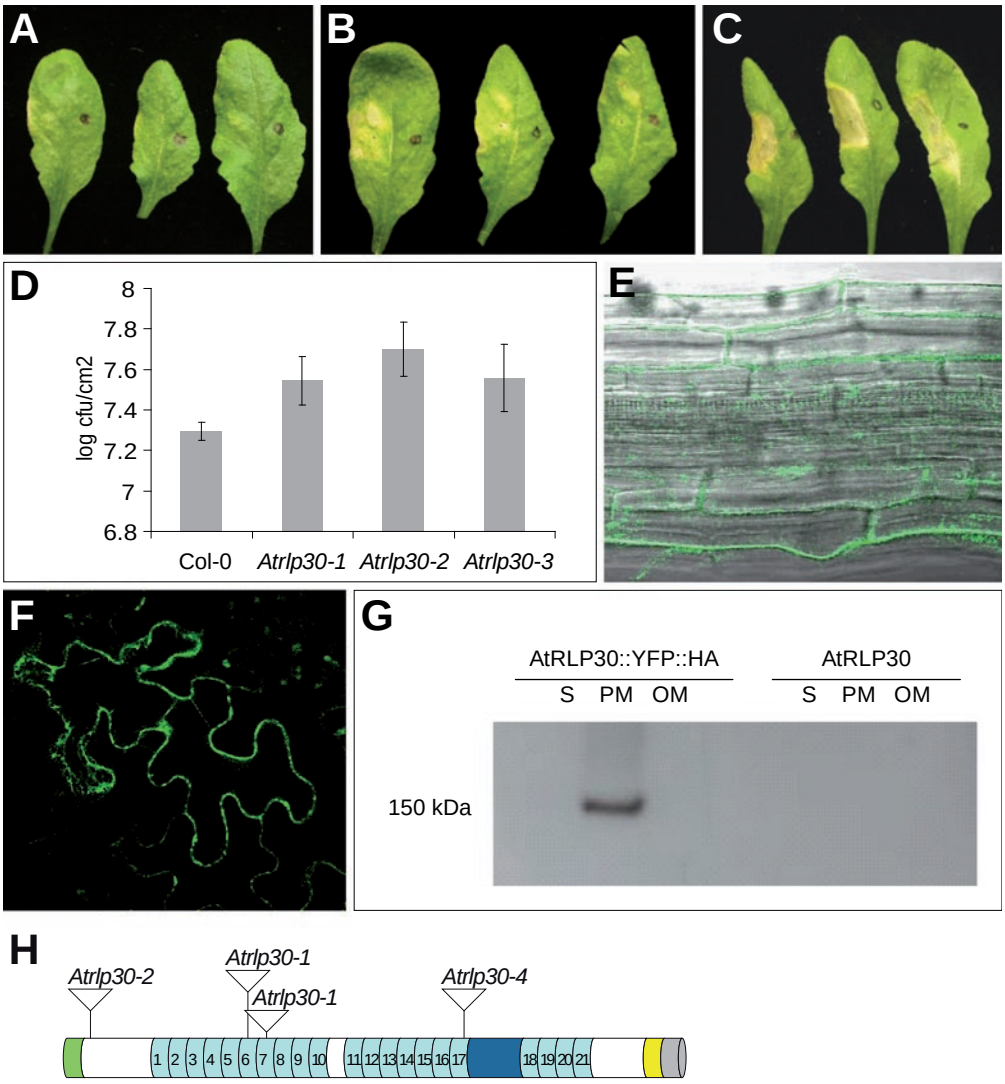

Figure 4. AtRLP30 is involved in bacterial resistance and localized at the plasma membrane.

A-C Symptom development in Arabidopsis leaves four days after inoculation with *Pseudomonas syringae* pv. *phaseolicola* (*Psp*). Areas in half leaves of Col-0 (A); *Atrlp30-1* (B) and Col-0 *fls2* (C) were syringe inoculated after wounding. Full details of symptom scores are recorded in Table 2.

D Comparative analysis of the multiplication of *Psp* 1448A in Col-0 and *Atrlp30* mutant plants. Infiltrated leaves were examined three days after inoculation; results are means from four replicates with SES. Statistical analysis using Student's t-test showed significantly higher numbers of bacteria in the mutants (P = 0.047, 0.014, and 0.088 for *Atrlp30-1*, *-2*, and *-3*, respectively).

E-G Localization of YFP-tagged AtRLP30 in leaf epidermis and petiole tissue as determined using confocal microscopy (E and F) and western blotting with an antibody directed against the HA tag (G).

H Locations of the T-DNA insertions in *AtRLP30*.

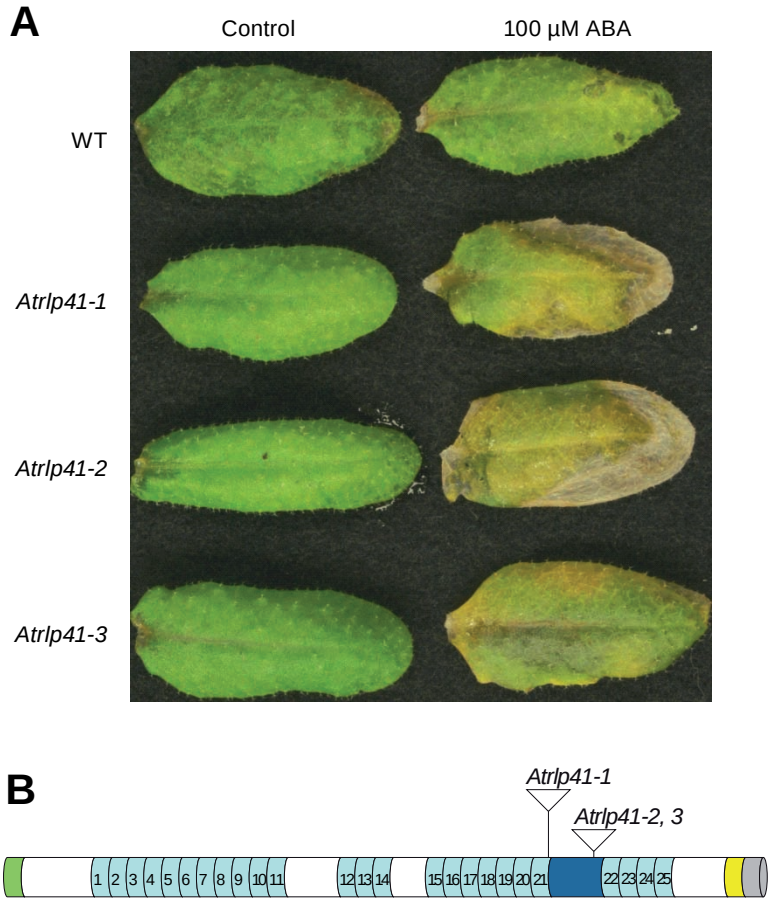

Figure 5. Characterization of the *AtRLP41* mutant alleles.
A Comparison of the leaf phenotype of wild-type with mutants, *Atrlp41-1*, *Atrlp41-2* and *Atrlp41-3* after exogenous application of ABA (right). **B** The location of T-DNA in *Atrlp41-1*, *Atrlp41-2* and *Atrlp41-3*.

To investigate the role of *AtRLP51* in activation of ISR, we tested the two T-DNA insertion mutants *Atrlp51-1* and *Atrlp51-2* for their ability to express ISR upon treatment with *P. fluorescens* WCS417r. After treatment, plants were inoculated with *Pst* DC3000 or with *B. cinerea*. While wild-type and mutant plants grown in non-infested control soil showed full susceptibility, both wild-type and the mutants developed similar levels of ISR towards these pathogens in soil infested with *P. fluorescens* indicating that *AtRLP51* is not involved in ISR (data not shown).

DISCUSSION

We have undertaken a reverse genetic approach to genome-wide study the role of *RLP* genes in Arabidopsis. Previously, a total of 56 *AtRLP* genes have been identified (Fritz-Laylin et al., 2005). In this study, we identified one additional putative *AtRLP* gene, *AtRLP5*, which corresponds to At1g34290. Although this gene carries only two eLRRs, it complies with the canonical RLP domain composition. Moreover, it has been noted that the number of LRR units of resistance genes and resistance gene analogs can be highly variable, ranging from one to over two dozen, which is likely to be caused by illegitimate recombination (Wicker et al., 2007). We assembled a genome-wide collection of T-DNA knock-out mutants that comprises at least one insertion mutant for 56 of the 57 *AtRLP* genes. We could not obtain any insertion line for just one of the *RLP* genes, *AtRLP19*, which may indicate that insertions in this specific *AtRLP* gene cause lethality. In total 77 homozygous insertion lines in *AtRLP* genes have been collected that have all been assessed for phenotypic alterations in plant growth and development, and for altered responsiveness to various external stimuli including abiotic stress triggers and microbial pathogens. Previously, biological roles have been assigned to only two *AtRLP* genes, *CLV2* and *TMM* (Jeong et al., 1999; Nadeau and Sack, 2002), while the biological functions of the remaining 55 *AtRLP* genes have remained elusive so far.

In this study, a number of additional novel phenotypes were found for insertion mutants in the *CLV2* and *TMM* genes. Previous studies have demonstrated that mutations in any of the three *CLV* genes result in enlargement of meristems and increased floral organ numbers (Clark et al., 1993, 1995). Our *CLV2* T-DNA insertion allele (*AtRLP10-1*) was found to grow slower, develop more rosette leaves and shorter stems, and develop flowers at a later stage than wild-type plants or *clv2-3* mutants. Furthermore, the meristem of the main inflorescence was found to terminate flowering for a short period, upon which flowering resumed, resulting in an irregular distribution of siliques over the main stem. These novel phenotypes were found to be linked to the T-DNA insertion in *CLV2* and can be complemented by introduction of the wild-type *CLV2* gene (G. Wang, unpublished results). Possibly, they may be attributed to the genetic background of the mutation as the T-DNA insertion is a mutant of the Col-0 ecotype, while all other previously described *clv2* mutants are backcrossed into the Landsberg *erecta* (L*er*) ecotype (Kayes and Clark, 1998). The progeny of crosses between *AtRLP10-1* and L*er* wild-type plants developed a strong carpel phenotype that is comparable to *clv2* alleles in the L*er* ecotype: more rosette leaves and reduced height without transient termination of the main inflorescence (G. Wang, unpublished results). This suggests that the transient termination of the main inflorescence in *AtRLP10-1* is most likely due to interplay within the genetic background of Col-0.

Previously, *TMM* has been shown to control the initiation of stomatal precursor cells and determine the orientation of the asymmetric divisions that pattern stomata (Geisler et al., 2000; Nadeau and Sack, 2002). In our *TMM* T-DNA insertion mutant (*AtRLP17-1*), we also observed the typical stomatal clustering phenotype. In addition, we found that mutations in *TMM* also displayed altered sensitivity to ABA. Growth of the *TMM* mutants was reduced upon exogenous application of ABA, while the induced chlorosis that is observed in control plants after ABA treatment was not observed. It has long been known that during early stages of drought, plant roots produce ABA that is transported with the transpiration stream and acts as a physiological signal to close stomata (Davies and Zhang, 1991). The actual closure is established by an increase of the Ca^{2+}-concentration in the guard cell cytoplasm (Schroeder and Hagiwara, 1989). At present it is not known how *TMM* regulates stomatal distribution, but ABA sensitivity might be a crucial factor in this process. Apart from *TMM*, a visible altered phenotype upon ABA treatment could be identified for *AtRLP41*, since the corresponding mutants *AtRLP41-1* to *AtRLP41-3* showed enhanced sensitivity to exogenous application of ABA. Nevertheless, for these mutants no abnormalities in stomatal patterning could be observed. *AtRLP41* appeared to be highly induced during plant senescence, and, since ABA is known to be able to act as an inducer of senescence it is tempting to speculate that *AtRLP41* is involved in ABA-induced senescence responses, although *AtRLP41* mutants did not show any phenotypic alterations at this stage. However, ABA also plays important roles in other processes, including seed development and dormancy (Christmann et al., 2006), which might explain why expression at senescence stages has been reported. Although ABA receptors have not been identified yet, it has been demonstrated that an RLK called RPK1 is involved in early ABA perception in Arabidopsis (Osakabe et al., 2005). Reminiscent to the situation as occurs with the RLK CLV1 that interacts with the RLP CLV2, RPK1 may interact with TMM1 or AtRLP41 to constitute an ABA receptor complex.

Interestingly, it was recently shown that TMM negatively regulates three RLKs during the process of stomatal differentiation, one of which is ERECTA that also controls organ size and shape (Torii et al., 1996; Shpak et al., 2005). In addition, it was recently found that *ERECTA* also regulates plant transpiration efficiency, as *ERECTA* was found to modulate stomatal density through a role in epidermal pavement cell expansion (Masle et al., 2005). Possibly, TMM functions as an anchor protein for multiple RLKs in different signaling processes. A similar situation has recently been demonstrated for the RLK protein BAK1/SERK3 that not only interacts with the RLK BRI1 to modulate brassinosteroid signaling and thus regulate brassinosteroid-dependent growth (Li et al., 2002; Russinova et al., 2004), but also interacts with the RLK FLS2 that acts as a PAMP receptor for bacterial flagellin and

functions in innate immunity in a brassinosteroid-independent manner (Chinchilla et al., 2007; Heese et al., 2007). It is anticipated that BAK1 interacts with additional innate immune receptors since it also regulates full responses to PAMPs that are not related to flagellin, the containment of microbial infection-induced cell death, and restriction of various bacterial, fungal and oomycete infections (Chinchilla et al., 2007; Heese et al., 2007; Kemmerling et al., 2007). The participation of specific receptor proteins in different receptor complexes may explain why some of these receptors play roles in processes as diverse as plant development and pathogen defense. This is not only the case for BAK1, but also for ERECTA that, in addition to development (Torii et al., 1996; Masle et al., 2005; Shpak et al., 2005) also plays a role in defense (Godiard et al., 2003; Llorente et al., 2005).

Remarkably, among the genome-wide collection of *AtRLP* T-DNA insertion mutants, visibly altered phenotypes were observed for only the four genes *CLV2*, *TMM*, *AtRLP41*, and *AtRLP30*, even though a wide range of developmental stages and treatments were tested. In other plant species, by far most *RLP* genes have been implicated in mediating microbial perception, mostly as pathogen resistance genes (Kruijt et al., 2005). In Arabidopsis, *AtRLP52* has been implicated in resistance against the powdery mildew pathogen *Erysiphe cichoracearum* (Ramonell et al., 2005). Interestingly, it was observed that this specific AtRLP is also required for full resistance against the barley pathogen *Blumeria graminis* f. sp. *hordei* (J. Mansfield, unpublished data). However, in this study, it is rather surprising that only two of the T-DNA insertion lines in the *AtRLP* genes, *AtRLP18* and *AtRLP30*, displayed altered susceptibility upon pathogen challenge. Four independent mutations in *AtRLP30* were found to affect Arabidopsis nonhost defense against the non-adapted bean pathogen *Psp*, although the mutants were not as susceptible as *fls2* mutants defective in the perception of bacterial flagellin. This suggests that, rather than acting as a true resistance gene like all other *RLPs* that have been characterized in plant defense, both AtRLP18 and AtRLP30 act as components of basal defense. Interestingly, defense against another non-adapted *P. syringae* strain (pv. *tabaci*) was not compromised, while defense against weakly pathogenic *Pst* strains (*hrpA*, *hrcC*, and coronatine mutants) also appeared to be intact. In tomato, the *RLP* genes *Ve1* and *Ve2* have been implicated in resistance against race 1 strains of the vascular pathogen *V. dahliae* (Kawchuk et al., 2001), which also is a pathogen of Arabidopsis (Fradin and Thomma., 2006). Nevertheless, none of the *AtRLP* insertion lines was found to display altered *V. dahliae* susceptibility. Based on sequence comparison and bioinformatic analysis it has been suggested that the vast majority of the *AtRLP* genes were likely to act as disease resistance genes. Despite screening a broad spectrum of pathogens with different colonization and feeding styles, we have so far not been able to support this hypothesis. Possibly, this is the consequence of not having used the correct

pathogen strains against which these genes are active. Alternatively, the *AtRLP* genes may not act as race-specific disease resistance genes, but rather play a role in nonhost resistance or basal host defense. In such case the array of potential microbial targets may be dramatically increased and the response to more microbes or even insects and nematodes should be tested (Stout et al., 2006).

The lack of identification of biological functions for *AtRLP* genes may also be explained by functional redundancy, a phenomenon that typically obscures studies employing reverse genetics strategies as has been described for MADS-box transcription factors (Pãrenicová et al., 2003) and *RLK* gene family members (Albrecht et al., 2005; DeYoung et al., 2006; Hord et al., 2006). It has been suggested that CLV1 and CLV2 heterodimerize to form a receptor complex for the secreted CLV3 signaling peptide (Jeong et al., 1999; Ogawa et al., 2008). However, when compared to *clv1* and *clv3* alleles, *clv2* mutants display relatively weak phenotypes, since fasciation in *clv2* mutants is rarely observed and only under short day growth conditions (Kayes and Clark, 1998). This may suggest that the role of CLV2 is indeed redundant, although the finding that CLV2, but not CLV1, can perceive the conserved CLE motif of CLV3-like peptides argues against this hypothesis (Fiers et al., 2005). Current strategies employ RNA interference experiments to interfere with the expression of multiple *AtRLP* genes at the same time, and thus possibly overcome functional redundancy among *AtRLP* genes. The RNA interference lines that are silenced for multiple *AtRLP* genes can be screened with the various abiotic and biotic stress factors to find biological roles for these *AtRLP* genes.

Materials and Methods

Bioinformatic Analysis

To investigate the structure of *AtRLP* genes, BLAST queries were performed using Arabidopsis CLV2 and TMM and tomato Cf-9 predicted protein sequences to search translated sequences from the Arabidopsis genome. SMART (http://smart.embl-heidelberg.de), PFAM (http://pfam.janelia.org), SignalP (http://www.cbs.dtu.dk/services/SignalP), and TMHMM (http://www.cbs.dtu.dk/services/TMHMM) were used for domain predictions. The exon/intron boundaries were investigated using GenScan (http://genes.mit.edu/GENSCAN.html), refined using SeqViewer at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org) and visualized using Jellyfish software (Riethof and Balakrishnan, 2001).

Identification and Analysis of T-DNA Insertion Mutants

The database at the SALK Institute Genome Analysis Laboratory (SIGnAL; Alonso et al., 2003; http://signal.salk.edu) was searched to identify putative T-DNA insertion mutants, of which the available lines of interest were obtained from the Nottingham Arabidopsis Stock Center (NASC; http://www.arabidopsis.info), GABI-Kat (Rosso et al., 2003; http://www.gabi-kat.de/), or Genoplante FLAGdb/FST (Balzergue et al., 2001; http://urgi.infobiogen.fr). Correct insertion of the T-DNA in these lines was determined with PCR. Genomic DNA was isolated from individual plants that belong to the respective T-DNA insertion lines and used in two separate PCR reactions with different primer sets (Supplemental Table S2). One contained a gene-specific primer and a T-DNA specific primer to check for the presence of the insertion, and the second PCR contained two gene-specific primers spanning the proposed insertion site to check for non-disrupted alleles. Plants for which the PCR with a gene-specific primer and a T-DNA specific primer yielded a product, while the PCR with the two gene-specific primers did not yield a product were considered homozygous insertion lines, which was confirmed in plants from the subsequent generation.

Plant Growth Conditions

Arabidopsis plants of the ecotypes Columbia (Col), Wassilewskija (Ws) and Landsberg *erecta* (L*er*) were used. Soil-grown plants were cultured either in a growth chamber at 22ºC, 72% relative humidity, and usually a 16 h photoperiod, or in a greenhouse at 21°C during the 16 h day period and 19°C during the night period at 72% relative humidity. In the greenhouse, supplemental light (100 Wm^{-2}) was used when the sunlight influx intensity was below 150 Wm^{-2}.

For *in vitro* growth of Arabidopsis, seeds were surface-sterilized and sown on Murashige and Skoog (MS) medium (Duchefa, Haarlem, NL) solidified with 1.5% plant agar (Duchefa, Haarlem, NL). After sowing, the plates were incubated at 4°C in the dark for three days and subsequently transferred to the growth chamber.

Phenotypic Evaluations of Plant Growth and Development

For phenotypic evaluations of plant growth and development, Arabidopsis plants were grown on half-strength MS medium, supplemented with 1% sucrose and 0.5 g/L MES (2-(N-morpholino) ethane-sulfonic acid), pH 5.8. After two weeks, plants were transferred to soil for further observations. To assess seed morphology, siliques from the primary inflorescences were screened for seed abortion using a dissection microscope (Tzafrir et al., 2004). Seeds at different developmental stages were mounted in clearing solution (Sabatini et al., 1999) and cleared samples were observed using a Nikon optiphot microscope equipped with Normarski optics. To score vascular patterning and stomatal distribution, cotyledon and rosette leaves were cleared by immersion in ethanol:acetic acid (3:1), subsequently rinsed in 70% ethanol and incubated in 100% ethanol at 4°C overnight (Jun et al., 2002). The leaves were observed using a dissecting microscope for vascular patterning and Normaski optics for the stomatal distribution. Finally, root geotropism was studied by growing seedlings on vertically oriented half-strength MS plates that were rotated 90° after six days of growth. After ten hours, the bending angle of the root was measured (Sedbrook et al., 2002).

Conditional Phenotype Assays

To assess susceptibility toward abiotic stress, seeds were sown on MS plates amended with NaCl (100 or 150 mM), LiCl (20 or 30 mM), mannitol (150 or 200 mM) or H_2O_2 (3.3 or 6.7 mM) and evaluated for aberrant growth. To assay heavy metal resistance, plants were grown vertically on half strength MS medium amended with 2% (w/v) sucrose and 85 µM $CdCl_2$ (Lee et al., 2003).

To test whether *AtRLP* genes are involved in responsiveness to hormones, the sterilized seeds were grown on vertically oriented half-strength MS plates containing different hormones at different concentration (Supplemental Table S3).

To screen whether *AtRLP* genes are involved into leaf senescence, detached leaves were floated on 3 mM MES (2-(N-morpholino) ethanesulfonic acid monohydrate) buffer, pH 5.8, in the presence of 50 µM or 100 µM abscisic acid (ABA), 50 µM methyl jasmonate (MeJA), 5 µM ethylene or 1 µM epibrassinolide (He et al., 2001).

Pathogen Cultivation

Alternaria brassicicola (strain MUCL20297; Mycotheque Université Catholique de Louvain, Louvain-la-Neuve, Belgium), *Cladosporium cucumerinum*, *C. fulvum*, *Plectospaerella cucumerina* (Thomma et al., 2000), *Sclerotinia sclerotium* strain ND30 and *Verticillium dahliae* strain ST37.01, were maintained on potato dextrose agar (Oxoid, Hampshire, UK). *Botrytis cinerea* (Brouwer et al., 2003) was grown on half-strength potato dextrose agar amended with 5 g/L agar and 150 g/L blended tomato leaves. *Colletotrichum destructivum* (strain IMI349061; CABI Bioscience, Egham, UK) was grown on Mathur's agar (Mathur et al., 1950). All fungal *in vitro* cultures were grown at 22°C. *Oidium neolycopersisi* (Bai et al., 2005) was maintained on Moneymaker tomato plants in the greenhouse. Two GFP transformants of the oomycete *Phytophthora infestans* strains 14.3 (Dr. Govers, Wageningen University, The Netherlands) and 208M2 (Dr. S. Kamoun, Ohio State University) were maintained on rye-agar (Caten and Jinks, 1968) at 18°C in the dark. Isolates of *Hyaloperonospora parasitica* were maintained as described (Tör et al, 2002). *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 with or without *avrRpt2*, *avrRpm1* or *avrRps4* were grown on King's B agar (King et al., 1954) supplemented with the appropriate antibiotics (25 µg/mL rifampicin and 100 µg/mL kanamycin). *Pectobacterium atrosepticum strain* LMG 6669 (Coordinated Collections of Micro-organisms, Ghent, Belgium) was maintained on nutrient agar (Oxoid, Hampshire, UK). *Xanthomonas campestris* pv. *campestris* (strain 568) was grown on Kado's medium agar (Kado and Heskett, 1970). All bacterial strain were grown overnight at 28°C.

Pathogen Inoculations

All pathogen (except *V. dahliae* and *H. parasitica*) inoculations were performed using soil-grown plants with fully expanded rosette leaves. Inoculum of all *in vitro* cultured fungi (except *S. sclerotiorum*) was prepared as previously described (Broekaert et al., 1990) and used as a suspension of 10^6 conidia per mL in water. Inoculations with *A. brassicicola*, *B. cinerea*, *C. destructivum* and *P. cucumerina* were performed by placing a 6-µl drop of the conidial suspensions on each expanded leaf (Thomma et al., 1998, 2000; Brouwer et al., 2003; O'Connell et al., 2004). *C. fulvum* and *C. cucumerinum* suspensions were sprayed as a mist on the adaxial sides of the leaves. For *V. dahliae* inoculations, two-week-old Arabidopsis plants were up-rooted, root tips were cut off, and incubated in the conidial suspension for one minute. Subsequently, the plants were re-planted into fresh soil. For *S. sclerotiorum*, three mycelium plugs from a culture plate were placed in a 300-mL flask containing 100 mL of potato dextrose broth (Difco, Detroit, USA) and grown for three days at 22°C with 150 rpm. Afterwards, the mycelium was homogenized in a blender. Leaves were inoculated by placing a 10-µl drop of

mycelium fragments (OD_{600} = 3.5) on each of the fully expanded leaves. For *P. infestans*, a rye-agar plate with 10-day old mycelium was incubated with sterile water at 4°C for two hours to release zoospores from zoosporangia. One 5-µl drop of a suspension of 10^5 zoospores per mL in water was placed on each fully expanded leaf. To avoid background fluorescence from superficial growing *P. infestans*, the drops were removed by drying with tissue paper after 36 h. For *O. neolycopersici*, 10^5 conidia per mL were used. The inoculation was performed as described by Bai et al. (2005). Inoculations of Arabidopsis seedlings with *H. parasitica* were performed as described (Tör et al, 2002).

For all bacterial inoculations, bacteria were grown overnight at 28°C in the appropriate medium supplemented with the appropriate antibiotics. Strains of *Ps. syringae* (except *P. syringae* pv. *phaseolicola*) and *P. atrosepticum* were spray-inoculated with a bacterial suspension of OD_{600} 0.3 supplemented with 0.05% [v/v] Silwet L-77 (van Meeuwen Chemicals BV, Weesp, NL). For *X. campestris*, two different inoculation methods were carried out (Meyer et al., 2005): infiltration of a concentrated bacterial suspension or wound inoculation.

For *P. syringae* pv. *phaseolicola* strain 1448A, three half leaves on eight plants were infiltrated with bacteria at OD_{600} 0.25 (approx 2×10^8 cells per mL). Symptom development was scored after four and six days and sites assigned to each progressive category; 0, no symptoms; 1, very pale yellowing; 2, pale yellowing; 3, yellowing over most of the area infiltrated; 4, pale yellowing with patchy collapse; 5, yellow with patchy collapse; 6, collapse of more than 50% of infiltration site; 7, collapse of all the infiltrated area. Bacterial numbers were recorded as described in de Torres et al. (2006).

For all inoculations, except those with *O. neolycopersici* and *V. dahliae*, plants were kept in boxes with transparent lids at high relative humidity for the remainder of the experiment. As positive control for the inoculations with *A. brassicicola*, *B. cinerea* and *P. cucumerina*, *pad3-1* mutant plants were used (Thomma et al., 1999, 2000; Kliebenstein et al., 2005). For *P. infestans*, the *pen2-1* mutant was used (Lipka et al., 2005), while for the Pseudomonas strains the genotypes *NahG* and *npr1-1* were used (Thomma et al., 1998). Finally, for *X. campestris* the ecotype Kas was used as positive control (Xu et al., 2008).

To test whether *AtRLP51* is involved in ISR expression, the ISR bioassay was performed as described in Pieterse et al. (1996) except for the challenge inoculation. For *P. syringae* and for *B. cinerea* the inoculations were performed as mentioned previously. Except for *P. syringae*, a lower concentration of a bacterial suspension of OD_{600} 0.3 five times diluted was used.

Response to Pathogen Elicitors

Flg22-induced seedling growth inhibition assays (Gomez-Gomez *et al*, 1999) were performed essentially as described (Pfund et al., 2004). After germination of Arabidopsis seeds for five days at 22˚C, two seedlings were transferred to 750 mL liquid MS medium in a 25 well plate either with or without 2 mg/L flg22 peptide (sequence; TRLSSGKINSAKDDAAGL). Each treatment was replicated five times. After two weeks further growth, the weights of the seedlings were recorded. Wassilewskija-0, Col-0 *fls2* (insensitive to flg22) and Col-0 (susceptible to flg22 growth inhibition) were used as controls in each experiment. Leaves of Arabidopsis plants were pressure infiltrated with the *Botrytis cinerea* elicitor protein BcNEP1 that was isolated from a *Pichia pastoris* culture heterologously expressing *BcNEP1*. A raw protein extract from culture filtrate containing the BcNEP1 protein was isolated as described (Schouten et al., 2008) and was ten times diluted in MMA (5 g/L MS salts (Duchefa, Haarlem, NL), 1.9 g/L MES (2-(N-morpholino) ethane-sulfonic acid)).

Localization of AtRLP30

AtRLP30 is predicted to contain a single exon, which was confirmed by sequencing full-length cDNA from Col-0 amplified using RT-PCR. The resulting cDNA was cloned into the gateway entry vector pDONR/Zeo using BP clonase (Invitrogen, Carlsbad, CA) and subsequently transferred to the gateway compatible binary vector pEarleyGate101 (Earley et al., 2006) using LR clonase (Invitrogen, Carlsbad, CA). This resulted in a plasmid with *AtRLP30* fused to the coding sequence of YFP::HA and expression was driven by the CaMV 35S promoter. The T-DNA insertion line Salk_122528, homozygous for the insertion in *AtRLP30*, was transformed with this plasmid using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on soil soaked with 150 mg/L Basta herbicide (glufosinate-ammonium, Bayer CropScience) and confirmed by PCR. Plants were checked for fluorescence using an Olympus IX70 microscope equipped with a Fluroview 300 confocal laser scanning unit. AtRLP30::GFP::HA fluorescence was excited with a 488 nm argon laser and fluorescence was detected between 510 nm and 530 nm.

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SUPPLEMENTAL DATA

Supplemental Figure S1. cDNA, EST and MPSS expression data for *AtRLP* genes.
The MPSS (17) and MPSS (20) abbreviations are: CAF/CAS, callus tissue culture; INF, inflorescence; LEF/LES, leaves; ROF/ROF, root; SIF/SIS, silique; AP1, ap1-10 infloresence; AP3, ap3-6 infloresence; AGM, agamous infloresence; INS, infloresence; SAP, sup/ap1 infloresence; S04, leaves, 4 hr after salicylic acid reatment; S52, leaves, 52 hr after salicylic acid treatment; GSE, germinating seedlings.

AGI code	Gene name	cDNA
At1g07390	AtRLP1	
At1g17240	AtRLP2	
At1g17250	AtRLP3	
At1g28340	AtRLP4	+
At1g34290	AtRLP5	
At1g45616	AtRLP6	
At1g47890	AtRLP7	
At1g54480	AtRLP8	+
At1g58190	AtRLP9	
At1g65380	AtRLP10	+
At1g71390	AtRLP11	
At1g71400	AtRLP12	+
At1g74170	AtRLP13	
At1g74180	AtRLP14	
At1g74190	AtRLP15	
At1g74200	AtRLP16	+
At1g80080	AtRLP17	+
At2g15040	AtRLP18	+
At2g15080	AtRLP19	+
At2g25440	AtRLP20	
At2g25470	AtRLP21	
At2g32660	AtRLP22	+
At2g32680	AtRLP23	
At2g33020	AtRLP24	
At2g33030	AtRLP25	
At2g33050	AtRLP26	+
At2g33060	AtRLP27	
At2g33080	AtRLP28	
At2g42800	AtRLP29	+
At3g05360	AtRLP30	+
At3g05370	AtRLP31	+
At3g05650	AtRLP32	+
At3g05660	AtRLP33	
At3g11010	AtRLP34	+
At3g11080	AtRLP35	
At3g23010	AtRLP36	
At3g23110	AtRLP37	
At3g23120	AtRLP38	+
At3g24900	AtRLP39	
At3g24954	AtRLP40	
At3g25010	AtRLP41	
At3g25020	AtRLP42	
At3g28890	AtRLP43	+
At3g49750	AtRLP44	+
At3g53240	AtRLP45	+
At4g04220	AtRLP46	
At4g13810	AtRLP47	+
At4g13880	AtRLP48	
At4g13900	AtRLP49	+
At4g13920	AtRLP50	+
At4g18760	AtRLP51	
At5g25910	AtRLP52	+
At5g27060	AtRLP53	+
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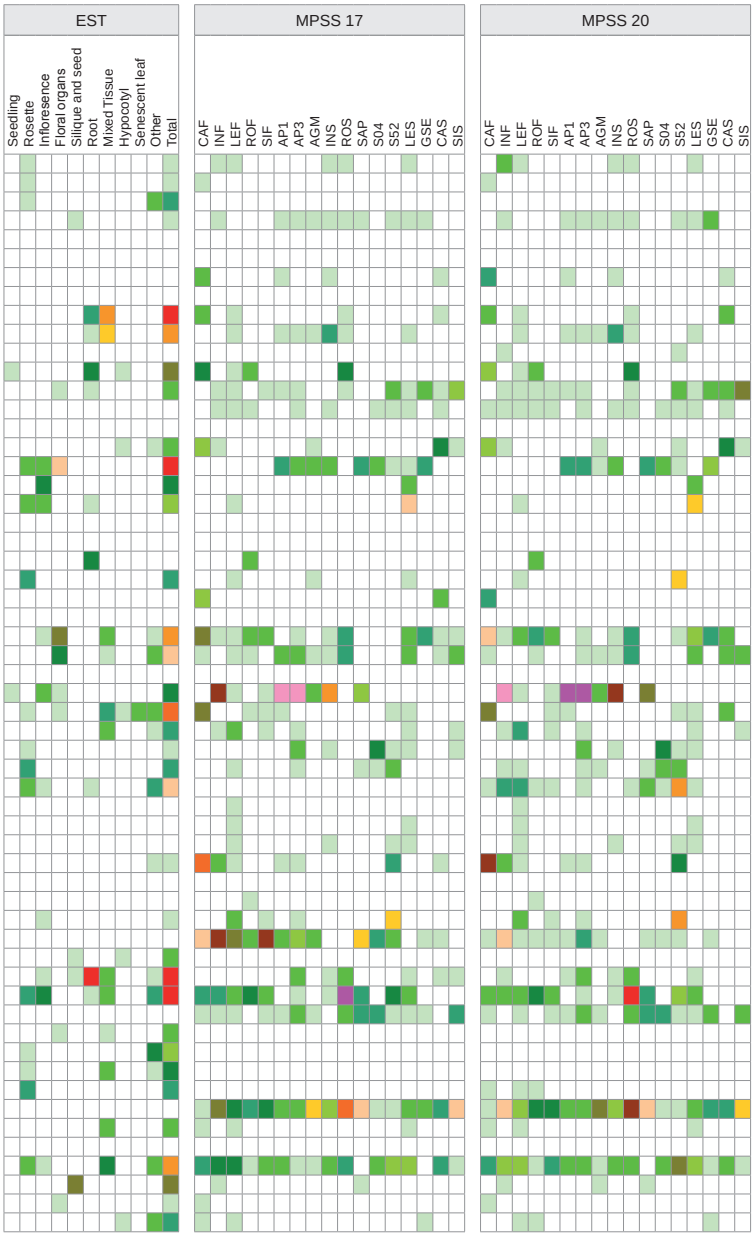

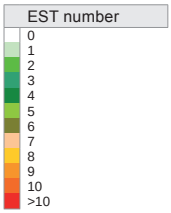

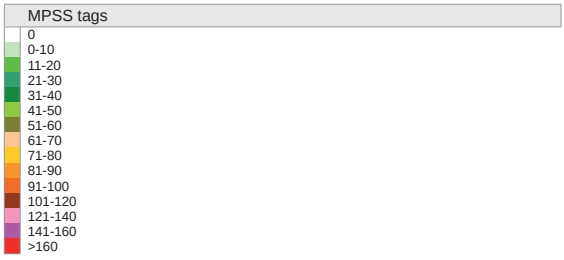

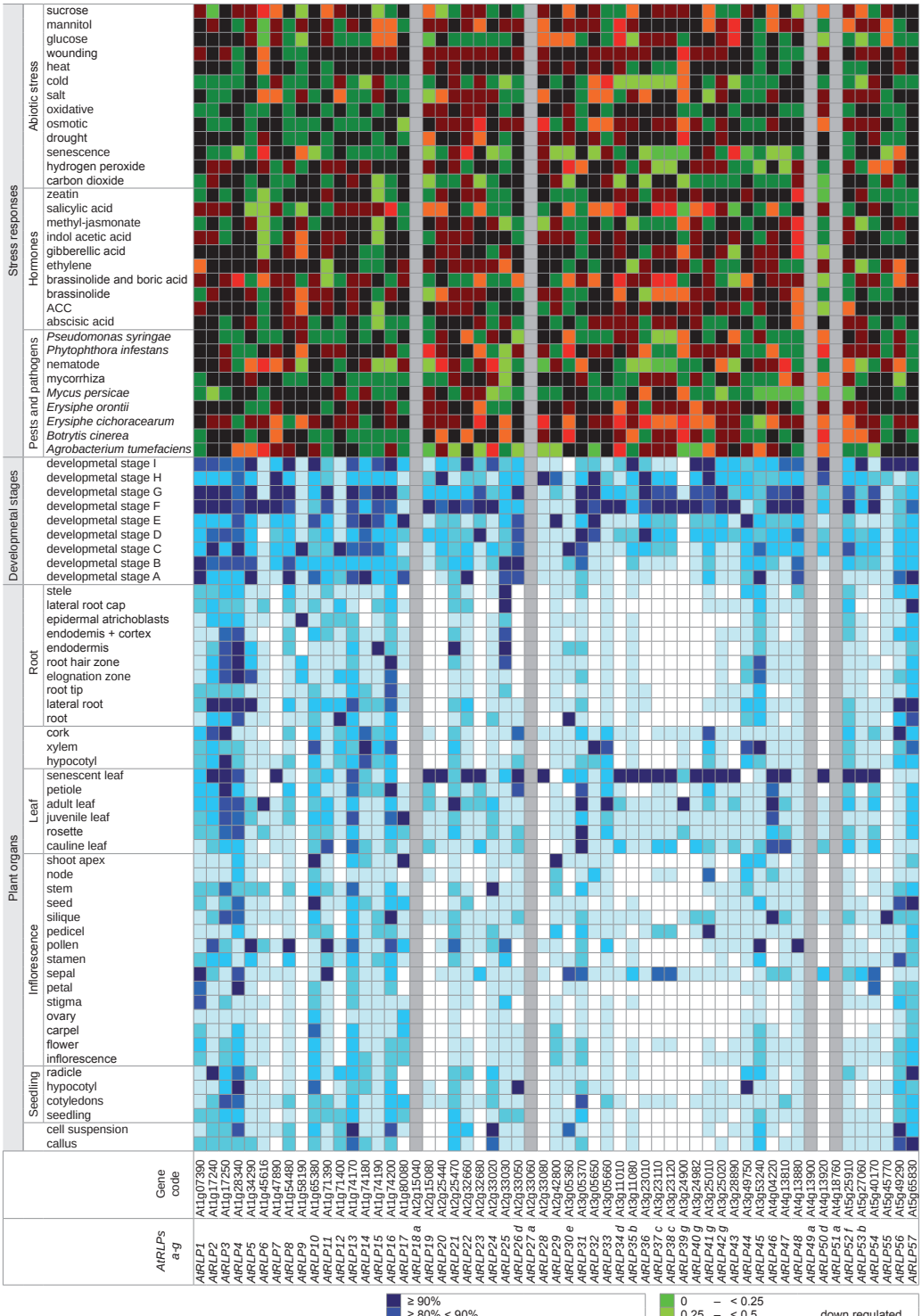
Stress responses
Abiotic stress
sucrose
mannitol
glucose
wounding
heat
cold
salt
oxidative
osmotic
drought
senescence
hydrogen peroxide
carbon dioxide
Hormones
zeatin
salicylic acid
methyl-jasmonate
indol acetic acid
gibberellic acid
ethylene
brassinolide and boric acid
brassinolide
ACC
abscisic acid
Pests and pathogens
Pseudomonas syringae
Phytophthora infestans
nematode
mycorrhiza
Mycus persicae
Erysiphe orontii
Erysiphe cichoracearum
Botrytis cinerea
Agrobacterium tumefaciens
Developmetal stages
developmetal stage I
developmetal stage H
developmetal stage G
developmetal stage F
developmetal stage E
developmetal stage D
developmetal stage C
developmetal stage B
developmetal stage A
Plant organs
Root
stele
lateral root cap
epidermal atrichoblasts
endodemis + cortex
endodermis
root hair zone
elongnation zone
root tip
lateral root
root
Leaf
cork
xylem
hypocotyl
Inflorescence
senescent leaf
petiole
adult leaf
juvenile leaf
rosette
cauline leaf
shoot apex
node
stem
seed
silique
pedicel
pollen
stamen
sepal
petal
stigma
ovary
carpel
flower
inflorescence
Seedling
radicle
hypocotyl
cotyledons
seedling
cell suspension
callus
Gene code
At1g07390
At1g17240
At1g17250
At1g28340
At1g34290
At1g45616
At1g47890
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At1g74200
At1g80080
At2g15040
At2g15080
At2g25440
At2g25470
At2g32660
At2g32680
At2g33020
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At2g33050
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At2g33080
At2g42800
At3g05360
At3g05370
At3g05650
At3g05660
At3g11010
At3g11080
At3g23010
At3g23110
At3g23120
At3g24900
At3g24982
At3g25010
At3g25020
At3g28890
At3g49750
At3g53240
At4g04220
At4g13810
At4g13880
At4g13900
At4g13920
At4g18760
At5g25910
At5g27060
At5g40170
At5g45770
At5g49290
At5g65830
AtRLPs a-g
AtRLP1
AtRLP2
AtRLP3
AtRLP4
AtRLP5
AtRLP6
AtRLP7
AtRLP8
AtRLP9
AtRLP10
AtRLP11
AtRLP12
AtRLP13
AtRLP14
AtRLP15
AtRLP16
AtRLP17
AtRLP18 a
AtRLP19
AtRLP20
AtRLP21
AtRLP22
AtRLP23
AtRLP24
AtRLP25
AtRLP26 d
AtRLP27 a
AtRLP28
AtRLP29
AtRLP30 e
AtRLP31
AtRLP32
AtRLP33
AtRLP34 d
AtRLP35 b
AtRLP36
AtRLP37 c
AtRLP38 c
AtRLP39 g
AtRLP40 g
AtRLP41 g
AtRLP42 g
AtRLP43
AtRLP44
AtRLP45
AtRLP46
AtRLP47
AtRLP48
AtRLP49 a
AtRLP50 d
AtRLP51 a
AtRLP52 f
AtRLP53 b
AtRLP54
AtRLP55
AtRLP56
AtRLP57
≥ 90%
≥ 80% < 90%
≥ 74% < 80%
≥ 55% < 74%
≥ 40% < 55%
≥ 10% < 40%
≥ 0% < 10%
0 – < 0.25
0.25 – < 0.5
0.5 – < 0.9
0.9 – < 1.1
1.1 – < 2.0
2.0 – < 4.0
>4.0
down regulated
induced
no data available

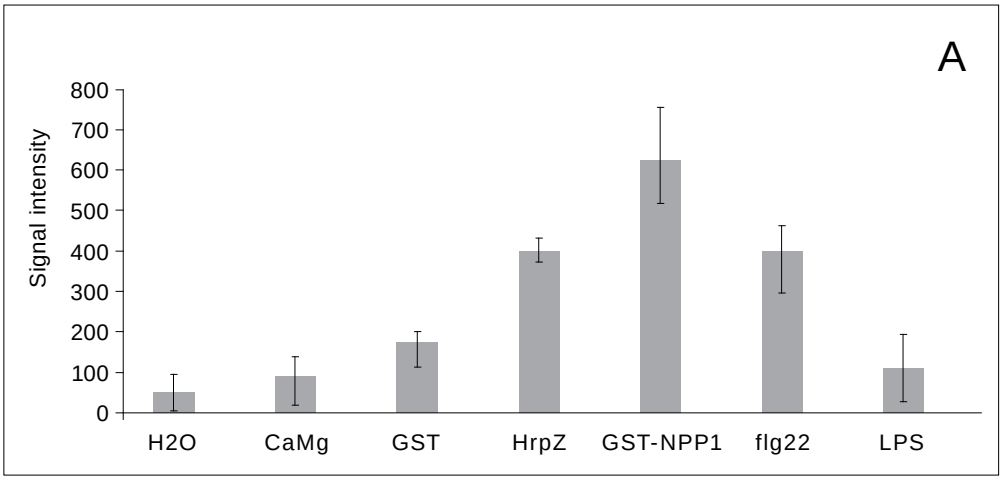
Supplemental Figure S2. Expression profile of *AtRLP* genes in various organs, growth stages and upon stress responses.

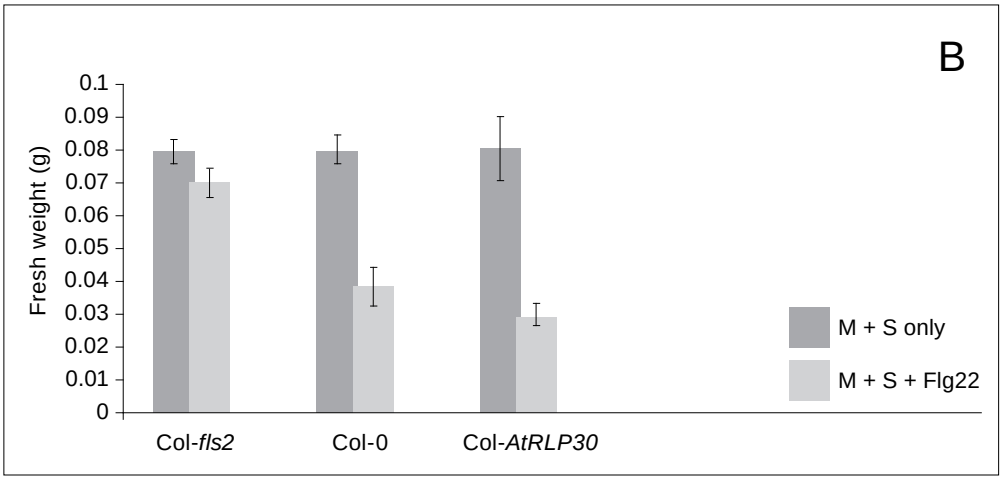
The figure was modified from an output of Meta-Analyzer of Genevestigator (Zimmermann et al., 2004). Heat maps are rendered either in blue-white for gene expression patterns for plant organs and developmental stages or in red-green for gene expression patterns upon stress responses. For the blue-white scheme on the left panel, absolute signal intensities of one gene for all plant organs or for all developmental stages were compared with each other and normalized that the highest signal intensity value obtained the value 100% (dark blue) and the absence of signals obtained value 0% (white). For the red-green scheme on the right panel, signal intensity values for a gene upon one treatment were compared with the corresponding control and given as linear ratio values. Red, orange and dark red indicate that the signal intensity of the treatment is higher than signal intensity of the corresponding control, and green, lime and bright green mean the opposite. Black indicates no difference in signal intensity between treatment and control.

Plant organs, developmental stages and stress responses are listed on top.

a For *AtRLP18*, *AtRLP27*, *AtRLP49* and *AtRLP51* no probesets are present on the Affymetrix ATH1 22k array chip.
b *AtRLP35* and *AtRLP53* have the same probeset (254741_at) and thus have the same values.
c *AtRLP37* and *AtRLP38* hybridize to the same probeset (257763_s_at) that is representing two or more closely related genes.
d *AtRLP26*, *AtRLP34* and *AtRLP50* hybridize to probesets (267596_s_at, 256431_s_at and 254741_s_at, respectively) that are representing two or more closely related genes.
e *AtRLP30* hybridizes to two different probesets (265993_at and 259297_at) of which only the data of one (259297_at) was included in the figure.
f *AtRLP52* hybridizes to two different probesets (265893_at and 246916_at) of which only the data of one (246916_at) was included in the figure.
g *AtRLP39*, *40*, *41* and *42* cross-hybridize to three different probesets (257100_at, 257591_at and 257592_at). In addition, *AtRLP39*, *41* and *42* cross-hybridize to another probeset (257101_at). For *AtRLP39* the data of probeset 257592_at, for *AtRLP40* the data of probeset 257100_at, for *AtRLP41* the data of probeset 257101_at and for *AtRLP42* the data of probeset 257591_at were included in the figure.

Abbreviations: ACC: 1-aminocyclopropane-1-carboxylic acid





Supplemental Figure S3. Expression of *AtRLP30* after PAMP treatment.

A Data obtained using the Genevestigator software derived from the AtGenExpress experiment "Response to bacterial-(LPS, HrpZ, Flg22) and oomycete-(NPP1) derived elicitors". More details of this experiment are available at http://www.arabidopsis.org. Expression level of *AtRLP30* was increased by the PAMPs HrpZ, flg22 and NPP1 when compared to control treatments (H_2O, CaMg and GST). LPS did not increase the level of expression.

B Effect of flg22 on seedling growth. The addition of flg22 to MS growth media causes a significant reduction in weight of seedlings that can detect flg22 (Col-0) but not in mutants in the flg22 perception pathway (Col-0 *fls2*). Col-*AtRLP30* shows a wild-type response to flg22, indicating that it is not involved in flg22 perception.

Supplemental Table S1. Pairwise alignment of AtRLP amino acid sequences. Amino acid similarities (lower triangle) and identities (upper triangle) are shown in percentages for pairwise alignments of the predicted full length protein sequences. A grey background indicates >70 % similarity or identity, respectively.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
01.	AtRLP1		24	23	17	11	27	22	23	24	26	22	23	34	34	35	12	18	22	24	26	28	9	24	25	16	24	25	26	27	26	27	27	22	27	27	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
02.	AtRLP2	39		68	18	14	25	26	25	18	26	28	29	24	26	26	18	22	24	26	26	26	26	26	29	28	22	29	28	26	27	26	27	27	28	25	29	28	27	27	13	28	28	28	15	28	27	28	29	29	19	14	26	28	22	27	15
03.	AtRLP3	37	80		19	13	17	16	20	12	20	19	19	17	17	19	16	18	17	17	18	17	19	19	13	20	19	30	25	22	30	28	27	26	27	11	29	30	25	22	30	28	27	28	25	29	28	27	28	30	14	28	28	27	28	21	30	15
04.	AtRLP4	30	35	36		13	17	16	20	12	20	19	19	17	17	19	16	18	17	17	18	17	19	19	13	20	19	18	20	19	19	17	18	19	17	18	19	18	15	18	12	27	19	18	20	19	19	21	17	19	13	18	20	19	18	15
05.	AtRLP5	16	22	21	22		11	11	16	7	13	12	12	9	10	11	17	15	10	10	13	11	16	11	11	21	11	12	13	19	12	12	11	11	11	15	12	12	11	23	11	10	13	22	10	11	13	12	13	11	17	22	10	12	17	12	20
06.	AtRLP6	46	38	40	30	16		62	22	20	24	37	38	26	28	27	13	19	35	45	30	28	29	38	37	12	36	37	32	19	36	37	43	42	45	29	35	35	38	11	37	37	38	12	11	23	28	28	37	36	38	40	35	15	27	12
07.	AtRLP7	46	40	40	28	17	74		20	21	24	36	39	28	28	27	12	18	35	43	30	28	28	38	37	12	35	36	32	18	34	37	41	41	42	44	28	36	34	39	12	38	38	35	12	27	29	36	35	37	37	15	10	44	34	17	28	13
08.	AtRLP8	33	40	38	38	25	31	30		16	26	21	23	26	37	30	29	24	21	22	25	41	28	23	22	14	26	25	21	24	24	22	24	22	23	22	22	24	23	17	23	23	25	19	32	22	25	25	25	22	23	15	21	23	21	38	18
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14.	AtRLP14	53	40	41	29	17	45	46	45	37	40	42	45	62																																																						
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41.	AtRLP41	44	43	43	33	17	53	53	34	29	42	51	52	41	44	45	22	29	48	53	59	46	49	79	79	20	67	68	63	29	49	53	57	58	56	56	56	42	50	48	90	23	86	36	13	29	39	38	35	37	40	18	11	40	41	42	18	17
42.	AtRLP42	43	42	43	33	16	53	54	34	28	41	53	54	44	45	45	21	29	48	53	58	47	50	79	78	20	67	68	63	29	49	52	56	58	57	58	42	51	48	89	24	91		36	15	30	30	39	37	40	18	11	40	41	18	30	13	
43.	AtRLP43	38	45	46	31	20	49	47	40	23	49	56	53	36	39	40	25	33	47	55	55	41	53	48	51	21	53	53	54	35	57	51	63	61	65	63	52	54	57	48	22	49	49		14	27	32	40	43	43	38	19	14	62	38	21	27	15	
44.	AtRLP44	17	21	21	23	18	18	21	19	17	18	17	17	16	17	22	20	27	20	20	22	34	22	19	20	17	18	17	18	25	20	20	23	24	22	19	20	19	39	19	22		13	14	15	15	16	14	24	29	12	14	21	13	80	
45.	AtRLP45	50	42	43	31	17	45	43	42	34	42	43	47	57	63	63	23	29	41	44	37	64	38	46	47	14	44	44	40	27	44	47	44	45	45	44	38	47	45	46	15	47	48	41	18		28	27	26	28	29	17	11	28	26	16	47	12	
46.	AtRLP46	44	44	45	35	21	48	48	41	26	46	57	53	38	43	42	25	32	46	50	55	44	53	54	56	17	56	57	56	32	48	49	49	48	47	47	47	47	20	44	48		30	33	30	31	31	30	18	35	20	29	15	
47.	AtRLP47	39	46	46	35	21	48	48	41	26	46	57	53	38	43	42	25	32	46	50	55	44	53	54	56	17	56	57	56	32	48	49	49	48	47	47	47	47	20	44	48	61	71	58	18	13	40	41	20	30	15				
48.	AtRLP48	37	47	45	35	21	48	48	39	24	48	55	52	35	41	39	25	33	43	49	53	42	51	50	51	20	55	55	54	33	54	50	56	55	52	54	49	52	55	49	21	49	49	61	22	42	48	74		65	58	19	13	39	40	20	27	16	
49.	AtRLP49	39	46	47	36	21	50	50	38	25	46	59	55	39	43	42	22	34	44	50	54	44	53	55	55	20	57	58	55	31	56	53	56	53	54	50	52	55	53	21	52	53	61	22	44	49	81	74		57	19	13	39	42	19	29	17	
50.	AtRLP50	43	44	43	32	17	55	55	33	29	43	56	59	43	47	46	22	29	50	60	47	49	45	60	59	17	53	54	50	28	55	55	61	62	62	43	56	53	59	17	58	60	52	19	49	48	68	65	68		17	10	46	38	19	31	14	
51.	AtRLP51	15	20	20	29	31	27	25	24	23	9	18	18	17	13	15	16	39	25	14	13	20	17	22	16	17	19	18	17	27	18	16	16	15	14	22	16	17	16	38	15	15	19	43	15	17	19	17	17	26		18	17	18	60	16	23	
52.	AtRLP52	15	20	20	30	27	13	14	23	9	18	18	17	13	15	16	39	25	14	13	20	17	22	16	17	19	18	17	18	27	18	16	16	15	14	22	16	17	16	38	15	15	19	43	15	17	19	17	17	26	29		18	17	18	60	16	23	
53.	AtRLP53	45	40	41	31	16	61	58	32	30	40	53	57	45	46	45	19	27	63	74	44	46	41	56	56	16	51	53	48	27	53	55	72	72	83	83	41	55	52	55	16	56	55	67	18	44	45	53	53	52	62	26	13		35	17	29	12	
54.	AtRLP54	42	42	44	34	19	49	49	35	26	47	54	52	38	41	42	24	32	45	48	55	42	51	58	58	19	64	65	58	31	55	51	54	53	51	47	52	55	57	55	58	53	29	18	49		18	28	14							
55.	AtRLP55	25	33	30	32	29	24	26	37	15	32	30	28	23	26	26	36	40	24	25	35	27	35	27	27	28	28	30	32	44	32	26	28	27	26	27	32	28	29	29	29	27	28	31	34	27	28	31	29	31	28	75	31	25	29		17	24
56.	AtRLP56	51	41	42	32	18	43	45	47	34	43	44	45	59	76	66	23	29	40	47	39	78	38	47	45	14	46	44	42	29	43	48	47	47	48	46	36	46	45	47	15	47	46	41	19	63	47	45	44	46	49	27	16	46	44	29		13
57.	AtRLP57	18	23	23	24	41	19	18	29	11	23	21	20	16	19	18	40	29	16	17	23	18	25	19	19	38	20	21	23	34	20	20	19	18	19	19	25	19	19	22	36	19	18	22	88	18	22	23	23	24	19	33	42	17	21	35	20	

Supplemental Table S2. Primers used to check for the presence of the predicted T-DNA insertions.

Gene name	AGI code	T-DNA line ordered	Mutant name	Gene-specific primer pair		
				Forward primer	Reverse primer	T-DNA primer
AtRLP1	at1g07390	SALK_059920	*Atrlp1-1*	TGCGTTCATCATATTCTACAGTTC	CTCCGCCGTCTCTTTCCAGTC	TGGTTCACGTAGTGGGCCATCG
		SALK_116923	*Atrlp1-2*	ATTTCTTACACTCTTATTTGATTA	CTGCCCATATGACCATTAGAAGAC	TGGTTCACGTAGTGGGCCATCG
AtRLP2	at1g17240	SALK_049366	*Atrlp2-1*	TCAGCCGAGAGTCAGTCAGAGA	ATACATTTTTGCAGCCATTTTG	TGGTTCACGTAGTGGGCCATCG
AtRLP3	at1g17250	SALK_051677	*Atrlp3-1*	GGCTCTGATGTCCCAAAAGGC	GCCGTGATCTTGCTGTCCAAC	TGGTTCACGTAGTGGGCCATCG
		SAIL_204_D01	*Atrlp3-2*	AACTTTTCCCCACCTTTTCTA	GTATCCGCTTTATTTGTGGTTGTA	TTCATAACCAATCTCGATACAC
AtRLP4	at1g28340	SALK_039264	*Atrlp4-1*	GCGGGGCTAATTGTCAGTT	AATTCAGTTTCAGGGTTCAGA	TGGTTCACGTAGTGGGCCATCG
AtRLP5	at1g34290	SALK_112291	*Atrlp5-1*	CCAGAAACGCAGATCAAGGTATT	TGGCGAAGGAACAAGAAGG	TGGTTCACGTAGTGGGCCATCG
AtRLP6	at1g45616	SALK_080898	*Atrlp6-1*	TCTTTCGAGCAATGCTTTCAC	TGAGACGAATCTTCTGGGAGACA	TGGTTCACGTAGTGGGCCATCG
		SAIL_84_E01	*Atrlp6-2*	CCGTAACAATAGCCTCGATGG	TCTAGGCGCGTGGATATCTCC	TTCATAACCAATCTCGATACAC
		SALK_020071	*Atrlp6-3*	CCGTAACAATAGCCTCGATGG	TCTAGGCGCGTGGATATCTCC	TGGTTCACGTAGTGGGCCATCG
AtRLP7	at1g47890	SALK_030269	*Atrlp7-1*	GGAGATGGAACGTGTCCTTACA	TGGTATGGAACCTACAAGCTGGT	TGGTTCACGTAGTGGGCCATCG
AtRLP8	at1g54480	SM_3_38632	*Atrlp8-1*	TTCTCCGGGAAGCTACC	CACAGAGCTGCCTTGAC	TACGAATAAGAGCGTCCATTTTAGAGTGA
		SM_3_20200	*Atrlp8-2*	TTCTCCGGGAAGCTACC	CACAGAGCTGCCTTGAC	TACGAATAAGAGCGTCCATTTTAGAGTGA
AtRLP9	at1g58190	SALK_061979	*Atrlp9-1*	AAATGCCGCTTCTTCGCTTAG	GAAACCCTCAAACTTATTATCTG	TGGTTCACGTAGTGGGCCATCG
		SALK_023419	*Atrlp9-2*	CTTTGGCAGGACTTTGTCAAC	GGTAAGTTCCCCGAGAACTTG	TGGTTCACGTAGTGGGCCATCG
AtRLP10	at1g65380	GABI_686A09	*Atrlp10-1*	GTCTAGCTTGTCAGAATCC	TTAAGAACCACCAATGG	CCCATTTGGACGTGAATGTAGACAC
AtRLP11	at1g71390	SALK_013218	*Atrlp11-1*	CTTTGGTAGGTGAAGTTCCAGC	GGCAACATCTAACAGTACGAGG	TGGTTCACGTAGTGGGCCATCG
AtRLP12	at1g71400	SALK_151456	*Atrlp12-1*	AAATTTGGGGGACAACAACTTC	ATAGCCAAGGGATTCAGGGA	TGGTTCACGTAGTGGGCCATCG
AtRLP13	at1g74170	SALK_020984	*Atrlp13-1*	GGCTCCATACCAACACAAG	ATATTTTTCCATGGGCAAGTCC	TGGTTCACGTAGTGGGCCATCG
AtRLP14	at1g74180	SAIL_513_A08	*Atrlp14-1*	GGTGTTATCCCAGCAGAG	CCCAGAGGCTATGAAAGC	TTCATAACCAATCTCGATACAC
		GABI_077G01	*Atrlp14-2*	ATGGAAAGGAAGGTGTTCTCGG	ACAACCTCTGGAGGCAGGAGA	CCCATTTGGACGTGAATGTAGACAC
AtRLP15	at1g74190	SALK_041143	*Atrlp15-1*	TTTGCGTCATGTTGATCTCTCC	GGTTCTCCTGAAAATTATTCTTGGA	TGGTTCACGTAGTGGGCCATCG
AtRLP16	at1g74200	SALK_032150	*Atrlp16-1*	TGCTTGACATTTCGAACAACAA	GCGAGGAATACTGCCTGTTAAA	TGGTTCACGTAGTGGGCCATCG
AtRLP17	at1g80080	FLAG_014F03	*Atrlp17-1*	GTTCACGAAGCGGTCGGA	CAACGATCCACAGCTTGTGAG	CGGCTATTGGTAATAGGACACTGG
		SAIL_165_F02	*Atrlp17-2*	GTTCACGAAGCGGTCGGA	CAACGATCCACAGCTTGTGAG	TTCATAACCAATCTCGATACAC
AtRLP18	at2g15040	SAIL_400_H02	*Atrlp18-1*	CAGTGTTTGCGACAGCAG	CGACTTTTCTCAACGGTC	TTCATAACCAATCTCGATACAC
AtRLP19	at2g15080	FLAG_524A03	*Atrlp19-1*	TGTCGATCGGACTATCCCTGTC	ACATCCCCGAACTTGGCATC	CGGCTATTGGTAATAGGACACTGG
AtRLP20	at2g25440	SALK_130147	*Atrlp20-1*	TCTATACTAGAATCACTGAAGC	GAGACACAACAAAGTAAGAGTAGC	TGGTTCACGTAGTGGGCCATCG
AtRLP21	at2g25470	SAIL_693_F05	*Atrlp21-1*	GGCTCTCTGGTGCTATTCC	GCCTATCAATGCGGTCAC	TTCATAACCAATCTCGATACAC
		SALK_133403	*Atrlp21-2*	TCTCCTTTAAATCTTTCTTTGCTCC	GCGGCATTAAGAAAAGGAAAT	TGGTTCACGTAGTGGGCCATCG
AtRLP22	at2g32660	SALK_125231	*Atrlp22-1*	TCACATTAGCGAAAGACATCGGA	CAAAGGAAACGTTCCTGATTGGA	TGGTTCACGTAGTGGGCCATCG
AtRLP23	at2g32680	SALK_034225	*Atrlp23-1*	ACAACAGAATTGAAGATACGTTTCC	CCAGTTCACAAAGTAGTTTGGTGG	TGGTTCACGTAGTGGGCCATCG
AtRLP24	at2g33020	SALK_046236	*Atrlp24-1*	CTCCTCATCAAGGTCCTCTCG	GCCTTCAAGAGACCAATGGATTC	TGGTTCACGTAGTGGGCCATCG
AtRLP25	at2g33030	SALK_048434	*Atrlp25-1*	TTCAAATGAGGATTTTGGTGG	TATTTACCCCCACCTTGAAGG	TGGTTCACGTAGTGGGCCATCG

Gene name	AGI code	T-DNA line ordered	Mutant name	Gene-specific primer pair		
				Forward primer	Reverse primer	T-DNA primer
AtRLP26	at2g33050	SALK_104127	*Atrlp26-1*	CGAACTCCAAGAAGTCCTTCC	TGACGTAACGATGATGACAATTC	TGGTTCACGTAGTGGGCCATCG
		SALK_026997	*Atrlp26-2*	TGCTCGCTACTCTTACTCTACT	TCTTACAGGTGGGAAACTAC	TGGTTCACGTAGTGGGCCATCG
AtRLP27	at2g33060	SALK_029443	*Atrlp27-1*	AGCTTCACTCTTGTTGTTGGACTT	GTCTAAAATGTTAAGCTGGCTAAGG	TGGTTCACGTAGTGGGCCATCG
AtRLP28	at2g33080	SM_3_1740	*Atrlp28-1*	CCTCGATCTTTCCGGTAACAGT	CCGCAGAGAAGGCTTTGATAGA	TACGAATAAGAGCGTCCATTTTAGAGTGA
AtRLP29	at2g42800	SALK_022220	*Atrlp29-1*	GATCGTTGGAGGGATTGTACCG	CGTGTCTCTGTCTTGTCTTGTCCC	TGGTTCACGTAGTGGGCCATCG
AtRLP30	at3g05360	SALK_122528	*Atrlp30-1*	GAATCTGGCGAGGTGGTTTCAC	GCCCAACTAAGTTGTTGTGG	TGGTTCACGTAGTGGGCCATCG
		SALK_008911	*Atrlp30-2*	TGACAAATCTTGAGACATTG	ACACAACTGACAGATATCAT	TGGTTCACGTAGTGGGCCATCG
		SALK_122536	*Atrlp30-3*	GAATCTGGCGAGGTGGTTTCAC	GCCCAACTAAGTTGTTGTGG	TGGTTCACGTAGTGGGCCATCG
		SALK_145342	*Atrlp30-4*	TCACTTGTTCTTCTGGTTCCG	TGGATCTTGGTTCGAATTCAC	TGGTTCACGTAGTGGGCCATCG
AtRLP31	at3g05370	SALK_058586	*Atrlp31-1*	TGGGACGTTGTATCAACC	CAATCCACAGACGACACCAGG	TGGTTCACGTAGTGGGCCATCG
		SALK_094160	*Atrlp31-2*	GAGTTTGGAAACATGTCTTCATCG	GTTGAAGCGATTATCGGACATAA	TGGTTCACGTAGTGGGCCATCG
AtRLP32	at3g05650	FLAG_588C11	*Atrlp32-1*	AATCAAAGGTCAGGTGC	GCGTAAGACAAGAACTTGC	CGGCTATTGGTAATAGGACACTGG
AtRLP33	at3g05660	FLAG_048F06	*Atrlp33-1*	AGAAGTCAATGAGTCTCATTCC	AAGGACTCCAAGATGGAGAGTG	CGGCTATTGGTAATAGGACACTGG
		SALK_087631	*Atrlp33-2*	TTTTAAAGGAGAAGCAAAACCTCA	CAAGAGTGCCGCTGAGTTGGT	TGGTTCACGTAGTGGGCCATCG
		SALK_085252	*Atrlp33-3*	GATTGGCATGAAACCATATAACC	ATTCCCAAACTCAAGAGTGCC	TGGTTCACGTAGTGGGCCATCG
AtRLP34	at3g11010	SALK_067155	*Atrlp34-1*	TTGGGACATACGAAGATGGGTC	TCCGAATCCTATTGCAGCTGC	TGGTTCACGTAGTGGGCCATCG
		SALK_085506	*Atrlp34-2*	CAAAAGCTACAAGTTCTTGTCCTTC	CAGGTCCGAATCCTATTGCA	TGGTTCACGTAGTGGGCCATCG
AtRLP35	at3g11080	SALK_096171	*Atrlp35-1*	CGGATGAACCCTTGATTG	GGACGGGATTTGACCTGAA	TGGTTCACGTAGTGGGCCATCG
		SALK_016143	*Atrlp35-2*	GCCAAAAGAAGATGGGATTTC	TCAGTTTCTGGACAAGCAACC	TGGTTCACGTAGTGGGCCATCG
AtRLP36	at3g23010	SALK_086147	*Atrlp36-1*	AACAAAGCTTTCTGAATTGTACCTC	GTCCGGAAAATGAGTTGTTATACA	TGGTTCACGTAGTGGGCCATCG
AtRLP37	at3g23110	SALK_041785	*Atrlp37-1*	GCGATTTTGGGTGTCTGAGAAC	GGTCCTTGGAGGGAATTTGAGC	TGGTTCACGTAGTGGGCCATCG
		SALK_012745	*Atrlp37-2*	TGCTCATGATTCCTTCGTTAGTC	TGTTGTAGGAAAGATCAAGGGAACT	TGGTTCACGTAGTGGGCCATCG
AtRLP38	at3g23120	SALK_017819	*Atrlp38-1*	ATCTACAAGGATTCGTGCCACG	TGCCGTGAGATTTCAGGTCAG	TGGTTCACGTAGTGGGCCATCG
		GT_5_105490	*Atrlp38-2*	TGGACTGAAATGGCAACG	CAGAACACACCAGGTCCAAAGG	ACCCGACCGGATCGTATCGGT
AtRLP39	at3g24900	SALK_126505	*Atrlp39-1*	CCCCCACAATTAGTAACCTCACC	GTATGTCGCCTCCGTATCTATTGT	TGGTTCACGTAGTGGGCCATCG
		SALK_126504	*Atrlp39-2*	GGTATGTCGCCTCCGTATCTA	TATGCCTTTCTTATCCTATCTTGA	TGGTTCACGTAGTGGGCCATCG
AtRLP40	at3g24982	GABI_564D03	*Atrlp40-1*	CTGGGTCTATATATGGTATATG	TTGTGTTCCTTGTGGTATTTCACCAA	CCCATTTGGACGTGAATGTAGACAC
AtRLP41	at3g25010	SALK_024020	*Atrlp41-1*	TGGTCCTCTATCTCCTCCAA	GCCTTCCCAGTTCAACACTTGTTCCTG	TGGTTCACGTAGTGGGCCATCG
		SM_3_20242	*Atrlp41-2*	TGGTCCTCTATCTCCTCCAA	GCCTTCCCAGTTCAACACTTGTTCCTG	TACGAATAAGAGCGTCCATTTTAGAGTGA
		SM_3_38956	*Atrlp41-3*	TGGTCCTCTATCTCCTCCAA	GCCTTCCCAGTTCAACACTTGTTCCTG	TACGAATAAGAGCGTCCATTTTAGAGTGA
		SALK_025749	*Atrlp41-4*	ACAATGCTTCCAAGGTCAATG	GCGTTTACGCAGTTCAAGAAC	TGGTTCACGTAGTGGGCCATCG
AtRLP42	at3g25020	SALK_080324	*Atrlp42-1*	GTCCGAAGGGAAATCTCTTTG	TGGAGTGTTACTTGGATTGGC	TGGTTCACGTAGTGGGCCATCG
		SALK_094190	*Atrlp42-2*	GGAATTAGGTGGGTTAGGAGG	TGGAGTGTTACTTGGATTGGC	TGGTTCACGTAGTGGGCCATCG
AtRLP43	at3g28890	SALK_041685	*Atrlp43-1*	AAATACTGAGGTCTCTAGATGTCGG	TCTTTCAATAGACCGATGGACTTT	TGGTTCACGTAGTGGGCCATCG
AtRLP44	at3g49750	SALK_097350	*Atrlp44-1*	GTTTGGATCGGCGGTGGTTA	GCTTTGCATTGGGCTTTACA	TGGTTCACGTAGTGGGCCATCG
		SALK_045246	*Atrlp44-2*	CGAGATACTGAATCTCCGGTG	GAGTGTGTCGCACTAAGGACC	TGGTTCACGTAGTGGGCCATCG

Table continues on next page.

Gene name	AGI code	T-DNA line ordered	Mutant name	Gene-specific primer pair		
				Forward primer	Reverse primer	T-DNA primer
AtRLP45	at3g53240	GABI_620G05	*Atrlp45-1*	GCATGGAACCATTCCCTC	CCCTCTCTAGATAACTCCCAG	CCCATTTGGACGTGAATGTAGACAC
		FLAG_339H12	*Atrlp45-2*	CCGTTAAAGTGGTGAAGACGAC	CCCAAGAGCAATTGACAGAGC	CGGCTATTGGTAATAGGACACTGG
AtRLP46	at4g04220	SALK_048207	*Atrlp46-1*	TCTTTGGAAGGCGAACTAGCG	TTCGAGAATGGAGACATGTAGA	TGGTTCACGTAGTGGGCCATCG
		SAIL_15_A02	*Atrlp46-2*	TGAGAATCTCATTGGAGGAGCG	GAGCATACTGGTGGATCCTCCA	TTCATAACCAATCTCGATACAC
AtRLP47	at4g13810	SALK_105921	*Atrlp47-1*	CTAGAGTGGATGAACTTCTCGC	CGTTGCAGACCAAATTCC	TGGTTCACGTAGTGGGCCATCG
AtRLP48	at4g13880	SALK_036842	*Atrlp48-1*	GTTCAACTCTCAGCTTTCCCTCAG	CCAGCTCCATATTTAATCCTTTGT	TGGTTCACGTAGTGGGCCATCG
AtRLP49	at4g13900	SALK_067372	*Atrlp49-1*	ACTGATTGCTGTTCTTGGGATGGT	GGTGAGGGAAGACTGACGGTTGA	TGGTTCACGTAGTGGGCCATCG
		SALK_116910	*Atrlp49-2*	AATTCCAACCAGGATGGAAAC	CTCAAGTTGAACTCGCTCACC	TGGTTCACGTAGTGGGCCATCG
AtRLP50	at4g13920	SALK_070876	*Atrlp50-1*	TTGGCTGCGGTGTGGTGTG	TCGGGGCTTGGGATAGAGAA	TGGTTCACGTAGTGGGCCATCG
AtRLP51	at4g18760	SALK_143038	*Atrlp51-1*	CGAAGTGTTCAAAATCGGTGGA	CCAGGCTGGATCTTTGATGGA	TGGTTCACGTAGTGGGCCATCG
		SAIL_740_C06	*Atrlp51-2*	TCTTCTTGTCCCCACCTCAATG	TGGGAGAGGTGGTTGGAGATG	TTCATAACCAATCTCGATACAC
AtRLP52	at5g25910	SALK_107922	*Atrlp52-1*	CCCATTGATGATGGGATGTGG	CACCGGAGAAATCCCAGAGTC	TGGTTCACGTAGTGGGCCATCG
		SALK_054976	*Atrlp52-1*	TTCCCGGCAAAACCTCTG	GGAAGCTTCACCGGCGTAAAC	TGGTTCACGTAGTGGGCCATCG
AtRLP53	at5g27060	SALK_124008	*Atrlp53-1*	TCAACTGGGCCTTGGGTGTTT	TAAATTAGCTTAAGAATGATG	TGGTTCACGTAGTGGGCCATCG
AtRLP54	at5g40170	SAIL_306_E09	*Atrlp54-1*	TCTGTTGCGTCTTTGTGACCAG	GGCAGAGTCCATAACAACTCAG	TTCATAACCAATCTCGATACAC
AtRLP55	at5g45770	SALK_139161	*Atrlp55-1*	CTCCGAGATGAAGAACCT	GTCCCTAAACTAACCCTATGT	TGGTTCACGTAGTGGGCCATCG
		SALK_076590	*Atrlp55-2*	CGCCGTAGATGCAAGACTCGT	CCTCAAACACTTGAATCTCGCTGA	TGGTTCACGTAGTGGGCCATCG
AtRLP56	at5g49290	SALK_129306	*Atrlp56-1*	TGGACTGGCGAGAAAAGAGGAG	CGACGCCAAGAACAATCAACAC	TGGTTCACGTAGTGGGCCATCG
		SALK_010565	*Atrlp56-2*	TTTCGCAGAGGCAATGAAAGC	AGGGGGAACAGTTTAACGGGA	TGGTTCACGTAGTGGGCCATCG
AtRLP57	at5g65830	SALK_077716	*Atrlp57-1*	GAGAGCGATTTGTGGTGAGA	AAAGGCTGAAAACGATAAAAAC	TGGTTCACGTAGTGGGCCATCG
AtRLP47	at4g13810	SALK_105921	*Atrlp47-1*	CTAGAGTGGATGAACTTCTCGC	CGTTGCAGACCAAATTCC	TGGTTCACGTAGTGGGCCATCG
AtRLP48	at4g13880	SALK_036842	*Atrlp48-1*	GTTCAACTCTCAGCTTTCCCTCAG	CCAGCTCCATATTTAATCCTTTGT	TGGTTCACGTAGTGGGCCATCG
AtRLP49	at4g13900	SALK_067372	*Atrlp49-1*	ACTGATTGCTGTTCTTGGGATGGT	GGTGAGGGAAGACTGACGGTTGA	TGGTTCACGTAGTGGGCCATCG
		SALK_116910	*Atrlp49-2*	AATTCCAACCAGGATGGAAAC	CTCAAGTTGAACTCGCTCACC	TGGTTCACGTAGTGGGCCATCG
AtRLP50	at4g13920	SALK_070876	*Atrlp50-1*	TTGGCTGCGGTGTGGTGTG	TCGGGGCTTGGGATAGAGAA	TGGTTCACGTAGTGGGCCATCG
AtRLP51	at4g18760	SALK_143038	*Atrlp51-1*	CGAAGTGTTCAAAATCGGTGGA	CCAGGCTGGATCTTTGATGGA	TGGTTCACGTAGTGGGCCATCG
		SAIL_740_C06	*Atrlp51-2*	TCTTCTTGTCCCCACCTCAATG	TGGGAGAGGTGGTTGGAGATG	TTCATAACCAATCTCGATACAC
AtRLP52	at5g25910	SALK_107922	*Atrlp52-1*	CCCATTGATGATGGGATGTGG	CACCGGAGAAATCCCAGAGTC	TGGTTCACGTAGTGGGCCATCG
		SALK_054976	*Atrlp52-1*	TTCCCGGCAAAACCTCTG	GGAAGCTTCACCGGCGTAAAC	TGGTTCACGTAGTGGGCCATCG
AtRLP53	at5g27060	SALK_124008	*Atrlp53-1*	TCAACTGGGCCTTGGGTGTTT	TAAATTAGCTTAAGAATGATG	TGGTTCACGTAGTGGGCCATCG
AtRLP54	at5g40170	SAIL_306_E09	*Atrlp54-1*	TCTGTTGCGTCTTTGTGACCAG	GGCAGAGTCCATAACAACTCAG	TTCATAACCAATCTCGATACAC
AtRLP55	at5g45770	SALK_139161	*Atrlp55-1*	CTCCGAGATGAAGAACCT	GTCCCTAAACTAACCCTATGT	TGGTTCACGTAGTGGGCCATCG
		SALK_076590	*Atrlp55-2*	CGCCGTAGATGCAAGACTCGT	CCTCAAACACTTGAATCTCGCTGA	TGGTTCACGTAGTGGGCCATCG
AtRLP56	at5g49290	SALK_129306	*Atrlp56-1*	TGGACTGGCGAGAAAAGAGGAG	CGACGCCAAGAACAATCAACAC	TGGTTCACGTAGTGGGCCATCG
		SALK_010565	*Atrlp56-2*	TTTCGCAGAGGCAATGAAAGC	AGGGGGAACAGTTTAACGGGA	TGGTTCACGTAGTGGGCCATCG
AtRLP57	at5g65830	SALK_077716	*Atrlp57-1*	GAGAGCGATTTGTGGTGAGA	AAAGGCTGAAAACGATAAAAAC	TGGTTCACGTAGTGGGCCATCG

Supplemental Table S3. Conditional phenotype assays for *AtRLP* mutants.

	Kingdom	Species	Strain	Concentration
Pathogens	fungi	*Alternaria brassicicola*	MUCL20297	10^6 spores/mL
		Botrytis cinerea	(Brouwer et al., 2003)	10^6 spores/mL
		Cladosporium cucumerinum		10^6 spores/mL
		Cladosporium fulvum	race 5	10^6 spores/mL
		Colletotrichum destructivum	IMI349061	10^6 spores/mL
		Fusarium oxysporum f. sp. *raphani*	815	10^6 budcells/mL
		Oidium neolycopersici	(Bai et al., 2005)	10^6 spores/mL
		Plectosphaerella cucumerina	(Thomma et al., 2000)	10^6 spores/mL
		Sclerotinia sclerotiorum	ND 30	mycelium fragments
		Verticillium dahliae	St12.01	10^6 spores/mL
			St17.01	10^6 spores/mL
			JR2	10^6 spores/mL
	oomycetes	*Phytophthora infestans*	14.3	10^5 spores/mL
		Phytophthora brassicae	HH	agar plugs
			CBS	agar plugs
	bacteria	*Pectobacterium atrosepticum*	LMG 6669	OD 0.3
		Pseudomonas syringae pv. *tomato* (*Pst*)	DC3000	OD 0.3
		Pst AvrRpm1	DC3000	OD 0.3
		Pst AvrRpt2	DC3000	OD 0.3
		Pst AvrRps4	DC3000	OD 0.3
		Xanthomonas campestris pv. *campestris*	568	OD 0.1

	Hormones	Agents	Hormone assay	Hypocotylalteration	Senescence assay
Hormones	auxin	2.4-D: 2.4-dichlorophenoxy acetic acid	0.1 µM 1 µM	5 µM	
	cytokinin	6-BA: 6-benzylaminopurine	1 µM		
	gibberellic acid	GA: gibberellic acid	1 µM 20 µM	20 µM	
	ethylene	ACC: 1-aminocyclopropane-1-carboxylic acid	1 µM	0.5 µM 10 µM	
	brassinolide	EBL: epibrassinolide	1 µM	1 µM	2 µM
	jasmonate	MeJA: methyl-jasmonate	1 µM		50 µM
	abscisic acid	ABA: abscisic acid	0.5 µM		100 µM

	Stress types	Agents	Concentration
Abiotic stress	salt stress	sodium chloride	100 mM 150 mM
		lithium chloride	20 mM 30 mM
	osmotic stress	mannitol	150 mM 200 mM
	reactive oxygen species	hydrogen peroxide	3.3 mM 6.7 mM
		paraquat	2.0 µM
	heavy metal test	cadmium chloride	85 µM

Supplemental Table S4. Interaction phenotypes of *Atrlp* mutants with isolates of *H. parasitica* that are virulent or avirulent on the Arabidopsis ecotypes Col-0 and Ws-0.

Gene name	Mutant name	Background	Cala2	Cand5	Emco1	Emoy2	Hiks1	Maks9	Noks1
AtRLP1	*Atrlp1-1*	Col-0	R	L5	H	L3	N	H	H
	Atrlp1-2	Col-0	R	L4	H	L3	N	H	H
AtRLP2	*Atrlp2-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP3	*Atrlp3-1*	Col-0	R	L4	H	L3	N	H	H
	Atrlp3-2	CS8846	R	L5	H	L4	N	H	H
AtRLP4	*Atrlp4-1*	Col-0	R	L5	H	L8	N	H	H
AtRLP5	*Atrlp5-1*	Col-0	NT	NT	NT	NT	NT	NT	NT
AtRLP6	*Atrlp6-1*	Col-0	R	L4	H	L3	N	H	H
	Atrlp6-2	CS8846	R	L4	H	L3	N	H	H
AtRLP7	*Atrlp7-1*	Col-0	R	L5	H	L3	N	H	H
AtRLP8	*Atrlp8-1*	Col-0	NT	NT	NT	NT	NT	NT	NT
	Atrlp8-2	Col-0	R	L4	H	L3	N	H	H
AtRLP9	*Atrlp9-1*	Col-0	R	L3	H	L4	N	H	H
	Atrlp9-2	Col-0	R	M11	H	L3	N	H	H
AtRLP10	*Atrlp10-1*	Col-0	R	L4	H	L3	N	H	H
(CLV2)	*clv2-3*	Col-0	L1	L4	N	L3	N	N	H
AtRLP11	*Atrlp11-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP12	*Atrlp12-1*	Col-0	R	L6	H	L3	N	H	H
AtRLP13	*Atrlp13-1*	Col-0	R	L5	H	L3	N	H	H
AtRLP14	*Atrlp14-1*	CS8846	R	L5	H	L3	N	H	H
AtRLP15	*Atrlp15-1*	Col-0	R	L5	H	L3	N	H	H
AtRLP16	*Atrlp16-1*	Col-0	R	L5	H	L3	N	H	H
AtRLP17	*Atrlp17-1*	WS-2	N	NT	N	NT	N	N	N
(TMM)	*tmm-1*	CS6140	R	L6	H	L3	N	H	H
AtRLP18	*Atrlp18-1*	CS8846	R	L4	H	L3	N	H	H
AtRLP19	*Atrlp19-1*	WS-2	NT	NT	NT	NT	NT	NT	NT
AtRLP20	*Atrlp20-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP21	*Atrlp21-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP22	*Atrlp22-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP23	*Atrlp23-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP24	*Atrlp24-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP25	*Atrlp25-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP26	*Atrlp26-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP27	*Atrlp27-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP28	*Atrlp28-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP29	*Atrlp29-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP30	*Atrlp30-1*	Col-0	R	L3	H	L3	N	H	H
	Atrlp30-2	Col-0	NT	NT	NT	NT	NT	NT	NT
	Atrlp30-3	Col-0	NT	NT	NT	NT	NT	NT	NT
	Atrlp30-4	Col-0	NT	NT	NT	NT	NT	NT	NT
AtRLP31	*Atrlp31-1*	Col-0	R	L3	H	L3	N	H	H
AtRLP32	*Atrlp32-1*	WS-2	R	NT	H	N	N	N	N

Gene name	Mutant name	Background	Cala2	Cand5	Emco1	Emoy2	Hiks1	Maks9	Noks1
AtRLP33	*Atrlp33-1*	WS-2	R	NT	H	N	N	N	N
	Atrlp33-2	Col-0	R	L4	H	L3	N	H	H
	Atrlp33-3	Col-0	NT	NT	NT	NT	NT	NT	NT
AtRLP34	*Atrlp34-1*	Col-0	R	L2	H	L3	N	H	H
AtRLP35	*Atrlp35-1*	Col-0	R	L2	H	L1	N	H	H
	Atrlp35-2	Col-0	R	NT	NT	NT	NT	NT	NT
AtRLP36	*Atrlp36-1*	Col-0	R	L2	H	L3	N	H	H
AtRLP37	*Atrlp37-1*	Col-0	R	L4	H	L3	N	H	H
	Atrlp37-2	Col-0	NT	NT	NT	NT	NT	NT	NT
AtRLP38	*Atrlp38-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP39	*Atrlp39-1*	Col-0	R	L3	H	L3	N	H	H
AtRLP40	*Atrlp40-1*	Col-0	R	L3	H	L3	N	H	H
AtRLP41	*Atrlp41-1*	Col-0	R	L3	H	L4	N	H	H
	Atrlp41-2	Col-0	R	L2	H	L4	N	H	H
	Atrlp41-3	Col-0	R	L3	H	L4	N	H	H
	Atrlp41-4	Col-0	NT	NT	NT	NT	NT	NT	NT
AtRLP42	*Atrlp42-1*	Col-0	R	L3	H	L3	N	H	H
	Atrlp42-2	Col-0	R	L3	H	L3	N	H	H
AtRLP43	*Atrlp43-1*	Col-0	L2	L3	H	L3	N	H	H
AtRLP44	*Atrlp44-1*	Col-0	R	L2	H	L3	N	H	H
	Atrlp44-2	Col-0	R	L3	H	L3	N	H	H
AtRLP45	*Atrlp45-1*	Col-0	R	L3	H	L3	N	H	H
	Atrlp45-2	WS-2	NT	NT	N	N	N	N	N
AtRLP46	*Atrlp46-1*	Col-0	R	L3	H	L3	N	H	H
AtRLP47	*Atrlp47-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP48	*Atrlp48-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP49	*Atrlp49-1*	Col-0	R	R	H	L3	N	H	H
	Atrlp49-2	Col-0	R	R	H	L3	N	H	H
AtRLP50	*Atrlp50-1*	Col-0	R	L3	H	L3	N	H	H
AtRLP51	*Atrlp51-1*	Col-0	L1	L3	H	L3	N	H	H
	Atrlp51-2	Col-0	L1	L3	H	L3	N	H	H
AtRLP52	*Atrlp52-1*	Col-0	R	L4	H	L3	N	H	H
AtRLP53	*Atrlp53-1*	Col-0	R	L3	H	L3	N	H	H
AtRLP54	*Atrlp54-1*	CS8846	R	L3	H	L3	N	H	H
AtRLP55	*Atrlp55-1*	Col-0	R	L3	H	L3	N	H	H
	Atrlp55-2	Col-0	R	L3	H	L4	N	H	H
AtRLP56	*Atrlp56-1*	Col-0	R	L3	H	L3	N	H	H
	Atrlp56-2	Col-0	R	L2	H	L3	N	H	H
AtRLP57	*Atrlp57-1*	Col-0	R	L2	H	L3	N	H	H
CONTROLS		Col-0	R	L4	H	L3	N	H	H
		Ws-0	N	N	H	N	N	N	N

Approximately 50 7-day old seedlings were spray inoculated with the conidiospores of the pathogen. Asexual sporulation was quantified by counting sporangiophores 7 days after inoculation as described previously (Tör et al., 2002). N, no sporulation; R, rare sporangiophores (<1 per cotyledon); L, low sporulation (1-10 sporangiophores); M, medium (11-16 sporangiophores); H, heavy (>16 sporangiophores); NT, not tested.

Supplemental Table S5. Screening *AtRLP* mutants with flg22 using seedling assays.

Gene name	Mutant name	Ecotype	Replicates on MS only					Mean±SE (MS only)	Replicates on MS+flg22					Mean±SE (MS+flg22)
AtRLP1	*Atrlp1-1*	Col-0	0,193	0,195	0,193	0,206	0,184	0.0970±0.0025	0,047	0,063	0,050	0,075	0,055	0.0290±0.0036
	Atrlp1-2	Col-0												
AtRLP2	*Atrlp2-1*	Col-0	0,137	0,141	0,157	0,158	0,158	0.0751±0.0033	0,095	0,079	0,091	0,094	0,099	0.0458±0.0024
AtRLP3	*Atrlp3-1*	Col-0	0,135	0,152	0,153	0,153	0,119	0.0712±0.0048	0,082	0,076	0,105	0,032	0,096	0.0391±0.0089
	Atrlp3-2	CS8846												
AtRLP4	*Atrlp4-1*	Col-0	0,168	0,152	0,149	0,161	0,163	0.0793±0.0025	0,061	0,065	0,028	0,039	0,047	0.0240±0.0048
AtRLP5	*Atrlp5-1*	Col-0												
AtRLP6	*Atrlp6-1*	Col-0	0,088	0,125	0,096	0,081	0,192	0.0582±0.0144	0,071	0,099	0,033	0,041	0,061	0.0305±0.0083
	Atrlp6-2	CS8846												
AtRLP7	*Atrlp7-1*	Col-0	0,152	0,178	0,149	0,155	0,150	0.0784±0.0038	0,074	0,061	0,072	0,073	0,069	0.0349±0.0017
AtRLP8	*Atrlp8-1*	Col-0												
	Atrlp8-2	Col-0	0,176	0,157	0,162	0,163	0,179	0.0837±0.0030	0,09	0,051	0,057	0,093	0,057	0.0348±0.0064
AtRLP9	*Atrlp9-1*	Col-0												
	Atrlp9-2	Col-0	0,189	0,148	0,132	0,153	0,143	0.0765±0.0068	0,103	0,078	0,096	0,096	0,087	0.0460±0.0031
AtRLP10	*Atrlp10-1*	Col-0	0,130	0,150	0,145	0,151	0,139	0.0715±0.0027	0,08	0,087	0,07	0,094	0,08	0.0411±0.0028
(CLV2)	*clv2-3*	Col-0												
AtRLP11	*Atrlp11-1*	Col-0	0,178	0,159	0,165	0,146	0,137	0.0785±0.0051	0,114	0,06	0,072	0,092	0,061	0.0399±0.0073
AtRLP12	*Atrlp12-1*	Col-0	0,141	0,154	0,148	0,148	0,159	0.0750±0.0022	0,085	0,082	0,064	0,083	0,092	0.0406±0.0033
AtRLP13	*Atrlp13-1*	Col-0	0,164	0,176	0,171	0,166	0,157	0.0834±0.0023	0,076	0,049	0,06	0,092	0,101	0.0378±0.0068
AtRLP14	*Atrlp14-1*	CS8846	0,148	0,170	0,135	0,144	0,143	0.0740±0.0042	0,082	0,068	0,07	0,085	0,074	0.0379±0.0023
AtRLP15	*Atrlp15-1*	Col-0	0,154	0,152	0,150	0,147	0,149	0.0752±0.0009	0,11	0,11	0,071	0,036	0,074	0.0446±0.0098
AtRLP16	*Atrlp16-1*	Col-0	0,169	0,153	0,156	0,157	0,147	0.0782±0.0025	0,068	0,076	0,071	0,092	0,075	0.0382±0.0029
AtRLP17	*Atrlp17-1*	WS-2	0,142	0,158	0,140	0,159	0,169	0.0768±0.0039	0,145	0,151	0,143	0,145	0,141	0.0725±0.0012
(TMM)	*tmm-1*	CS6140	0,145	0,142	0,152	0,149	0,134	0.0722±0.0022	0,084	0,028	0,072	0,119	0,083	0.0429±0.0103
AtRLP18	*Atrlp18-1*	CS8846	0,179	0,165	0,156	0,149	0,139	0.0788±0.0048	0,101	0,093	0,064	0,061	0,07	0.0389±0.0057
AtRLP19	*Atrlp19-1*	WS-2												
AtRLP20	*Atrlp20-1*	Col-0	0,154	0,153	0,157	0,149	0,149	0.0762±0.0011	0,075	0,071	0,086	0,071	0,082	0.0385±0.0021
AtRLP21	*Atrlp21-1*	Col-0	0,170	0,155	0,168	0,168	0,151	0.0812±0.0028	0,078	0,049	0,052	0,055	0,089	0.0323±0.0056
AtRLP22	*Atrlp22-1*	Col-0	0,121	0,163	0,174	0,161	0,143	0.0762±0.0066	0,089	0,081	0,068	0,085	0,053	0.0376±0.0047
AtRLP23	*Atrlp23-1*	Col-0	0,155	0,182	0,153	0,158	0,155	0.0803±0.0038	0,089	0,071	0,082	0,08	0,087	0.0409±0.0022
AtRLP24	*Atrlp24-1*	Col-0	0,169	0,155	0,157	0,161	0,138	0.0780±0.0036	0,095	0,047	0,069	0,031	0,056	0.0298±0.0076
AtRLP25	*Atrlp25-1*	Col-0	0,185	0,180	0,187	0,178	0,189	0.0919±0.0015	0,078	0,054	0,094	0,061	0,066	0.0353±0.0050
AtRLP26	*Atrlp26-1*	Col-0	0,189	0,183	0,164	0,182	0,147	0.0865±0.0055	0,062	0,07	0,097	0,038	0,077	0.0344±0.0068
AtRLP27	*Atrlp27-1*	Col-0	0,121	0,163	0,174	0,161	0,143	0.0762±0.0066	0,089	0,081	0,068	0,085	0,053	0.0376±0.0047
AtRLP28	*Atrlp28-1*	Col-0	0,170	0,184	0,180	0,174	0,171	0.0879±0.0019	0,050	0,058	0,044	0,052	0,020	0.0225±0.0047
AtRLP29	*Atrlp29-1*	Col-0	0,141	0,161	0,154	0,152	0,161	0.0769±0.0026	0,082	0,071	0,082	0,07	0,092	0.0397±0.0029
AtRLP30	*Atrlp30-1*	Col-0	0,169	0,157	0,156	0,160	0,154	0.0796±0.0019	0,086	0,066	0,04	0,067	0,04	0.0299±0.0062
	Atrlp30-2	Col-0												
	Atrlp30-3	Col-0												
	Atrlp30-4	Col-0												
AtRLP31	*Atrlp31-1*	Col-0	0,154	0,137	0,139	0,108	0,134	0.0672±0.0053	0,047	0,077	0,058	0,026	0,054	0.0262±0.0058
	Atrlp31-2	Col-0												

Gene name	Mutant name	Ecotype	Replicates on MS only					Mean±SE (MS only)	Replicates on MS+flg22					Mean±SE (MS+flg22)
AtRLP32	*Atrlp32-1*	WS-2	0,164	0,149	0,127	0,134	0,142	0.0716±0.0045	0,025	0,036	0,049	0,082	0,029	0.0221±0.0073
AtRLP33	*Atrlp33-1*	WS-2												
	Atrlp33-2	Col-0	0,177	0,180	0,189	0,181	0,178	0.0905±0.0015	0,062	0,062	0,068	0,054	0,06	0.0306±0.0016
	Atrlp33-3	Col-0												
AtRLP34	*Atrlp34-1*	Col-0	0,190	0,165	0,160	0,153	0,175	0.0843±0.0046	0,054	0,066	0,075	0,079	0,07	0.0344±0.0030
AtRLP35	*Atrlp35-1*	Col-0	0,157	0,162	0,174	0,148	0,163	0.0804±0.0030	0,031	0,034	0,054	0,066	0,064	0.0249±0.0052
	Atrlp35-2	Col-0												
AtRLP36	*Atrlp36-1*	Col-0	0,175	0,154	0,168	0,096	0,155	0.0748±0.0099	0,042	0,072	0,048	0,062	0,067	0.0291±0.0040
AtRLP37	*Atrlp37-1*	Col-0	0,157	0,185	0,145	0,180	0,179	0.0846±0.0055	0,043	0,062	0,049	0,051	0,029	0.0234±0.0038
AtRLP38	*Atrlp38-1*	Col-0	0,151	0,159	0,170	0,165	0,157	0.0802±0.0023	0,058	0,03	0,053	0,054	0,076	0.0271±0.0052
AtRLP39	*Atrlp39-1*	Col-0	0,176	0,177	0,161	0,169	0,159	0.0842±0.0026	0,042	0,077	0,068	0,068	0,07	0.0325±0.0042
AtRLP40	*Atrlp40-1*	Col-0	0,180	0,183	0,176	0,171	0,182	0.0892±0.0016	0,049	0,022	0,065	0,058	0,043	0.0237±0.0052
AtRLP41	*Atrlp41-1*	Col-0												
	Atrlp41-2	Col-0	0,184	0,173	0,141	0,179	0,165	0.0842±0.0053	0,033	0,033	0,032	0,017	0,038	0.0153±0.0025
	Atrlp41-3	Col-0												
AtRLP42	*Atrlp42-1*	Col-0	0,196	0,157	0,171	0,190	0,192	0.0906±0.0053	0,084	0,079	0,084	0,058	0,108	0.0413±0.0056
	Atrlp42-2	Col-0												
AtRLP43	*Atrlp43-1*	Col-0	0,183	0,179	0,188	0,160	0,170	0.0880±0.0035	0,069	0,05	0,056	0,06	0,045	0.0280±0.0029
AtRLP44	*Atrlp44-1*	Col-0	0,178	0,163	0,186	0,180	0,185	0.0892±0.0029	0,072	0,08	0,072	0,067	0,064	0.0355±0.0019
	Atrlp44-2	Col-0												
AtRLP45	*Atrlp45-1*	Col-0	0,176	0,156	0,163	0,157	0,162	0.0814±0.0025	0,071	0,036	0,066	0,059	0,037	0.0269±0.0052
	Atrlp45-2	WS-2												
AtRLP46	*Atrlp46-1*	Col-0	0,175	0,179	0,172	0,162	0,167	0.0855±0.0021	0,033	0,054	0,055	0,069	0,06	0.0271±0.0042
AtRLP47	*Atrlp47-1*	Col-0	0,177	0,161	0,165	0,158	0,167	0.0828±0.0023	0,039	0,054	0,042	0,044	0,068	0.0247±0.0037
AtRLP48	*Atrlp48-1*	Col-0	0,042	0,176	0,053	0,043	0,044	0.0358±0.0185	0,073	0,032	0,028	0,032	0,03	0.0195±0.0060
AtRLP49	*Atrlp49-1*	Col-0	0,158	0,160	0,163	0,159	0,179	0.0819±0.0028	0,068	0,037	0,063	0,087	0,036	0.0291±0.0069
	Atrlp49-2	Col-0												
AtRLP50	*Atrlp50-1*	Col-0	0,155	0,181	0,169	0,149	0,164	0.0818±0.0039	0,051	0,056	0,05	0,045	0,049	0.0251±0.0013
AtRLP51	*Atrlp51-1*	Col-0												
	Atrlp51-2	Col-0	0,186	0,185	0,194	0,174	0,177	0.0916±0.0025	0,075	0,063	0,077	0,059	0,072	0.0346±0.0025
AtRLP52	*Atrlp52-1*	Col-0	0,177	0,177	0,175	0,162	0,175	0.0866±0.0020	0,072	0,088	0,076	0,073	0,06	0.0369±0.0032
AtRLP53	*Atrlp53-1*	Col-0	0,163	0,180	0,174	0,170	0,186	0.0873±0.0028	0,045	0,048	0,062	0,051	0,071	0.0277±0.0034
AtRLP54	*Atrlp54-1*	CS8846	0,188	0,183	0,168	0,201	0,192	0.0932±0.0039	0,086	0,09	0,066	0,076	0,052	0.0370±0.0049
AtRLP55	*Atrlp55-1*	Col-0	0,172	0,174	0,172	0,182	0,207	0.0907±0.0047	0,059	0,035	0,04	0,041	0,043	0.0218±0.0029
	Atrlp55-2	Col-0												
AtRLP56	*Atrlp56-1*	Col-0	0,131	0,170	0,165	0,176	0,138	0.0780±0.0064	0,056	0,08	0,052	0,065	0,034	0.0287±0.0054
	Atrlp56-2	Col-0												
AtRLP57	*Atrlp57-1*	Col-0	0,171	0,162	0,136	0,151	0,129	0.0749±0.0055	0,052	0,054	0,051	0,077	0,053	0.0287±0.0035
		Ler-Fls2	0,125	0,151	0,129	0,141	0,130	0.0676±0.0034	0,139	0,117	0,112	0,145	0,152	0.0665±0.0056
		Col-Fls2	0,161	0,151	0,153	0,153	0,171	0.0789±0.0026	0,145	0,144	0,143	0,136	0,128	0.0696±0.0023
		Ler-0	0,151	0,138	0,155	0,148	0,160	0.0752±0.0026	0,028	0,052	0,028	0,048	0,052	0.0208±0.0040
		Col-0	0,158	0,157	0,143	0,176	0,161	0.0795±0.0037	0,084	0,07	0,066	0,076	0,086	0.0382±0.0027
		Ws-4	0,159	0,167	0,113	0,166	0,165	0.0770±0.0073	0,137	0,153	0,157	0,141	0,157	0.0745±0.0030

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Chapter 3

Gene Silencing to Investigate the Roles of Receptor-Like Proteins in Arabidopsis

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ABSTRACT

Receptor-like proteins (RLPs) are cell surface receptors that play important roles in various processes. In several plant species RLPs have been found to play a role in disease resistance, including the tomato Cf and Ve proteins and the apple HcrVf proteins that mediate resistance against the fungal pathogens *Cladosporium fulvum*, *Verticillium* spp., and *Venturia inaequalis*, respectively. The Arabidopsis genome contains 57 *AtRLP* genes. Two of these, *CLV2* (*AtRLP10*) and *TMM* (*AtRLP17*), have well-characterized functions in meristem and stomatal development, respectively, while *AtRLP52* is required for defense against powdery mildew. We recently reported the assembly of a genome-wide collection of T-DNA insertion lines for the Arabidopsis *AtRLP* genes. This collection was functionally analyzed with respect to plant growth, development and sensitivity to various stress responses including pathogen susceptibility. Only few new phenotypes were discovered; while *AtRLP41* was found to mediate abscisic acid sensitivity, *AtRLP30* (and possibly *AtRLP18*) was found to be required for full nonhost resistance to a bacterial pathogen. Possibly, identification of novel phenotypes is obscured by functional redundancy. Therefore, RNA interference (RNAi) to target the expression of multiple *AtRLP* genes simultaneously was employed followed by functional analysis of the RNAi lines.

INTRODUCTION

Receptor-like proteins (RLPs) are cell surface receptors that typically consist of an extracellular leucine-rich repeat (eLRR) domain, a single-pass transmembrane domain and a short cytoplasmatic tail that lacks obvious motifs for intracellular signaling except for the putative endocytosis motif found in some members (Jones and Jones, 1997; Joosten and de Wit, 1999; Kruijt et al., 2005). In several plant species RLPs play important roles in development and pathogen defense. Arabidopsis CLAVATA2 (CLV2; AtRLP10) and its maize ortholog FASCIATED EAR2 are required for maintaining the meristematic stem cell population in shoot apical meristems, while Arabidopsis TOO MANY MOUTHS (TMM; AtRLP17) controls the initiation of stomatal precursor cells (Jeong et al., 1999; Geisler et al., 2000; Taguchi-Shiobara et al., 2001; Nadeau and Sack, 2002). The *RLP* disease resistance gene family comprises the tomato *Cf* and *Ve* genes that provide resistance against *Cladosporium fulvum* and *Verticillium* spp., respectively (Kawchuk et al., 2001; Thomma et al., 2005; Fradin and Thomma, 2006), *LeEIX* genes that encode receptors for the ethylene inducible xylanase

produced by *Trichoderma* biocontrol fungi (Ron and Avni, 2004), apple *HcrVf* genes that confer resistance to the scab fungus *Venturia inaequalis* (Malnoy et al., 2008), and an Arabidopsis *RLP* gene (*AtRLP52*) that provides resistance against the powdery mildew pathogen *Erysiphe cichoracearum* (Ramonell et al., 2005). We recently reported the assembly of a genome-wide collection of T-DNA insertion lines for the 57 Arabidopsis *RLP* genes (*AtRLP*) in the Arabidopsis genome (Wang et al., 2008). This collection was functionally analyzed with respect to plant growth, development and sensitivity to various stress responses including pathogen susceptibility. Only few novel phenotypes were discovered; while *AtRLP41* was found to mediate abscisic acid sensitivity, *AtRLP30* (and possibly *AtRLP18*) was found to influence nonhost resistance towards *Pseudomonas syringae* pv. *phaseolicola* (Wang et al., 2008).

Results and discussion

The lack of identification of biological functions for the majority of the *AtRLP* genes may be caused by functional redundancy. Here, we describe a reverse genetics strategy by employing RNA interference (RNAi) to target the expression of multiple *AtRLP* genes simultaneously, and thus possibly overcome functional redundancy among *AtRLP* genes. To select suitable fragments for RNAi silencing, the *AtRLP* genes were aligned and sequence stretches of a few hundred base pairs (bp) containing minimum one 21 bp stretch with 100% identity to minimum one other *AtRLP* gene were identified. Specificity of the selected fragments was verified with BLAST searches against the Arabidopsis genome (Altschul et al., 1997). Seven *AtRLP* gene fragments, varying in length between 238 and 407 bp, were PCR-amplified such that the PCR products contained a 5' *Bam*HI or *Hin*dIII site and a 3' *Eco*RI and *Not*I site (Table 1; Fig. 1A) and cloned into the pGEM-T Easy vector (Promega, Leiden, NL). The resulting plasmids were digested in two separate reactions with *Hin*dIII (or *Bam*HI for RNAi constructs 2 and 5) in combination with *Not*I and in combination with *Eco*RI. Both inserts were cleaned from gel using the QIAquick gel extraction kit (Qiagen, Venlo, NL) and subsequently ligated with a *Not*I- and *Eco*RI-digested 129 bp spacer segment from the *Pichia pastoris Aox-1* gene into the *Hin*dIII-digested (or *Bam*HI for RNAi constructs 2 and 5) pGreen plasmid (Hellens et al., 2000) to obtain inverted repeat constructs driven by the CaMV 35S promoter that target expression of multiple *AtRLP* genes (Fig. 1B). The resulting seven plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, transformed to Arabidopsis (Clough and Bent, 1998), and multiple homozygous single-insertion T_3 lines were selected on MS plates supplemented with 100 µg/mL kanamycin that were used for functional analysis.

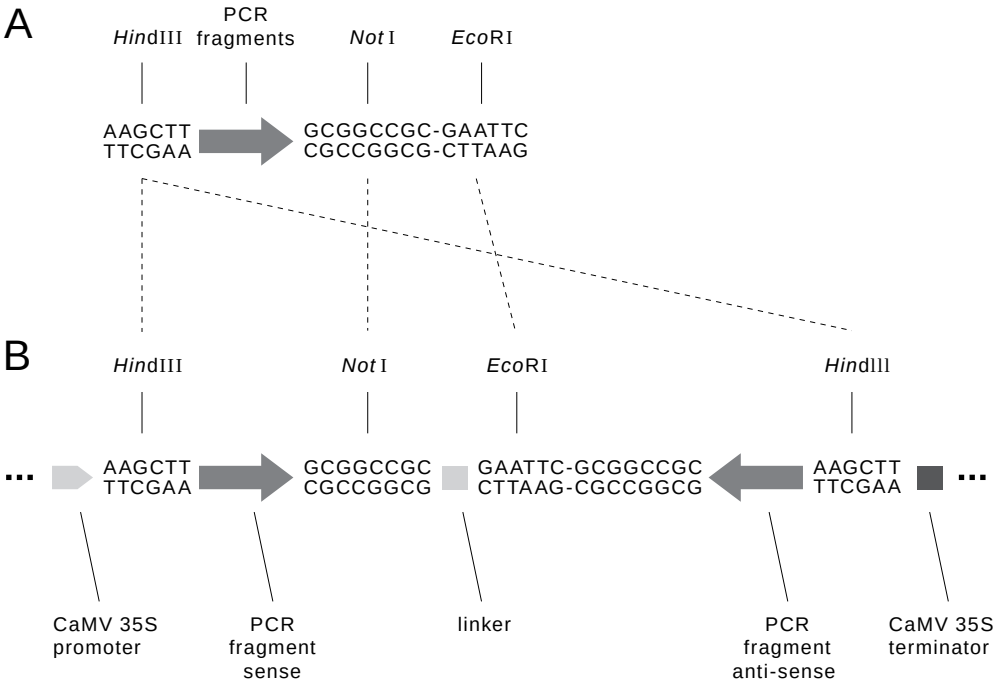

Figure 1. Cloning strategy for RNAi constructs.

A PCR fragments of specific *AtRLP* fragments are generated with 5'*Hin*dIII (or *Bam*HI for RNAi construct 2 and 5) and 3' *Not*I and *Eco*RI restriction sites.

B Inverted repeat constructs are generated by ligating *Hin*dIII (or *Bam*HI for RNAi constructs 2 and 5) and *Not*I digested PCR fragment and *Hin*dIII (or *Bam*HI for RNAi constructs 2 and 5) and *Eco*RI digested PCR fragment together with a *Not*I- and *Eco*RI-digested 129 bp spacer segment from the *Pichia pastoris Aox-1* gene into the *Hin*dIII-digested (or *Bam*HI for RNAi constructs 2 and 5) pGreen backbone. The fragments are not drawn to scale.

Similar as the individual *AtRLP* insertion lines (Wang et al., 2008), also the RNAi lines were analyzed with respect to plant development and sensitivity to various abiotic and biotic stress factors. Development of roots, rosettes, leaf cuticle and flowers as well as stomatal patterning were examined, but no developmental anomalies were observed. In addition, the RNAi lines were assayed for altered sensitivity to plant hormones and abiotic stress factors. The only consistently altered phenotype was observed for lines containing RNAi construct 5 upon exogenous application of the plant hormone abscisic acid (ABA), as leaves of the RNAi lines bleached while wild-type leaves remained green (Fig. 2). Since RNAi construct 5 is predicted to target *AtRLP41* of which a knock-out has been shown to result in enhanced ABA susceptibility (Wang et al., 2008) this phenotype was expected. Moreover, this observation confirms that RNAi-mediated gene silencing can be used as a mechanism to investigate

the function of RLP receptors. To determine whether *AtRLP* genes play a role in recognition of plant pathogens, similar as the individual *AtRLP* insertion lines (Wang et al., 2008) the collection of *AtRLP* RNAi lines was assessed for altered phenotypic responses upon challenge with a range of diverse host-adapted and non-adapted necrotrophic and biotrophic pathogens (Thomma et al., 2001; Wang et al., 2008). In addition to the previously used pathogens (Wang et al., 2008), we included *Fusarium oxysporum* f. sp. *raphani* strain 815, the *Verticillium dahliae* strains St12.01, St17.01 and JR2, as well as the two oomycete strains *Phytophthora brassicae* HH/CBS782.97 and CBS686.95 in our analysis. Interestingly, no significant differences were identified when the responses of the RNAi lines were compared to those of the parental Col-0 line upon inoculation with any of the pathogens used.

The Arabidopsis genome harbors 24 loci containing a single *AtRLP* gene and 13 loci comprising multiple, between two and five, *AtRLP* genes (Fritz-Laylin et al., 2005; Wang et al., 2008). Often, the most homologous *AtRLP* genes reside at the same locus (Fritz-Laylin et al., 2005; Wang et al., 2008), and therefore crossing individual T-DNA insertion lines to obtain knock-out lines for multiple *AtRLP* genes is nearly impossible. RNAi-mediated gene silencing currently is the most suitable strategy to target expression of several highly homologous genes simultaneously.

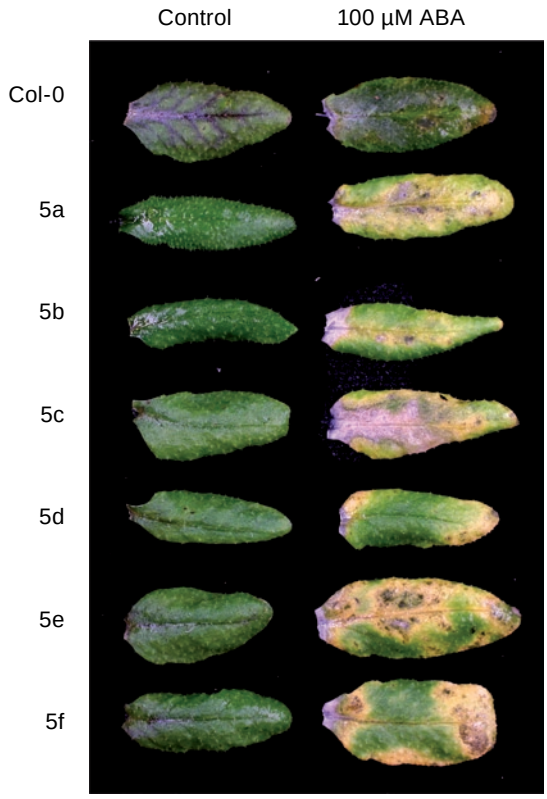

Figure 2. RNAi construct 5 triggers ABA-induced chlorosis.
Comparison of the leaf phenotype of six independent transgenic lines containing RNAi construct 5 (a to f) with the parental line Col-0 three days after application of 100 mM abscisic acid (ABA).

Table 1. RNAi constructs to target homologous *AtRLP* genes.

RNAi construct	Target gene [a]	Primer name	Restriction site	PCR product	Primer sequence (5'-3') [b]	TF No.[c]	Homology to [d]		
1	***AtRLP8***	At1g54480F	*Hin*dIII	301 bp	AAGCTT-GGTTATCCCAGCAGAGC	5	*AtRLP14*	(At1g74180)	21 bp
	(At1g54480)	At1g54480R	*Eco*RI/*Not*I		GAATTC-GCGGCCGC-ATTGGT-CGGTGGTCCAC		*AtRLP21*	(At2g25470)	28 bp
2	***AtRLP53***	At5g27060F	*Bam*HI	407 bp	GGATCC-AAAGGTGTAGCGATGGAGCTGG	8	*AtRLP19*	(At2g15080)	20 + 33 bp
	(At5g27060)	At5g27060R	*Eco*RI/*Not*I		GAATTC-GCGGCCGC-GCTGGCGTGTG-AATATCTCTGC		*AtRLP34*	(At3g11010)	22 + 24 + 26 + 28 + 44 + 59 bp
							AtRLP35	(At3g11080)	21 + 43 + 55 + 102 bp
							AtRLP43	(At3g28890)	22 + 24 + 25 + 27 + 29 bp
3	***AtRLP36***	At3g23010F	*Hin*dIII	336 bp	AAGCTT-CCGATTCTCCGGACATATCCCT	7	*AtRLP37*	(At3g23110)	25 bp
	(At3g23010)	At3g23010R	*Eco*RI/*Not*I		GAATTC-GCGGCCGC-GGCACATGATGG-CTTTCTCCAC		*AtRLP38*	(At3g23120)	28 bp
4	***AtRLP15***	At1g74190F	*Hin*dIII	289 bp	AAGCTT-CCAGACACATTGCTTGC	4	*AtRLP13*	(At1g74170)	22 bp
	(At1g74190)	At1g74190R	*Eco*RI/*Not*I		GAATTC-GCGGCCGC-CATCAGAAGGG-AAAGAAATGC				
5	***AtRLP41***	At3g25010F	*Bam*HI	312 bp	GGATCC-CCGAAATTGCAAGTCCTTCTCC	9	*AtRLP23*	(At2g32680)	24 bp
	(At3g25010)	At3g25010R	*Eco*RI/*Not*I		GAATTC-GCGGCCGC-GGCTGAGG-AAGTAAGAACC		*AtRLP39*	(At3g24900)	22 + 24 + 54 + 56 bp
							AtRLP40	(At3g24954)	25 bp
							AtRLP42	(At3g25020)	24 + 24 + 26 + 27 + 32 + 36 bp
							PGIP	(At3g24982)	25 bp
6	***AtRLP47***	At4g13810F	*Hin*dIII	269 bp	AAGCTT-CCTCTCTGGTATTTTTCCAG	5	*AtRLP48*	(At4g13880)	22 + 24 + 27 bp
	(At4g13810)	At4g13810R	*Eco*RI/*Not*I		GAATTC-GCGGCCGC-TTCGCAACCTG-GAGAAACTTAAAG		*AtRLP49*	(At4g13900)	33 + 36 + 59 + 63 bp
							AtRLP50	(At4g13920)	23 + 41 bp
							PGIP	(At4g13820)	21 bp
7	***AtRLP2***	At1g17240F	*Hin*dIII	238 bp	AAGCTT-TACCAGTCGAAGTTGGCCAG	8	*AtRLP3*	(At1g17250)	28 bp

a Target gene used as template for PCR amplification

b Restriction sites are underlined.

c Number of homozygous single-insert lines tested.

d Stretches of base pair identities (> 20 bp) of the *AtRLP* fragment in the RNAi construct with the most homologous *AtRLP* genes indicated.

Based on the sequence comparison between Arabidopsis and rice RLP genes, and building on the hypothesis that developmental genes are less likely to be duplicated and undergo diversifying selection than are disease resistance genes (Leister, 2004), most *AtRLP* genes were proposed to be candidate disease resistance genes (Fritz-Laylin et al., 2005). Remarkably, despite an extensive list of pathogens tested, including adapted and non-adapted pathogens of Arabidopsis, we have been able to identify only one *AtRLP* gene with a role in basal nonhost resistance against the non-adapted bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola* when screening a genome-wide collection of T-DNA insertion lines in the *AtRLP* genes (Wang et al., 2008). It was hypothesized that the lack of identification of biological functions for *AtRLP* genes may be explained by functional redundancy (Wang et al., 2008). In the experiments presented in this manuscript we employed RNA interference to interfere with the expression of multiple *AtRLP* genes at the same time to overcome functional redundancy among *AtRLP* genes. Nevertheless, no biological functions could be assigned to additional *AtRLP* genes. Obviously, the targeted *AtRLP* genes might function in defense against pathogens that have not yet been assayed. As suggested previously (Wang et al., 2008), if *AtRLP* genes are active in nonhost resistance or basal defense, the array of potential microbial targets may be significantly increased and the response to more microbes or even insects and nematodes should be tested (Stout et al., 2006). Furthermore, it may be questioned whether the knock-down established by RNAi is sufficiently strong to compromise RLP receptor activity, although gene silencing has been successfully used to compromise the activity of RLP-type disease resistance genes in tomato (Gabriëls et al., 2006). Also, the observation that transformants expressing RNAi construct 5 phenocopies the *AtRLP41* T-DNA insertion allele with respect to ABA responsiveness argues against this possibility. Possibly, however, the RNAi constructs do not silence all redundant *AtRLP* homologs as efficiently or target all the redundant *AtRLP* homologs. For instance, RNAi construct 4 that is derived from *AtRLP15* is predicted to silence expression of *AtRLP13*, but not of *AtRLP16* which is also close homologue of *AtRLP15*. Finally, redundant *AtRLP* genes are not necessarily those with the highest overall homology, since ligand specificity may be determined by only a small sequence stretch, making it difficult to design the most potent RNAi constructs. Therefore, a more extensive analysis using many more RNAi constructs is needed to exclude the possibility that the lack of phenotypes can be explained by a high degree of functional redundancy among the *AtRLP* genes. Overall, the RNAi lines developed in our studies provide a useful tool for further investigation into roles of the *AtRLP* genes.

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Chapter 4

RNA Silencing

is Required for Arabidopsis Defense against *Verticillium* Wilt Disease

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Abstract

RNA silencing is a conserved mechanism in eukaryotes that plays an important role in various biological processes including regulation of gene expression. RNA silencing also plays a role in genome stability and protects plants against invading nucleic acids such as transgenes and viruses. Recently, RNA silencing has been found to play a role in defense against bacterial plant pathogens in Arabidopsis through modulating host defense responses. In this study, we show that gene silencing plays a role in plant defense against multicellular microbial pathogens; vascular fungi belonging to the *Verticillium* genus. Several components of RNA silencing pathways were tested, of which many were found to affect *Verticillium* defense. Remarkably, no altered defense towards other fungal pathogens that include *Alternaria brassicicola*, *Botrytis cinerea* and *Plectosphaerella cucumerina*, but also the vascular pathogen *Fusarium oxysporum*, was recorded. Since the observed differences in *Verticillium* susceptibility cannot be explained by notable differences in root architecture, it is speculated that the gene silencing mechanisms affect regulation of *Verticillium*-specific defense responses.

Introduction

Plant defense against pathogens is activated through specific host signaling mechanisms (Chisholm et al., 2006; Jones and Dangl, 2006). Microbial intruders can be recognized by extracellular receptor molecules that detect the presence of pathogen-associated molecular patterns (PAMPs) and subsequently activate PAMP-triggered immunity (PTI) as a basal defense response. Virulent pathogen strains are able to interfere with, or suppress, PTI by utilizing effector molecules (Bolton et al., 2008; van Esse et al., 2007; 2008). In turn, some plant genotypes have developed specific receptor molecules, the resistance proteins, to detect the presence of the pathogen effector molecules and activate effector-triggered immunity (ETI; Chisholm et al., 2006; Jones and Dangl, 2006). Only in few cases, a direct interaction of the host resistance protein with the pathogen effector molecule has been observed (Scofield et al., 1996; Tang et al., 1996; Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006; Burch-Smith et al., 2007). More often, however, the resistance protein monitors the status of a host target of the pathogen effector molecule in compliance with the guard hypothesis (Dangl and Jones, 2001; Mackey et al., 2002; Shao et al., 2003).

Nearly twenty years ago, the phenomenon of RNA silencing was discovered in experiments with plants transgenic that showed silencing of a transgene, and in a number of

cases also of homologous endogenous genes (Napoli et al., 1990; van der Krol et al., 1990). The gene silencing was found to result from inhibition of gene transcription (transcriptional gene silencing, TGS) or from post-transcriptional degradation of RNA (post-transcriptional gene silencing, PTGS), and correlated with the accumulation of small double-stranded RNA segments of 20 to 27 nucleotides, so-called small RNAs (sRNAs). These corresponded to the promoter of the silenced gene, or to the degraded RNA in TGS and PTGS, respectively (Hamilton and Baulcombe, 1999; Mette et al., 2000).

RNA silencing is now known as a conserved regulatory mechanism in most eukaryotic organisms that plays a determinant role in various biological processes, including regulation of endogenous gene expression, genome stability, taming of transposons, heterochromatin formation and defense against viruses (Brodersen and Voinnet, 2006; Vaucheret, 2006). The key characteristic of RNA silencing is the formation of the sRNAs that are produced by RNaseIII-like Dicer enzymes (Bernstein et al., 2001). These sRNAs can be divided into two major types, the small interfering RNAs (siRNAs) and the micro RNAs (miRNAs), based on their origin and formation. Subsequently, a selected sRNA strand is incorporated into an effector complex that is targeted towards partially or fully complementary RNA or DNA stretches. This so-called RNA-induced silencing complex (RISC) contains an Argonaute (Ago) protein that has an sRNA-binding domain and endonucleolytic activity to cleave target RNAs (Martinez et al., 2002).

Several studies have shown that PTGS mechanisms are an RNA-based host defense system to control nucleic acid invaders of various nature through the action of cis-acting si-RNAs that derive from, and target, the invaders (Vance and Vaucheret, 2001; Bartel, 2004; Baulcombe, 2004; Dunoyer and Voinnet, 2005). These invaders may be endogenous, such as transposons, or exogenous, such as transgenes and viral pathogens. Thus, RNA silencing has been implicated in pathogen defense through its role in viral defense. Upon virus infection, the accumulation of virus-derived sRNAs has been observed (Hamilton and Baulcombe, 1999). Moreover, plant mutants defective in PTGS are often hyper-susceptible to viral infection (Mourrain et al., 2000; Dalmay et al., 2001; Qu et al., 2005; Schwach et al., 2005).

Apart from viral defense, evidence accumulates for RNA silencing to play a role in interactions with other pathogen types, more specifically bacterial defense (Voinnet, 2008). The first example is a miRNA from Arabidopsis that contributes to basal defense against *Pseudomonas syringae* by regulating auxin signaling (Navarro et al., 2006). The miRNA was induced upon perception of flg-22, a PAMP that is derived from bacterial flagellin, and negatively regulated transcripts of a number of F-box auxin receptors. In turn, repression of auxin signaling was shown to restrict growth of the bacterium *P. syringae* (Navarro et al., 2006).

Another example is an endogenous Arabidopsis siRNA that is specifically induced by avirulent *P. syringae* carrying *AvrRpt2* (Katiyar-Agarwal et al., 2006). This siRNA contributes to RPS2-mediated disease resistance by repressing a putative negative regulator of the RPS2 resistance pathway. Recently, a novel class of small RNAs, long siRNAs (lsiRNAs that are 30-40 nt) that is induced by pathogen infection or under specific growth conditions was identified. One of the lsiRNAs, AtlsiRNA-1, was specifically induced by avirulent *P. syringae* carrying *AvrRpt2* and induction of AtlsiRNA-1 was found to silence a RAP-domain protein that is involved in disease resistance (Katiyar-Agarwal et al., 2007). Finally, in a forward genetics screen, an Arabidopsis mutant with enhanced disease susceptibility towards a compatible *P. syringae* strain, an incompatible strain carrying *AvrRpm1*, and non-adapted *P. syringae* pv. *tabaci* was isolated (Agorio and Vera, 2007). Positional cloning revealed a mutation in the *Argonaute* gene *AGO4*, that is associated with small interfering RNAs involved in RNA-directed DNA methylation (RdDM), showing that AGO4 plays a role in nonhost resistance, basal defense and effector-triggered immunity against bacterial pathogens (Agorio and Vera, 2007). In addition to *P. syringae*, it has been shown that RNA silencing mutants are hypersusceptible to the crown gall bacterium *Agrobacterium tumefaciens* (Dunoyer et al., 2006). Finally, RNA silencing has been shown to be required for the development of nodule differentiation on *Medicago truncatula* roots in the interaction with the nitrogen fixating *Rhizobium* bacteria (Combier et al., 2006; Boualem et al., 2008).

Recently it has been demonstrated that miRNAs are key components of plant basal defense as miRNA-deficient Arabidopsis mutants sustained growth of a non-pathogenic, type III secretion-defective *P. syringae* mutant, nonpathogenic *P. fluorescens* and *Escherichia coli* strains (Navarro et al., 2008). Interestingly, *P. syringae* effectors were identified that suppressed the transcriptional activation or activity of several PAMP-responsive miRNAs, demonstrating that these bacteria suppress RNA silencing to cause disease (Navarro et al., 2008).

In our research, *Arabidopsis thaliana* has been used as a host to investigate the biology of the vascular wilt pathogen *Verticillium dahliae* (Fradin and Thomma, 2006). To investigate the role of putative defense genes against *Verticillium* infection, we employ transgenic over-expression in wild-type (Col-0) Arabidopsis, but also in the PTGS mutant *sgs2* (Butaye et al., 2004). Previously, it has been shown that the inter-transformant variability of transgene expression is reduced in *sgs* mutants, as the incidence of highly expressing transformants increased from 20% in Col-0 to 100% in *sgs* mutants (Butaye et al., 2004). Intriguingly, it was observed in several of our experiments that non-transformed *sgs2* plants displayed significantly enhanced susceptibility towards *V. dahliae* when compared with the parental line Col-0. In this manuscript we investigate the role of RNA silencing in Arabidopsis defense against a number of fungal pathogens including *Verticillium dahliae*.

RESULTS

Sgs Mutants Display Enhanced Susceptibility towards *Verticillium dahliae*

Transgenic expression in the post-transcriptional gene silencing (PTGS) mutant *suppressor of gene silencing 2* (*sgs2*; Elmayan et al., 1998; Mourrain et al., 2000) reduces inter-transformant variability of transgene expression (Butaye et al., 2004). In several experiments to investigate putative defense genes against *V. dahliae* in Arabidopsis, transgenic overexpression in Col-0 as well as *sgs2-1* was performed. Remarkably, in subsequent disease susceptibility assays with *V. dahliae* strain JR2 it appeared that untransformed *sgs2-1* plants displayed more severe disease symptoms than Col-0 plants (Fig. 1, A and B). While Col-0 plants displayed only mild disease symptoms upon *V. dahliae* inoculation as visualized by rather slight stunting resulting in a reduced rosette diameter at three weeks post inoculation, inoculated *sgs2-1* plants showed severe stunting, wilting, anthocyanin accumulation and tissue necrosis (Fig. 1, A and B). Also the ratio of leaves displaying symptoms of disease was significantly more for *sgs2-1* plants than for Col-0 plants (Fig. 1, A and B)

In addition to *V. dahliae* strain JR2, our analysis was extended to include additional *Verticillium* pathogens of Arabidopsis (Fradin and Thomma, 2006). These included *V. dahliae* strain ST12.01, the *V. albo-atrum* strains VA1 and CBS451.88, and *V. longisporum* strain Vl43. All these *Verticillium* strains caused more disease symptoms on *sgs2-1* plants when compared with Col-0 plants (Supplemental Fig. S1), confirming that the enhanced susceptibility of the *sgs2-1* mutant broadly concerns plant pathogenic *Verticillium* species.

In addition to *sgs2-1*, reduced inter-transformant variability in transgene expression was similarly demonstrated in the non-allelic *sgs3-1* mutant (Butaye et al., 2004). To investigate the role of PTGS in Arabidopsis defense against *Verticillium* further, the two additional non-allelic PTGS mutants; *sgs1-1* and *sgs3-1* (Elmayan et al., 1998; Mourrain et al., 2000) were tested for their susceptibility towards *V. dahliae* strain JR2. Similar as for *sgs2-1* plants, also *sgs1-1* and *sgs3-1* plants consistently displayed enhanced disease development upon *V. dahliae* inoculation (Figs. 1, A and B).

To quantify *V. dahliae* colonization in the different Arabidopsis genotypes, the fungal biomass was measured with real-time PCR. Determination of the average fungal biomass revealed significantly enhanced fungal colonization in *V. dahliae*-inoculated *sgs1-1*, *sgs2-1* and *sgs3-1* plants when compared with the inoculated Col-0 plants (Fig. 1C), since at least double the amount of fungal biomass was detected in these mutants at three weeks post inoculation.

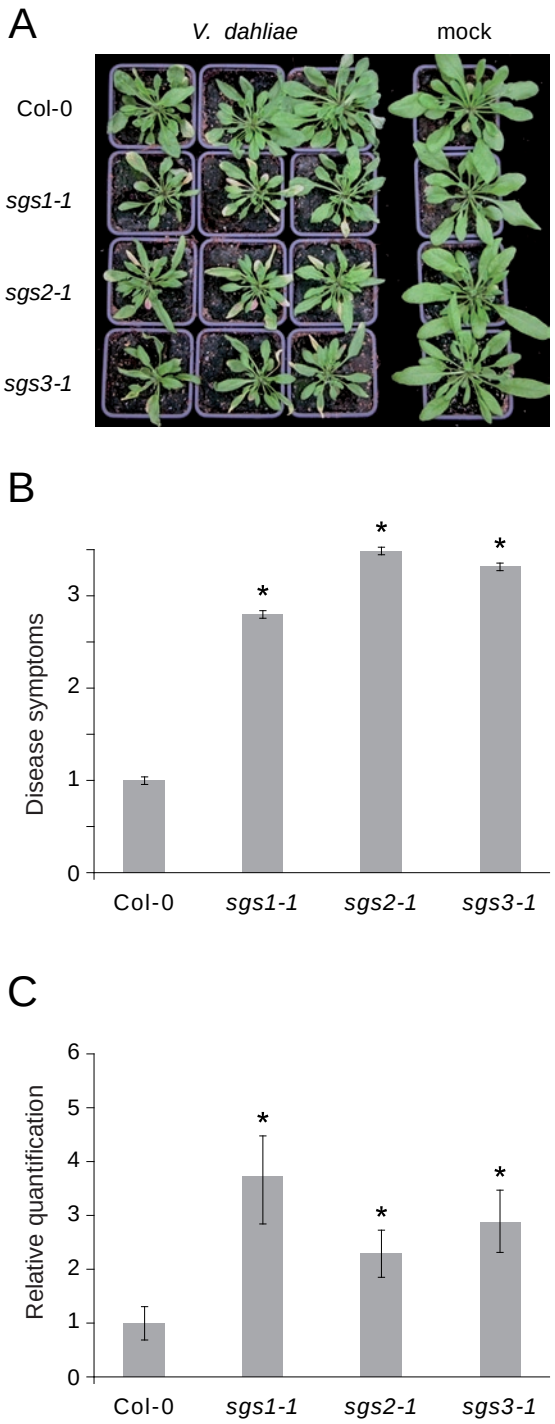

Figure 1. Arabidopsis *sgs* mutants display enhanced susceptibility towards *Verticillium dahliae*.

A Typical symptoms of *Verticillium dahliae* on Arabidopsis *sgs* mutants. The mutants *sgs1-1*, *sgs2-1*, *sgs3-1*, and the corresponding wild type Col-0 were inoculated with *V. dahliae* strain JR2 or mock-inoculated. *V. dahliae*-inoculated *sgs* mutants show enhanced symptom development, including more severe stunting, wilting, anthocyanin accumulation and tissue necrosis, when compared with Col-0 plants at 19 days post inoculation.

B Quantification of symptom development at 19 days post inoculation shown as ratio of diseased rosette leaves with standard deviation. The ratio of diseased rosette leaves for Col-0 is set to one. Asterisks indicate significant differences when compared with the wild type Col-0 ($P < 0.05$).

C Quantitative real-time PCR of fungal colonization by comparing *V. dahliae* internal transcribed spacer (ITS) transcript levels (as a measure for fungal biomass) relative to Arabidopsis Rubisco transcript levels (for equilibration) at 19 days post inoculation. The mutants *sgs1-1*, *sgs2-1*, *sgs3-1*, and the corresponding wild type Col-0 were inoculated with *V. dahliae* strain JR2 and the relative average fungal biomass is shown with standard errors. Asterisks indicate significant differences when compared with colonization of the wild type Col-0.

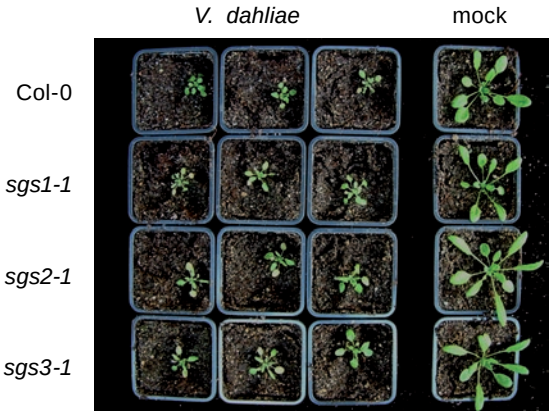

Figure 2. Typical symptoms caused by *Fusarium oxysporum* on Arabidopsis *sgs* mutants.
The mutants *sgs1-1*, *sgs2-1*, *sgs3-1*, and the corresponding wild type Col-0 were inoculated with *F. oxysporum* f.sp. *raphani*, or mock-inoculated. The picture was taken at 12 days post inoculation.

Sgs Mutants do not Display Enhanced Susceptibility towards Other Pathogens

To investigate whether the enhanced pathogen susceptibility phenotype of the *sgs* mutants extended to other pathogens in addition to *Verticillium* species, we tested the susceptibility of the *sgs1-1*, *sgs2-1* and *sgs3-1* mutants towards the vascular fungus *Fusarium oxysporum* f.sp. *raphani* (Diener and Ausubel, 2005). However, disease development on the three *sgs* mutants did not differ from disease development on Col-0 plants upon inoculation with this pathogen (Fig. 2). Furthermore, a number of additional fungal and bacterial pathogens was tested on the *sgs* mutants (Supplemental Table S1; Wang et al., 2008). These comprised the foliar fungal pathogens *Botrytis cinerea*, *Alternaria brassicicola* and *Plectosphaerella cucumerina*, and virulent and avirulent strains of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. However, for none of these pathogens altered susceptibility was observed in the *sgs* mutants when compared with Col-0 (data not shown). Thus, the enhanced susceptibility of the *sgs* mutants is specific for *Verticillium* pathogens and does not extend to other pathogens.

Sgs Mutants do not Display Altered Sensitivity towards Abiotic Stress

RNA silencing has also been implicated in abiotic stress resistance (Borsani et al., 2005; Sunkar et al., 2007). Therefore, the *sgs* mutants were screened for their responses towards treatment with different hormones (abscisic acid, auxin, brassinolide, cytokinin, ethylene, gibberellic acid and jasmonate) and sensitivity towards salt, heavy metal reactive oxygen and osmotic stress (Supplemental Table S1; Wang et al., 2008). However, none of the *sgs* mutants showed significantly altered phenotypes towards these treatments when compared with Col-0 plants (data not shown).

Table 1. Arabidopsis mutants used in this study.

Gene name	AGI code	Protein function	Mutant allele	Reference
AGO1	At1g48410	slicer in RISC	*ago1-25*	Morel et al, 2002
			ago1-27	Morel et al, 2002
AGO7	At1g69440	slicer in RISC	*ago7-2*	SALK_095997 [a]
DCL2	At3g03300	dicer	*dcl2-1*	Xie et al, 2004
DCL4	At5g20320	dicer	*dcl4-2*	Yoshikawa et al, 2005
HEN1	At4g20910	methyltransferase	*hen1-6*	Li et al, 2005
HST	At3g05040	transporter	*hst-1*	Telfer and Poethig, 1998
NRPD1a/SDE4	At1g63020	polymerase	*nrpd1a-3*	Herr et al, 2005
RDR2	At4g11130	RDR	*rdr2-4*	Smith et al, 2007
RDR6/SDE1/SGS2	At3g49500	RDR	*sgs2-1*	Elmayan et al, 1998
			rdr6-11	Peragine et al, 2004
			rdr6-15	Allen et al, 2004
SDE3	At1g05460	RNA helicase	*sde3-4*	Vazquez et al, 2004b
			sde3-5	SALK_003347 [a]
SGS1	Unknown	Unknown	*sgs1-1*	Elmayan et al, 1998
SGS3/SDE2	At5g23570	CC-domain	*sgs3-1*	Mourrain et al, 2000
		protein	*sgs3-11*	Peragine et al, 2004

a SALK T-DNA insertion mutant (Alonso et al., 2003)

Assessment of *Verticillium* Susceptibility in Additional Gene Silencing Mutants

The enhanced susceptibility phenotype of the *sgs* mutants upon *Verticillium* inoculation directed us to assess susceptibility towards this pathogen in additional gene silencing mutants. These comprised additional mutant alleles of *SGS2* (also known as *RDR6*), namely *rdr6-11* and *rdr6-15*, and for *SGS3*, namely *sgs3-11*. Furthermore, also mutants of other components of RNA silencing pathways were included (Table 1). These included mutants of genes that encode different enzyme families, such as the argonautes AGO1 and AGO7, the dicers DCL2 and DCL4, the methyltransferase HEN1, the putative sRNA transporter HST, the DNA dependent RNA polymerase NRPD1a, the RNA-dependent RNA polymerase RDR2 and the RNA helicase SDE3 that all have been implicated in different RNA silencing pathways (Table 1; Voinnet 2008). All mutants, derived from a Col-0 parental line, were challenged with *V. dahliae* strain JR2. As expected, additional mutant alleles of *SGS2* and *SGS3* (*rdr6-11*, *rdr6-15* and *sgs3-11*) were more susceptible than Col-0 plants upon *V. dahliae* inoculation (Fig. 3A), thus confirming the enhanced susceptibility observed in the *sgs2-1* and *sgs3-1* mutants.

The other PTGS mutants could be divided into three classes based on the phenotypes upon *V. dahliae* inoculation; those displaying enhanced susceptibility (Fig. 3A), mutants displaying enhanced resistance (Fig. 3B), and mutants displaying similar disease phenotypes as *Verticillium*-inoculated Col-0 plants (Fig. 3C). The mutants *ago7-2*, *dcl4-2*, *nrpd1a-3* and *rdr2-4* were found to be more susceptible to *V. dahliae* challenge by showing more severe stunting and necrosis when compared with inoculated Col-0 plants (Fig. 3A and Supplemental Fig. S2). In contrast, the mutants *ago1-25*, *ago1-27*, *hen1-6* and *hst-1* were found to be more resistant because they displayed less necrosis and no anthocyanin production when compared with Col-0 plants upon *V. dahliae* inoculation (Fig. 3B and Supplemental Fig. S2). Finally, the mutants *dcl2-1*, *sde3-4* and *sde3-5* showed a disease susceptibility phenotype that was similar to that of Col-0 with respect to severity of stunting, necrosis and anthocyanin production (Fig. 3C and Supplemental Fig. S2).

Table 2. Quantification of *Verticillium dahliae* biomass in Arabidopsis gene silencing mutants by real time PCR comparison of *V. dahliae* internal transcribed spacer (ITS) transcript levels (as a measure for fungal biomass) relative to Arabidopsis RuBisCo transcript levels (for equilibration) at 19 to 29 days post inoculation with *V. dahliae* strain JR2.

Gene name	Genotype	Symptom display [a]	Biomass fold change [b]	Significance [c]
	Col-0	-	1	-
AGO1	*ago1-27*	reduced	0.007	p<0.1
AGO7	*ago7-2*	enhanced	3.174	p<0.2
DCL2	*dcl2-1*	similar	0.829	no
DCL4	*dcl4-2*	enhanced	2.422	p<0.05
HEN1	*hen1-6*	reduced	0.045	p<0.1
HST	*hst-1*	reduced	0.039	p<0.05
NRPD1a/SDE4	*nrpd1a-3*	enhanced	1.816	p<0.2
RDR2	*rdr2-4*	enhanced	2.701	p<0.05
RDR6/SDE1/SGS2	*sgs2-1*	enhanced	2.279	p<0.05
	rdr6-15	enhanced	3.286	p<0.05
SDE3	*sde3-4*	similar	1.674	no
SGS1	*sgs1-1*	enhanced	3.729	p<0.05
SGS3/SDE2	*sgs3-1*	enhanced	2.938	p<0.05

a Symptom display upon *V. dahliae* inoculation when compared with Col-0 (also see Fig. 3).

b The relative average fungal biomass is indicated as relative fold-change when compared with fungal biomass in *V. dahliae*-inoculated Col-0 plants of which the average fungal biomass was set to one.

c Statistically significant differences are given as p-values according to a Student's t-test with a 95 to 80% confidence interval ($p < 0.05$ to 0.2).

Quantification of *Verticillium dahliae* Biomass *in Planta*

To quantify *V. dahliae* colonization in the different Arabidopsis genotypes, the fungal biomass was measured in individual plants with real-time PCR. For each of the genes tested, the average fungal colonization of at least one mutant allele was quantified with real-time PCR. This analysis demonstrated that the altered susceptibility phenotypes correlated with the degree of fungal colonization when compared with inoculated Col-0 plants (Table 2). The mutants displaying enhanced symptoms upon *Verticillium* inoculation (*sgs1-1*, *sgs2-1*, *sgs3-1*, *ago7-2*, *dcl4-2*, *nrpd1a-3*, *rdr2-4* and *rdr6-15*) accumulated significantly more fungal biomass when compared with inoculated Col-0 plants, while the mutants that showed reduced symptom development (*ago1-27*, *hen1-6* and *hst-1*) accumulated significantly less fungal biomass. In contrast, fungal biomass accumulation in *Verticillium*-inoculated *dcl2-1* and *sde3-4* plants was not significant different from that of inoculated Col-0 plants (Table 2).

Assessment of Root Development and Architecture

Being a root pathogen, differences in *Verticillium* susceptibility of the different mutants may be explained by differences in root architecture, the tissues that are inoculated. Although no obvious differences in root architecture were observed during uprooting and inoculation of the mutants, except for the *ago* mutants that developed shorter roots, root development and architecture was assessed upon *in vitro* growth on MS medium. However, apart from rather slight growth differences, no notable differences in root development and architecture were observed for the RNA silencing mutants that correlated with the differences in *Verticillium* susceptibility (Fig. 4). For all mutants, development of the primary, dominant, root was followed by production of lateral roots in a later stage.

Assessment of Basal Defense Responses

To investigate whether the altered *Verticillium* susceptibility phenotypes of the various PTGS mutants can be explained by defects in basal defense signaling pathways, the expression of molecular markers for salicylic acid- (SA-) and jasmonic acid- (JA-) mediated defense response pathways was assessed. Expression of the SA marker gene *PR-1* (Uknes et al., 1992) was clearly induced in Col-0 plants as well as in all PTGS mutants at 24 hours after drop-inoculation with 2 mM SA (Supplemental Figure S3). In non-treated plants, little to no *PR-1* expression was monitored in these genotypes (data not shown). Thus, the altered susceptibility phenotypes could not be correlated to changes in SA-mediated defense responses. Similarly, also the expression patterns of the JA-marker *PDF1.2* (Penninckx et al., 1996; Thomma et al., 1998) and the chitin elicitor-responsive marker *MPK3* (Wan et al., 2008) could not be correlated to the altered susceptibility phenotypes (data not shown).

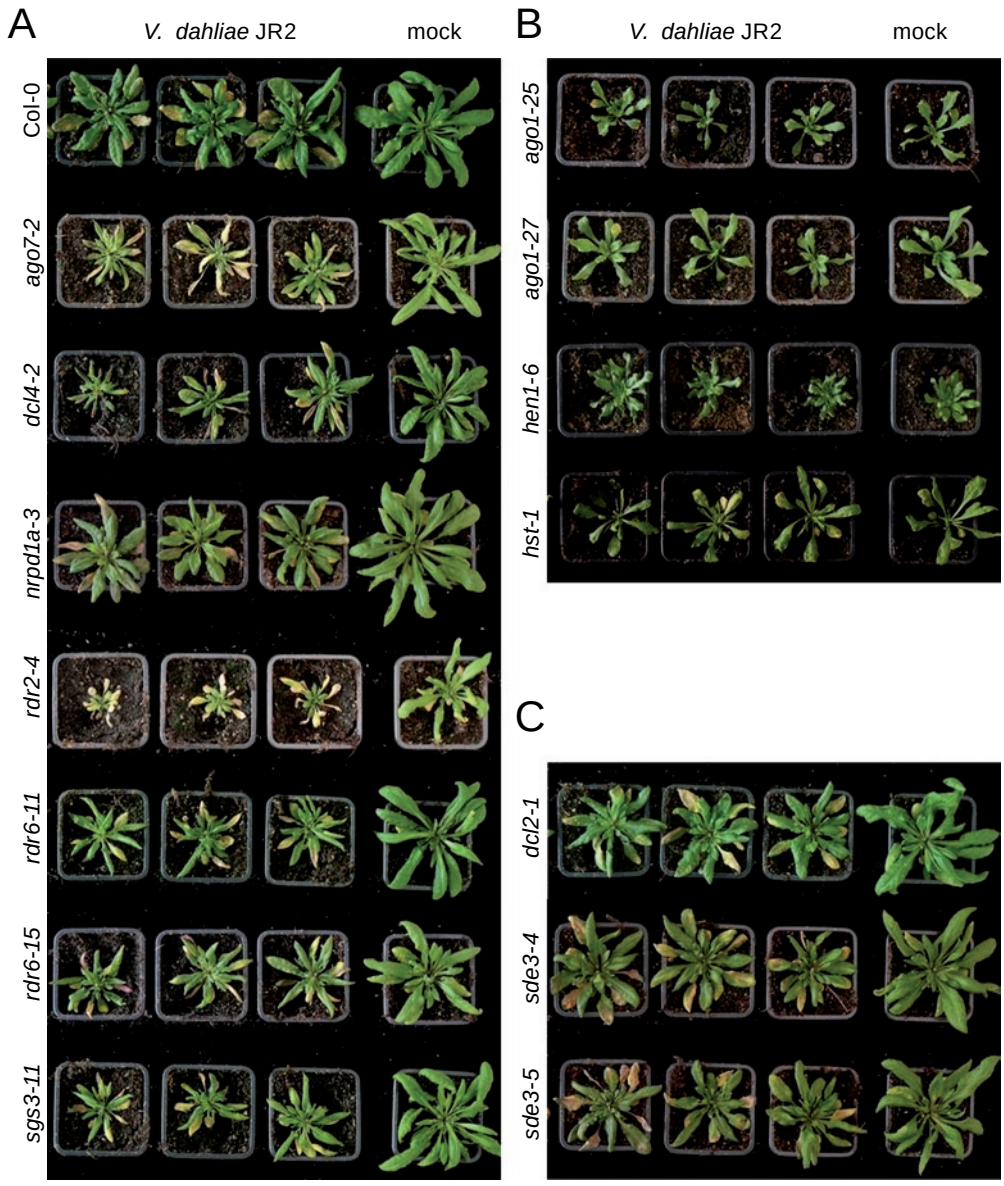

Figure 3. Typical symptoms caused by *Verticillium dahliae* on various Arabidopsis silencing mutants.
Arabidopsis gene silencing mutants and the corresponding wild type Col-0 were inoculated with *V. dahliae* strain JR2, or mock-inoculated.

A *V. dahliae*-inoculated *ago7-2*, *dcl4-2*, *rdr6-11*, *rdr6-15*, and *sgs3-11* plants show enhanced symptom development, including more severe stunting, wilting, anthocyanin accumulation and tissue necrosis, compared with inoculated Col-0 plants at 20 days post inoculation.

B *V. dahliae*-inoculated *ago1-25*, *ago1-27*, *hen1-6* and *hst-1* mutants develop fewer symptoms than inoculated Col-0 plants (panel A) at 20 days post inoculation.

C *V. dahliae*-inoculated *dcl2-1*, *sde3-4* and *sde3-5* mutants show similar disease symptoms as inoculated Col-0 plants (panel A) at 20 days post inoculation.

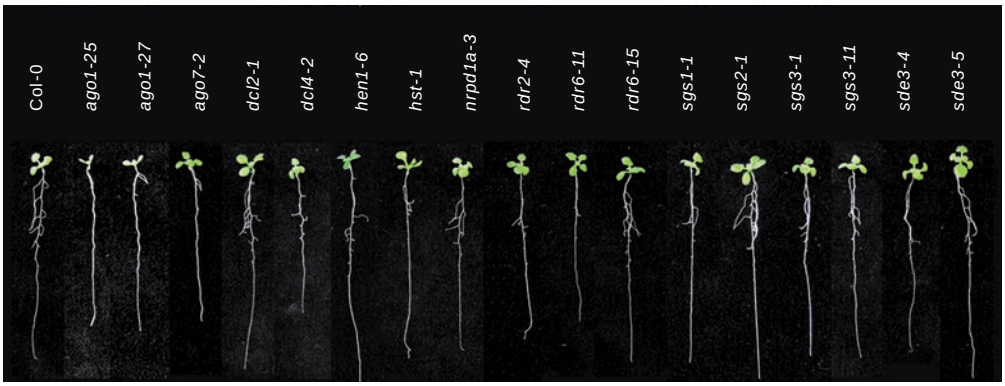

Figure 4. Typical root architecture of *in vitro*-grown Arabidopsis gene silencing mutants.
Roots were grown on vertically oriented MS plates and pictures were taken ten days after sowing.

DISCUSSION

Recent evidence indicates that, apart from defense against viruses, RNA silencing plays a role in defense against bacterial pathogens (Voinnet, 2008), and that similar to viruses also bacteria have developed mechanisms to suppress RNA silencing in order to cause disease (Navarro et al., 2008). Here, we show that RNA silencing is also important for defense against multicellular, eukaryotic, microbial pathogens; namely vascular fungi of the *Verticillium* genus. These include strains of the species *V. dahliae*, *V. albo-atrum* and *V. longisporum* that are all pathogenic on Arabidopsis (Fradin and Thomma, 2006). Various components of RNA silencing pathways were tested and most of them were found to affect *Verticillium* resistance, some positively and others negatively. Furthermore, our results show that PTGS is truly affecting *Verticillium* resistance and not merely symptom development or display, since altered symptom development of the *Verticillium* inoculated RNA silencing mutants correlated with altered *Verticillium* colonization in these mutants as shown by real-time PCR-based fungal biomass quantification (Table 2).

The altered susceptibility phenotypes of the RNA silencing mutants is specific for *Verticillium* defense as is shown for the *sgs* mutants. Inoculations of the *sgs* mutants with strains belonging to different pathogenic species of the *Verticillium* genus all resulted in a similar increased susceptibility phenotype. Inoculations with other pathogens that employ different colonization and feeding styles did not show altered susceptibility phenotypes. This suggests that the enhanced susceptibility is not due to defects in any of the well-known basal

defense signaling pathways (Thomma et al., 2001a). Indeed, in our analysis we were not able to correlate altered susceptibility to SA or JA signaling. However, this is not surprising because alterations in these basal defense responses would most likely be reflected in altered susceptibility towards some of the other pathogens that were tested. For instance, altered SA signaling would most likely lead to altered susceptibility towards *P. syringae* and *P. cucumerina*, while altered JA-signaling would be reflected in *A. brassicicola* and *B. cinerea* resistance (Thomma et al., 1998; 2001a; 2001b). Our assays also included the vascular fungal pathogen *F. oxysporum* f sp. *raphani* that displays a similar life style as *Verticillium* spp. Both *F. oxysporum* and *Verticillium* spp. infect plants through the roots and enter the xylem where they release conidia that spread upwards through the vessels with the transpiration stream (Di Pietro et al., 2001; Fradin and Thomma, 2006; Berrocal-Lobo and Molina, 2008). Despite these similarities in host colonization, the susceptibility of the RNA silencing mutants is specific towards *Verticillium* spp., suggesting that a highly specific disease mechanism is affected in these mutants. Since the different RNA silencing mutants did not show obvious alterations in root development or architecture that correlated with the altered susceptibility phenotypes, this mechanism could not be linked to root development.

In contrast to *SGS1*, both *SGS2* (also known as *RDR6* and *SDE1*) and *SGS3* were cloned and found to encode an RNA dependent RNA polymerase (RDR) and a protein of unknown function, respectively. SGS2 and SGS3 are required for the synthesis of dsRNA in different RNA silencing pathways (Dalmay et al., 2000; Mourrain et al., 2000; Brodersen and Voinnet, 2006; Vaucheret, 2006). Furthermore, our analysis comprised mutants for the argonautes AGO1 and AGO7, the dicers DCL2 and DCL4, the methyltransferase HEN1, the putative sRNA transporter HST, the DNA dependent RNA polymerase NRPD1a, the RNA dependent RNA polymerase RDR2 and the RNA helicase SDE3, all of which have been implicated in different RNA silencing pathways and regulate processes including TGS, PTGS, antiviral defense, plant development, hormone signaling, and abiotic and biotic stress tolerance (Brodersen and Voinnet, 2006; Vaucheret, 2006; Voinnet, 2008). While HEN1 methylates small RNA species and thus protects these sRNAs from degradation and polyuridylation (Chen et al., 2002; Li et al., 2005; Yu et al., 2005), HST possibly mediates the transport of miRNAs from the nucleus to the cytoplasm (Mallory and Vaucheret, 2006; Sunkar et al., 2007). SDE3 acts as an RNA helicase and may facilitate the synthesis of dsRNA by SGS2/RDR6/SDE1 (Dalmay et al., 2001). Although its precise function is unclear, NRPD1a is suggested to be a silencing-specific polymerase (Herr et al., 2005). In this study, as many as ten different RNA silencing components, namely AGO7, DCL4, NRPD1a, RDR2, SGS1, SGS2/RDR6/SDE1, SGS3, AGO1, HEN1 and HST were all shown to affect *Verticillium* defense.

The combination of RNA silencing components that is involved in altered *Verticillium* susceptibility does not comply with one single RNA silencing pathway among those that are currently discriminated. However, the identification and full characterization of such pathways is still in its infancy. Defense against *Verticillium* might trigger a novel RNA silencing pathway that is similar to the natural *cis*-antisense transcript-derived siRNA (nat-siRNAs) pathway that is induced upon stresses including bacterial infection (Borsani et al., 2005; Katiyar-Agarwal et al., 2006). In this case siRNAs might be specifically produced upon induction of NATs by the action of RDR6/SGS2/SDE1, SGS3 NRPD1a, RDR2, and DCL4 and incorporated in AGO7 to trigger a defense response by repression of AGO1, HEN1 and HST. Alternatively, the observed phenomena are the result of the cross-interaction of multiple RNA silencing pathways that influence the defense response. Furthermore, the presence of ten AGOs, four DCLs and six RDRs in Arabidopsis (Morel et al., 2002; Schauer et al., 2002; Yu et al., 2003) may reflect the versatility of these components in RNA silencing pathways.

Whatever the exact pathway that is involved, it is likely that RNA silencing is involved either in a highly specific defense response against *Verticillium* pathogens or, alternatively is involved in a developmental cue that is of particular importance for *Verticillium* infections. Interestingly, it was recently demonstrated that inoculation of Arabidopsis with non-pathogenic *P. syringae* that triggers a robust basal defense response in Arabidopsis leads to altered accumulation of several microRNAs, including those targeting multiple components of auxin signaling pathways (Fahlgren et al., 2007). Furthermore, it was recently suggested that the transcriptional regulation of resistance gene loci may be under the control of RNA silencing, as was demonstrated for the *RPP5*-locus for recognition of the oomycete downy mildew pathogen *Peronospora parasitica* (Yi and Richards, 2007). This demonstrates that RNA silencing may affect diverse pathogens by regulating various modulators of host defense (Voinnet, 2008). Relatively little is known about the biology of vascular wilt diseases, and processes that are involved in defense against these pathogens (Fradin and Thomma, 2006). This makes it difficult to identify the physiological process that is affected in the RNA silencing mutants and that explains the observed disease phenotypes. Possibly, microarray analyses on inoculated wild-type plants and RNA silencing mutants will facilitate the identification of this process. However, the main challenge will be to identify the small RNAs that are at the basis of the altered *Verticillium* susceptibility in these mutants.

Materials and Methods

Plant Growth Conditions

Soil-grown Arabidopsis plants were cultivated in a growth chamber at 22°C, 72% relative humidity, and a 16 h photoperiod, or in a greenhouse at 21°C during the 16 h day period and 19°C during the 8 h night period at 72% relative humidity. In the greenhouse, supplemental light (100 Wm^{-2}) was used when the sunlight influx intensity was below 150 Wm^{-2}.

For *in vitro* growth of Arabidopsis, seeds were surface-sterilized and sown on MS medium (Duchefa, Haarlem, NL) solidified with 1.5% plant agar (Duchefa, Haarlem, NL). For phenotypic evaluations of root growth and development, Arabidopsis plants were grown on vertically oriented half-strength MS plates, supplemented with 1% sucrose and 0.5 g/L MES (2-(N-morpholino) ethane-sulfonic acid), pH 5.8. After sowing, the plates were incubated at 4°C in the dark for three days and subsequently transferred to the growth chamber.

Conditional Phenotype Assays

To assess susceptibility toward abiotic stress and responsiveness to hormones, *in vitro* assays were performed (Wang et al., 2008; Table S1). For abiotic stress assays, seeds were sown on MS agar amended with 100 or 150 mM NaCl, 20 or 30 mM LiCl, 150 or 200 mM mannitol and 3.3 or 6.7 mM H_2O_2 (Table S1) and evaluated for aberrant growth. To assay heavy metal resistance, plants were grown on vertically oriented half strength MS plates amended with 2% (w/v) sucrose and 85 µM $CdCl_2$. To assay hormone responsiveness, the sterilized seeds were grown on vertically oriented half-strength MS plates containing different hormones (Table S1). All plates were incubated in the growth chamber. For hypocotyl length assays, plates were incubated in the dark.

Pathogen Cultivation

Verticillium dahliae strains JR2 and ST12.01, *Verticillium longisporum* strain 43, *Verticillium albo-atrum* strains VA1 and CBS451.88, *Fusarium oxysporum* f.sp. *raphani* strain 815 (Diener and Ausubel, 2005), *Alternaria brassicicola* strain MUCL20297 (Mycotheque Université Catholique de Louvain, Louvain-la-Neuve, Belgium) and *Plectosphaerella cucumerina* were maintained on potato dextrose agar (PDA; Oxoid, Hampshire, UK). *Botrytis cinerea* (Brouwer et al., 2003) was grown on half-strength PDA amended with 5 g/L agar and 150 g/L blended tomato leaves. All fungal cultures were grown at 22°C. The bacterial strains of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 with or without *avrRpt2*, *avrRpm1* or *avrRps4*, was grown on King's B agar (King et al., 1954) supplemented with the appropriate antibiotics (25 µg/mL rifampicin and 100 µg/mL kanamycin). All bacterial strains were grown overnight at 28°C.

Pathogen Inoculations

Inoculum of all fungi (except *F. oxysporum* f. sp. *raphani*) was prepared as previously described (Broekaert et al., 1990) and prepared as a suspension of 10^6 conidia/mL in water. For *Verticillium* inoculations, a minimum of eight two-week-old Arabidopsis plants were up-rooted and the roots were incubated in the conidial suspension for three minutes. Subsequently, the plants were re-planted into fresh soil. Inoculations with *F. oxysporum* f. sp. *raphani* were performed similar as the *Verticillium* inoculations, except for the budcell-inoculum that was prepared as described by Diener and Ausubel (2005). All other pathogens were inoculated onto a minimum of four approximately four-week-old soil-grown plants with fully expanded rosette leaves. Inoculations with *A. brassicicola*, *B. cinerea* and *P. cucumerina* were performed by placing 6-µl drops of the conidial suspensions on each expanded leaf (Thomma et al., 1998; Thomma et al., 2000; Brouwer et al., 2003; O'Connell et al., 2004).

For inoculations with *P. syringae*, bacteria were grown overnight at 28°C in liquid King's B medium supplemented with the appropriate antibiotics. Arabidopsis plants were spray-inoculated with a bacterial suspension of OD_{600} 0.3 supplemented with 0.05% [v/v] Silwet L-77 (van Meeuwen Chemicals BV, Weesp, NL).

For all inoculations, except those with *F. oxysporum* f. sp. *raphani* and *Verticillium* spp., plants were kept in boxes with transparent lids at high relative humidity for the remainder of the experiment. All inoculations have been performed a minimum of three times with similar results.

V. dahliae* Biomass Quantification *in Planta

Two-week-old Arabidopsis plants were inoculated with *V. dahliae* strain JR2 as described above. After visible symptom development at 19 to 29 days post-inoculation, per experiment and for each Arabidopsis genotype all above-ground tissues were harvested per plant and flash-frozen in liquid nitrogen. The samples were ground to powder, of which an aliquot of approximately 100 mg was used for DNA isolation (Fulton et al., 1995). Quantitative real-time PCR was conducted using an ABI7300 PCR machine (Applied Biosystems, Foster City, USA) with the qPCR Core kit for SYBR Green I (Eurogentec Nederland BV, Maastricht, NL). To measure *V. dahliae* biomass, the internal transcribed spacer region of the ribosomal DNA was targeted using the fungus-specific ITS1-F primer (AAAGTTTTAATGGTTCGCTAAGA; (Gardes and Bruns, 1993) in combination with the *V. dahliae*-specific reverse primer ST-VE1-R (CTTGGTCATTTAGAGGAAGTAA; (Lievens et al., 2006), generating a 200 bp amplicon. For sample equilibration, the Arabidopsis large subunit of the RuBisCo gene was targeted using the primer set At-RuBisCo-F3 and -R3 (GCAAGTGTTGGGTTCAAAGCTGGTG and

CCAGGTTGAGGAGTTACTCGGAATGCTG, respectively), generating a 120 bp amplicon. Real-time PCR conditions consisted of an initial 95°C denaturation step for four min, followed by 30 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C. The average fungal biomass was determined using at least four *Verticillium*-inoculated plants for each genotype.

Reverse Transcription PCR

Total RNA was extracted from plant tissue frozen in liquid nitrogen using the RNeasy Plant Mini kit (Qiagen, Venlo, the Netherlands). On-column DNaseI treatment was performed as described by the manufacturer using the RNase-free DNase Set (Qiagen, Venlo, the Netherlands). Approximately 1.5 µg of total RNA was used for cDNA synthesis using SuperScriptTM III Reverse Transcriptase and Oligo(dT)12-18 primers according to the manufacturer's protocol (Invitrogen, Breda, the Netherlands). PCR amplification of actin (with primer pair Actin2-F2 TAACTCTCCCGCTATGTATGTCGC, and Actin2-R2 GAGAGAAACCCTCGTAGATTGGC) and of PR-1 (with primer pair PR1-F1 AGGCTAACTACAACTACGCTGCG, and PR1-R1 GCTTCTCGTTCACATAATTCCCAC) consisted of an initial denaturing step at 94°C for 5 minutes, followed by 30 to 35 cycles of 20 sec at 94°C, 20 sec at 56°C and 20 sec at 72°C, followed by a final extension step for 5 minutes at 72°C. PCR products were visualized on ethidium bromide-stained 1% agarose gels.

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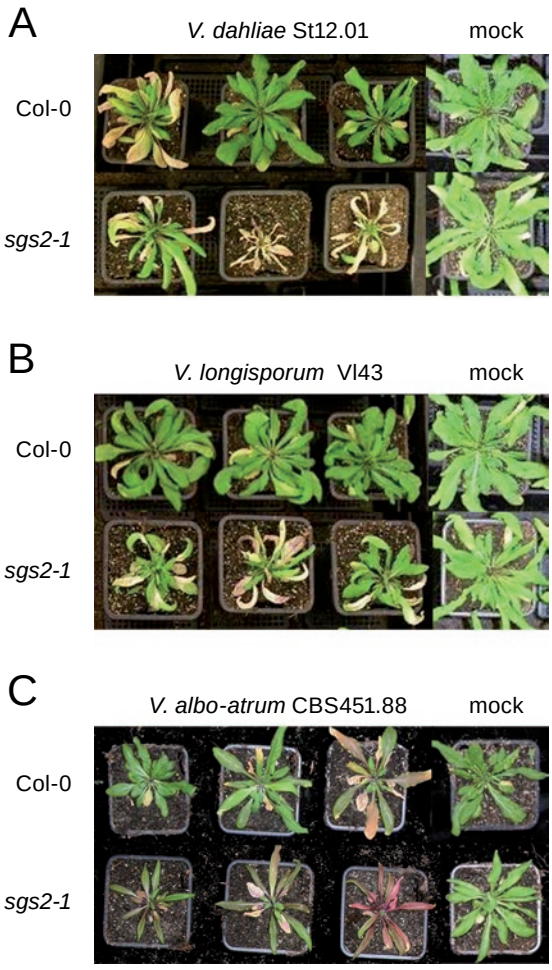
SUPPLEMENTAL DATA



Supplemental Figure S1. Typical symptoms of Arabidopsis *sgs2-1* mutants upon inoculation with plant pathogenic *Verticillium* species.

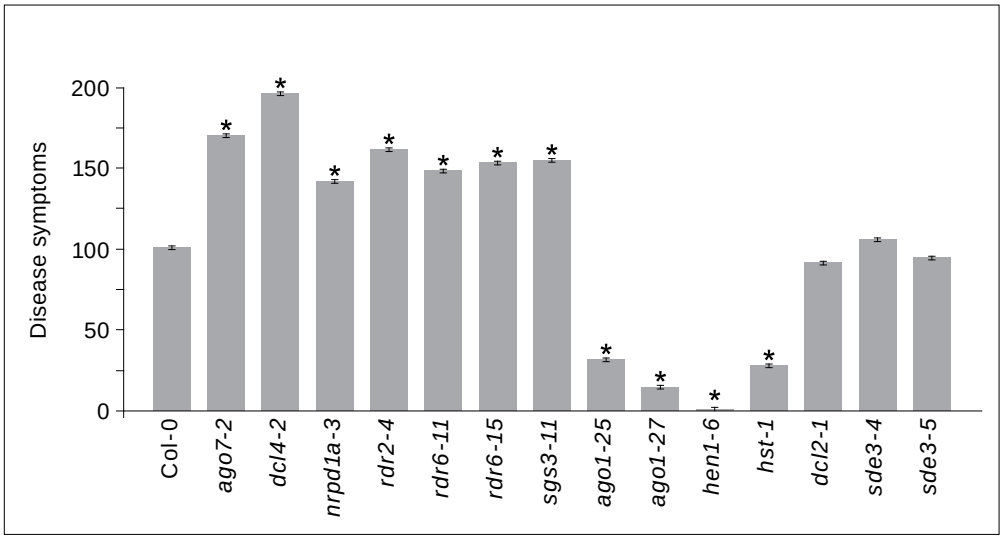
The mutant *sgs2-1* and the corresponding wild-type (Col-0) were inoculated with

A *V. dahliae* strain ST12.01

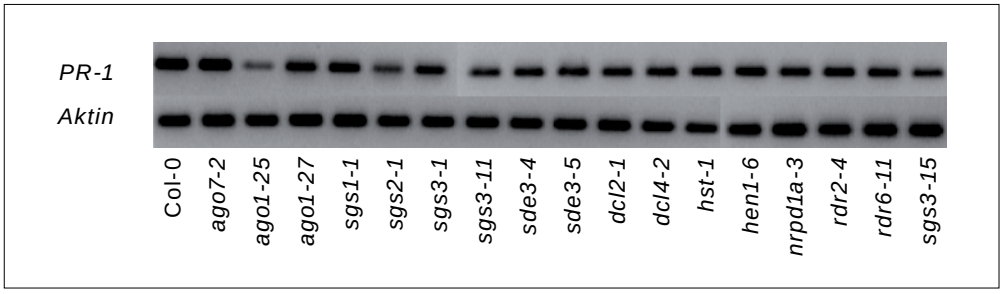
B *V. albo-atrum* strain CBS451.88

C *V. longisporum* strain Vl43.

The *Verticillium*-inoculated *sgs2-1* mutant shows enhanced symptom development upon inoculation with any of these *Verticillium* strains, including more severe stunting, wilting, anthocyanin accumulation and tissue necrosis, when compared with Col-0 plants at three weeks post inoculation.



Supplemental Figure S2. Quantification of symptom development at 20 days post inoculation shown as ratio of diseased rosette leaves with standard deviation. The ratio of diseased rosette leaves for Col-0 is set to one. Asterisks indicate significant differences when compared with the wild-type Col-0 ($P < 0.05$).



Supplemental Figure S3. Salicylic acid-induced *PR-1* expression in Arabidopsis gene silencing mutants. Wild type Columbia-0 (Col-0) and gene silencing mutants were treated with 2 mM salicylic acid and *PR-1* expression was analyzed with reverse transcription PCR after 24 hours. Equal loading of cDNA samples was verified by amplification of actin transcripts.

Supplemental Table S1. Conditional phenotype assays for *sgs1-1*, *sgs2-1* and *sgs3-1* mutants.

	Kingdom	Pathogen species	Strain	Concentration
Pathogens	fungi	*Alternaria brassicicola*	MUCL20297	10^6 spores/mL
		Botrytis cinerea	(Brouwer et al., 2003)	10^6 spores/mL
		Fusarium oxysporum f.sp. *raphani*	815	10^6 budcells/mL
		Plectosphaerella cucumerina	(Thomma et al., 2000)	10^6 spores/mL
		Verticillium albo-atrum	VA1 [b]	10^6 spores/mL
			CBS451.88 [b]	10^6 spores/mL
		Verticillium dahliae	JR2	10^6 spores/mL
			St12.01 [b]	10^6 spores/mL
		Verticillium longisporum	Vl 43 [b]	10^6 spores/mL
	bacteria	*Pseudomonas syringae* pv. *tomato*	DC3000	OD 0.3
		Pst AvrRpm1 [a]	DC3000	OD 0.3
		Pst AvrRpt2 [a]	DC3000	OD 0.3
		Pst AvrRps4 [a]	DC3000	OD 0.3

	Hormones	Agents	Hormone assay	Hypocotyl alteration
Hormones	auxin	2,4-D: 2,4-dichlorophenoxy acetic acid	0,1 µM 1 µM	5 µM
	cytokinin	6-BA: 6-benzylaminopurine	1 µM	
	gibberellic acid	GA: gibberellic acid	1 µM 20 µM	20 µM
	ethylene	ACC: 1-aminocyclopropane-1-carboxylic acid	1 µM	0,5 µM 10 µM
	brassinolide	EBL: epibrassinolide	1 µM	1 µM
	jasmonate	MeJA: methyl-jasmonate	1 µM	
	abcisic acid	ABA: abcisic acid	0,5 µM	

	Stress types	Agents	Concentration
Abiotic stress	salt stress	sodium chloride	100 mM 150 mM
		lithium chloride	20 mM 30 mM
	osmotic stress	mannitol	150 mM 200 mM
	reactive oxygen species	hydrogen peroxide	3.3 mM 6.7 mM
		paraquat	2.0 µM
	heavy metal	cadmium chloride	85 µM

a *Pst, Pseudomonas syringae* pv. *tomato*.

b These pathogens were only used on *sgs2-1* and Col-0 plants.

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Chapter 5

General Discussion

This thesis describes the first genome-wide functional investigation into roles of *AtRLP* genes in Arabidopsis. At the start of this thesis research, only two Arabidopsis RLP genes, *CLV2* and *TMM*, were functionally characterized (Jeong et al., 1999; Nadeau and Sack, 2002). Based on the bioinformatic analysis described in chapter 2, we identified *AtRLP5* in addition to the previously described 56 *AtRLP* genes in Arabidopsis (Fritz-Laylin et al., 2005). In a reverse genetics approach, several new developmental phenotypes for T-DNA insertion mutants of the *CLV2* and *TMM* genes were identified. In addition, a role in defense was identified for *AtRLP30* and *AtRLP18*, since corresponding T-DNA insertion mutants were found to affect non-host resistance against the non-adapted bacterial bean pathogen *Pseudomonas syringae* pv. *phaseolicola* (chapter 2). Based on sequence comparison and bioinformatic analysis, it was expected that the vast majority of the *AtRLP* genes would be disease resistance genes (Fritz-Laylin et al., 2005), but despite extensive disease assays with many different pathogens only two AtRLPs were found to be involved pathogen resistance. The lack of identification of novel phenotypes was thought to be due to functional redundancy. In chapter 3, an RNA interference (RNAi) strategy to target the expression of multiple *AtRLP* genes simultaneously is described. Unfortunately, no additional phenotypes in disease resistance were discovered in this analysis.

Apart from a role in viral defense, RNA silencing has recently been shown to play a role in host defense against bacterial plant pathogens (Voinnet, 2008). In chapter 4 it is demonstrated that RNA silencing is also important for defense against *Verticillium*. Several components of RNA silencing pathways were tested, of which many were found to affect *Verticillium* resistance. The mechanism is highly specific for *Verticillium*, since no altered defense was found towards other fungal pathogens, including *Alternaria brassicicola*, *Botrytis cinerea* and *Plectosphaerella cucumerina*, but also the vascular pathogen *Fusarium oxysporum* (chapter 4).

LRR-CONTAINING PLANT RECEPTORS

Plants cells use various receptor molecules to sense signals that are perceived from the environment, which may be signals released from other cells within the organism, signals from the abiotic environment, and signals from the biotic environment. The biotic environment comprises microbial organisms including symbionts, endophytes and pathogens. Of these, receptors for pathogen recognition have been most intensively studied, and various classes of intracellular and extracellular receptor molecules have been identified (Dangl and Jones, 2001).

Most receptors in plant innate immunity can be assigned to two classes, containing either extracellular or intracellular leucine-rich repeat (LRR) regions. In Arabidopsis, the largest class of pathogen receptors consists of the intracellular receptors, with a nucleotide-binding (NB) domain in addition to the C-terminal LRRs (NB-LRRs). The extracellular LRR-containing pathogen receptors can be divided into two groups. Both contain an extracellular (e)LRR domain and a single-pass transmembrane domain, but while the receptor-like kinases (RLK) contain a cytoplasmic serine/threonine kinase domain, the receptor-like proteins (RLP) only contain a short cytoplasmic tail without obvious signaling motifs except for the putative endocytosis motif found in some members (Fig. 1; Joosten and de Wit, 1999; Fritz-Laylin et al., 2005; Kruijt et al., 2005a; Wang et al., 2008). Structurally, RLPs can be divided into 7 conserved domains (A to G) with a signal peptide (A), a cysteine-rich domain (B), the LRR domain (C), a spacer (D), an acidic domain (E), the transmembrane domain (F), and a short cytoplasmic region (G). The eLRR-containing C domain is subdivided into three subdomains C1, C2 and C3, with C2 being a non-LRR island domain (Jones and Jones, 1997). However, not all RLPs contain the C2 island domain within the eLRR region (Fig. 1; Wang et al., 2008).

A recent review in MPMI extensively discusses the role of RLKs in plant defense (Afzal et al., 2008). In this review we focus on the role of the RLPs in plant defense. The absence of obvious cytoplasmic signaling motifs and the lack of family members in the model plant Arabidopsis involved in plant defense have hampered progress in research on made RLP signaling. However, recently considerable progress has been made in our understanding of RLP signaling and function.

THE HISTORY OF *RLP* GENES

Over the last two decades, several *RLP* genes that act as race-specific resistance genes have been identified in various plant species. The first *RLP* gene was discovered as the *Cf-9* resistance gene in tomato (*Solanum lycopersicum*) that governs resistance against strains of the biotrophic leaf mold fungus *Cladosporium fulvum* that secrete the effector protein Avr9 (Jones et al., 1994; Thomma et al., 2005). By now, all *Cf* resistance genes that have been cloned from tomato encode RLPs and can be grouped into two large gene families. The *Cf-4*, *Cf-4E*, *Cf-9* and *9DC* genes that mediate recognition of the cognate Avr4, Avr4E and Avr9 (both *Cf-9* and *9DC*) effectors of *C. fulvum*, respectively, are highly homologous and belong to the *Hcr9* (Homologues of *C. fulvum* resistance gene *Cf-9*) gene family (Jones et al., 1994; Thomas et al., 1997; Takken et al., 1999; Kruijt et al., 2004). Similarly, the *Cf-2* and *Cf-5* genes

that mediate recognition of the cognate Avr2 and Avr5 effectors of *C. fulvum*, respectively, belong to the *Hcr2* (Homologues of the *C. fulvum* resistance gene *Cf-2*) gene family (Dixon et al., 1996; 1998). Both classes contain *Cf* genes with demonstrated resistance specificities as well as members with currently unknown functions. For instance, the *Cf-4* cluster from *L. hirsutum* contains five *Hcr9* genes, two of which function as *C. fulvum* resistance genes. The *Hcr9-4D* homologue is the *Cf-4* gene that mediates Avr4 recognition (Thomas et al., 1997), while *Hcr9-4E* is the *Cf-4E* gene that mediates recognition of Avr4E elicitor (Takken et al., 1998; Westerink et al., 2004).

RLP genes have also been identified as resistance genes against other pathogens than *C. fulvum* in tomato (Kawchuk et al., 2001). The *Ve* locus that provides resistance against race 1 strains of the soil-borne vascular wilt pathogens *V. dahliae* and *V. albo-atrum* (Kawchuk et al., 1994; Kawchuk et al., 1998; Diwan et al., 1999) consists of two inversely oriented genes, *Ve1* and *Ve2*, that provide resistance when individually transferred into a susceptible potato cultivar (Kawchuk et al., 2001). However, recent functional characterization of the *Ve* genes shows that *Ve1*, but not *Ve2*, provides resistance against *Verticillium* spp. in tomato (Fradin et al., 2009), demonstrating that like most *Cf*-loci also the *Ve* locus is composed of active and non-active homologs.

Furthermore, in tomato the ethylene-inducing xylanase (EIX) that is produced by *Trichoderma* biocontrol fungi is perceived by receptors encoded by the *LeEIX* locus comprising at least two, but possibly three *LeEIX* genes, of which *LeEIX1* and *LeEIX2* have been cloned. While over-expression of either *LeEIX1* or *LeEIX2* genes in EIX-nonresponsive tobacco plants showed binding of EIX, overexpression of only *LeEIX2* did activate a hypersensitive response (Ron and Avni, 2004).

In addition to tomato, *RLP* genes have been identified in apple as *Vf* resistance genes against the scab fungus *Venturia inaequalis*. The *Vf* locus is derived from the crabapple species *Malus floribunda* and confers resistance to five races of *V. inaequalis*, while two new races of the fungus (races 6 and 7) have been identified that are able to overcome this resistance (Durel et al., 2003; Guerin et al., 2007). The *Vf* locus comprises a cluster of four RLP genes, *HcrVfa1* to *HcrVfa4* (for homologue of the *C. fulvum* resistance genes of the *Vf* region), of which three genes *HcrVfa1*, *HcrVfa2* and *HcrVfa4* encode typical RLPs while *HcrVfa3* contains an insertion at the end of the LRR motif, resulting in truncated transcripts (Vinatzer et al., 2001; Xu and Korban, 2002). Expression of *HcrVfa1* or *HcrVfa2*, but not of *HcrVfa4* in susceptible apple cultivars provided resistance against *V. inaequalis* strains that belong to races 1 to 5 (Belfanti et al., 2004; Malnoy et al., 2008).

Although some studies list the rice *Xa21D* resistance gene that provides resistance to the bacterial leaf blight pathogen *Xanthomonas oryzae* pv. *oryzae* as an *RLP* homolog (Afzal et al., 2008) the predicted encoded protein lacks a transmembrane domain (Wang et al., 2008). Thus, Xa21D structurally resembles the *S* locus glycoprotein (Nasrallah et al., 1994) and polygalacturonase inhibitor proteins (De Lorenzo et al., 1994), LRR-containing proteins that are secreted into the extracellular matrix.

THE *RLP* FAMILY IN ARABIDOPSIS

The first *RLP* genes that were identified in tomato were found to encode pathogen receptors (Jones et al., 1994). The genome of Arabidopsis was found to contain 57 *RLP* genes (*AtRLP*) assembled in 34 loci (Wang et al., 2008). These AtRLPs comply with the typical RLP domain structure, although only 45 of them are predicted to contain a C2 island domain nested in between two eLRR blocks (C1 and C3). The AtRLPs display low overall sequence identity, with only 10 pairwise combinations that share over 70% identity (Wang et al., 2008). Furthermore, the predicted sizes of the AtRLPs range from 218 amino acids (for AtRLP25) to 1,784 amino acids (for AtRLP9), whereas the eLRR numbers vary from two (for AtRLP5) to 49 (for AtRLP9). This suggests that the AtRLPs may have very diverse functions.

Until recently, only two Arabidopsis *RLP* genes had been characterized in detail. *TOO MANY MOUTHS* (*TMM*; *AtRLP17*) is an *RLP* gene that regulates stomatal distribution across the epidermis by initiation of stomatal precursor cells (Nadeau and Sack, 2002). CLAVATA2 (CLV2; AtRLP10) is an RLP that, together with CLV1 and CLV3, is involved in the restriction of stem cell proliferation and promotes differentiation (Jeong et al., 1999). CLV2 was proposed to stabilize the RLK CLV1 (Jeong et al., 1999), which acts as a receptor for extracellular peptide ligand CLV3 (Ogawa et al., 2008). It was recently demonstrated that the receptor kinase CORYNE (CRN) and CLV2 act in concert, in parallel with CLV1, to perceive the CLV3 signal. Mutations in *CRN* cause stem cell proliferation, similar to *clv1*, *clv2*, and *clv3* mutants, but CRN also has additional functions during plant development that are shared with CLV2, including floral organ development. Since the CRN protein lacks a distinct extracellular domain it was proposed that CRN and CLV2 interact via their transmembrane domains to establish a functional receptor (Müller et al., 2008). The maize gene *FASCIATED EAR* (*FEA2*; Taguchi-Shiobara et al., 2001) is characterized as a CLV2 homolog.

Based on the notion that *R* genes are under strong diversifying selection pressure to produce highly divergent sequences with distinct recognition capacities (Leister, 2004) while developmental genes are under purifying selection to reduce sequence drift and maintain a conserved function, it has been suggested that the majority of the Arabidopsis *RLPs* play a role in pathogen defense rather than in plant development (Fritz-Laylin et al., 2005). However, only in 2005 the first Arabidopsis *RLP* with a role in pathogen defense was identified. One of the genes that were induced in Arabidopsis plants upon treatment with the fungal PAMP chitin appeared to be an *RLP* gene (*AtRLP52*) that was found to be required for resistance against the powdery mildew pathogen *Erysiphe cichoracearum* (Ramonell et al., 2005).

To further characterize the *RLP* gene family in Arabidopsis, a genome-wide T-DNA insertion collection for the *RLP* genes was assembled (Wang et al., 2008). This collection was functionally analyzed with respect to plant growth, development and sensitivity to various stress responses including challenge inoculation with a diverse range of host-adapted and non-adapted necrotrophic or biotrophic pathogens. Remarkably, besides new alleles of *clv2* and *tmm* that displayed previously not yet described phenotypes, only few new phenotypes were discovered using the T-DNA insertion collection. *AtRLP41* was found to mediate abscisic acid (ABA) sensitivity since *AtRLP41* mutants were bleached upon submergence in ABA while

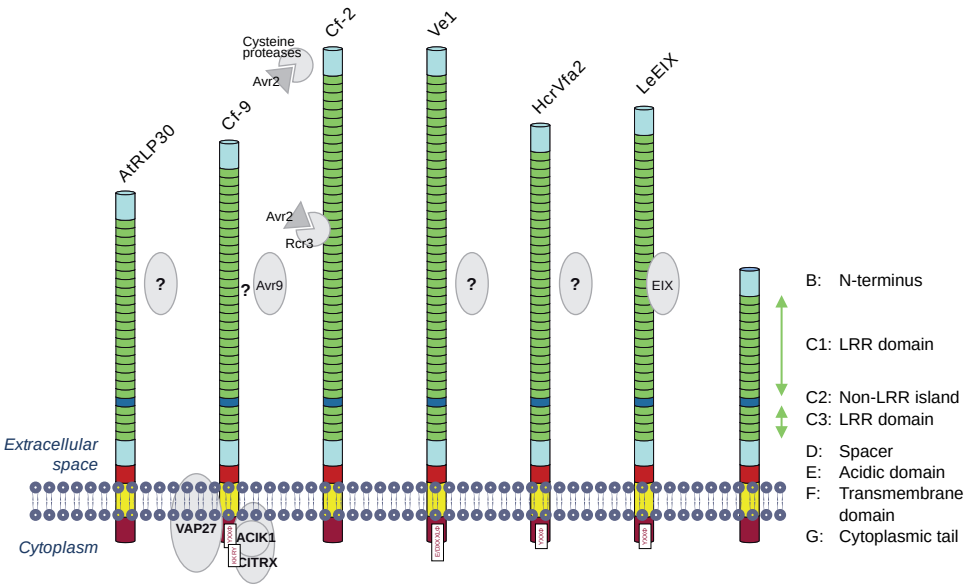

Figure 1. Schematic representation of plant RLPs involved in pathogen defense with typical domain structures. See text for further details.

wild-type leaves remained green. Only one gene, *AtRLP30*, could be implicated in host defense against pathogens as *Atrlp30* mutants showed consistently enhanced symptom development and increased bacterial multiplication upon inoculation with the non-adapted bacterial bean pathogen *Pseudomonas syringae* pv. *phaseolicola* (Wang et al., 2008). *AtRLP18* may play a similar role in non-host resistance, but this observation could not be confirmed due to absence of additional T-DNA insertion alleles for this gene (Wang et al., 2008). To overcome possible functional redundancy among *AtRLP* genes RNA interference (RNAi) was used to target the expression of multiple *AtRLP* genes simultaneously. However, also this analysis failed to uncover additional processes in which the *AtRLPs* play a role (Ellendorff et al., 2008).

ARABIDOPSIS RLPS FUNCTION IN BASAL DEFENSE

In Arabidopsis, three *AtRLPs* have been implicated in pathogen defense so far. *AtRLP30* and *AtRLP18* were identified by testing the genome-wide *AtRLP* T-DNA insertion collection for susceptibility to several non-adapted pathogens including fungi (*C. fulvum*, *Cladosporium cucumerinum*), bacteria (*P. syringae* pv. *phaseolicola*) and an oomycete (*Phytophthora infestans*; Wang et al., 2008). *Atrlp30* and possibly *Atrlp18* T-DNA mutants were found to be compromised in non-host resistance against *P. syringae* pv. *phaseolicola*, which demonstrates that *AtRLP30* and possibly *AtRLP18* play a role in basal defense.

Another *AtRLP*, the chitin responsive *AtRLP52*, was evaluated for altered levels of susceptibility to virulent strains of the powdery mildew pathogen *Erysiphe cichoracearum* by challenging the Col-0 wild-type and T-DNA insertion mutants for *AtRLP52* with a low-density inoculum. Interestingly, the *Atrlp52* mutants displayed more severe macroscopic disease symptoms that were accompanied with increased production of conidiophores compared to Col-0 wild-type plants (Ramonell et al., 2005). The fact that Col-0 wild-type is susceptible and that *Atrlp52* is even more susceptible towards *E. cichoracearum* when compared to Col-0 clearly indicates a role for *AtRLP52* in basal defense. Also *AtRLP52* was demonstrated to be required for resistance against the non-adapted barley (*Hordeum vulgare*) pathogen *Blumeria graminis* f. sp. *hordei* (J. Mansfield, unpublished data). Recognition of a non-adapted pathogen shows that *AtRLP52, AtRLP30* and possibly *AtRLP18,* function in basal defense.

Nowadays, two types of plant immune responses are distinguished (Jones and Dangl, 2006). Primary immunity involves plant cell surface receptors that recognize invariant microbial non-self molecules, also referred to as pathogen-associated molecular patterns (PAMPs; Chisholm et al., 2006; Jones and Dangl, 2006). Recognition of PAMPs by these so-called

pattern recognition receptors (PRRs) leads to the activation of basal defense, also called PAMP-triggered immunity (PTI). To overcome PTI, pathogen strains may develop effectors that suppress host defense and thus become virulent (He et al., 2007; Shan et al., 2008; van Esse et al., 2008; Xiang et al., 2008). With secondary immunity, resistant plant genotypes have evolved race-specific disease resistance (R) proteins that specifically detect the (activity of) pathogen effectors and subsequently activate effector-triggered immunity (ETI), turning the effectors into avirulence (Avr) molecules.

The genome-wide functional analysis of the Arabidopsis *RLP* genes for potential roles in plant defense comprised the screening of virulent and avirulent strains of host-adapted pathogens including fungi (*Alternaria brassicicola, Botrytis cinerea, Colletotrichum destructivum, Oidium neolycopersici, Plectosphaerella cucumerina and Verticillium dahliae*), bacteria (*Pseudomonas syringae* pv. *tomato*, *Xanthomonas campestris* pv. *campestris*) and oomycetes (*Hyaloperonospora parasitica*, *Phytophthora brassicae*). However, none of the *AtRLP* T-DNA insertion lines displayed altered susceptibility upon inoculation with this diverse range of host-adapted pathogens. Thus, so far no *AtRLP* gene has been identified to function as a race-specific *R* gene that mediated ETI (Ellendorff et al., 2008; Wang et al., 2008).

Unlike AtRLPs, tomato RLPs have been found to play roles in both PTI and ETI. For instance, the LeEIX2 receptor directly binds the Ethylene-Inducing Xylanase (EIX) from *T. viride* (Ron and Avni, 2004). EIX elicits ethylene biosynthesis, which results in defense induction in plants (Bailey et al., 1993; Ron et al., 2000). Xylanase is a PAMP that is commonly produced by many pathogenic and nonpathogenic fungi, and it can thus be stated that LeEIX functions as a PRR in basal defense. Thus, a role of RLPs in basal defense has been established in different plant species including at least tomato and Arabidopsis. However, most of the functionally characterized *RLP* genes that play a role in plant defense, including the tomato *Cf* and *Ve* genes as well as the apple *Vf* genes, encode R proteins that recognize specific effectors secreted by races of the target pathogens. Thus, although many *RLPs* have been found to function in *R* gene-mediated defense in tomato and apple, in Arabidopsis no AtRLPs have been found to act as *R* genes, whereas a few candidate genes were identified that play a role in mediating basal defense.

DEVELOPMENT OF THE TOMATO *RLP* GENE FAMILY

In tomato, the *LeEIX* locus, the *Ve* locus, and loci that provide resistance against *C. fulvum* all were found to be members of *RLP* gene clusters, containing genes with a demonstrated role in resistance as well as genes of unknown function. To date, the cloned *RLP* genes from tomato can be divided into four *RLP* gene families, located on different chromosomes. The two smallest *RLP* gene families each comprise one gene cluster. While the *LeEIX* cluster that consists of two, possibly three, genes is located on the short arm of chromosome 7 (Ron et al., 2000), the *Ve* cluster containing two genes is positioned on the short arm of chromosome 9 (Diwan et al., 1999). The largest tomato *RLP* gene families are the two *Cf* gene families *Hcr2* and *Hcr9*. Five loci on chromosome 1 comprise the *Hcr9* gene family (Jones et al., 1993; Parniske et al., 1997; Haanstra et al., 1999; Takken et al., 1999; Yuan et al., 2002; Kruijt et al., 2004), while one locus on chromosome 6 harbors the *Hcr2* gene family (Dickinson et al., 1993; Dixon et al., 1996; 1998). The four tomato *RLP* gene families contain over 40 *RLP* members in total (Kruijt et al., 2005a), that have all been identified by targeted sequencing of resistance gene loci. Moreover, a survey by Caicedo and Schaal (2004) of *Cf-2* variation in natural populations of the wild tomato *S. pimpinellifolium* uncovered at least 26 additional *Cf-2* homologues. The plethora of tomato *RLP* genes that has been identified only based on family members that play a role in resistance against a single pathogen, *C. fulvum*, could point towards an expanded *RLP* gene family in tomato compared to, for instance, the Arabidopsis *RLP* family that comprises 57 genes. *R* genes usually belong to tightly linked gene families, and their evolution is driven by selection on allelic variants originating from mutations and recombination between alleles or different gene family members. The interaction between tomato and *C. fulvum* has been proven a useful model system to study *R* gene evolution (Parniske et al., 1997; 1999; Thomas et al., 1997; Parniske and Jones, 1999; van der Hoorn et al., 2001a; Kruijt et al., 2004) and gene-for-gene interactions (Jones et al., 1993; Balint-Kurti et al., 1994; Jones and Jones, 1997; Parniske et al., 1997; Thomas et al., 1997; Laugé et al., 1998; Takken et al., 1998; Kruijt et al., 2004). In tomato, *Hcr2* and *Hcr9* genes were most likely derived from a common ancestral gene, and later physically separated by translocation to two different chromosomes, where they underwent independent extensive evolution by duplication, recombination and diversification resulting in the expanded two distinct groups known today (Dixon et al., 1996). For instance, the *Cf-2* locus harbors two genes, *Cf-2-1* and *Cf-2-2*, encoding proteins that differ only by three amino acids and both confer resistance to *C. fulvum* isolates that produce the wild-type Avr2 elicitor (Dixon et al., 1996; Luderer et al., 2002). Like the *Cf-2* genes, the *Ve* and *LeEIX* genes likely resulted from recent gene

duplication of a *Ve* and *LeEIX* progenitor gene, respectively, albeit the members of each family do not share identical activities (Kawchuk et al., 2001; Ron and Avni, 2004; Fradin et al., 2009). By contrast, three *9DC* genes of the *9DC* cluster, which have the same recognition specificity as *Cf-9*, evolved by intragenic recombination of *Cf-9* and another *Hcr9* homologue of the *Cf-9* cluster (van der Hoorn et al., 2001a; Kruijt et al., 2004). This supports an earlier suggestion that the major mechanism for generating (novel) variation in the *Hcr9* genes appears to be sequence exchange between the various homologues rather than accumulation of point mutations (Parniske et al., 1997; 1999; Parniske and Jones, 1999). Interestingly, screening diverse *Solanum* species for responsiveness to *C. fulvum* effectors demonstrated maintenance of many functional *Cf* genes throughout the *Solanum* genus (Laugé et al., 2000; van der Hoorn et al., 2001a; Wulff et al., 2001; Kruijt et al., 2004; 2005b), which suggests that *C. fulvum* is an ancient pathogen of Solanaceous plants, whereby tomato-*C. fulvum* coevolution has caused an extensive development of gene-for-gene relationships. Thus, it seems that tomato harbors more *RLP* genes than Arabidopsis. While no indications have been found of pathogen-Arabidopsis coevolution resulting in the development of *RLP* genes functioning as *R* genes, extensive coevolution between tomato and *C. fulvum* has resulted in many tomato *Cf* genes. When the tomato genome is fully annotated, comparisons with genomes of wild species will enable uncovering of additional *RLP* gene families and their interrelationships. This will show whether in tomato *RLP* gene families are much more expanded when compared to other plant species such as Arabidopsis.

MOLECULAR MECHANISMS OF RLP FUNCTION AND SIGNALING

Heterodimer Formation

Little is known about how RLPs relay extracellular signals into intracellular responses. Probably the best studied RLP model is CLV2 in Arabidopsis. CLV2 was shown to stabilize the RLK CLV1 (Jeong et al., 1999), and moreover to act together with the receptor kinase CRN and in parallel with CLV1 to perceive the CLV3 signal (Müller et al., 2008). Although biochemical evidence for complex formation between CLV2 and the receptors CLV1 and CRN is missing, CLV2 was proposed to act as co-receptor for both CLV1 and CRN (Jeong et al., 1999; Müller et al., 2008). Since the CRN protein lacks a distinct extracellular domain it was proposed that CRN and CLV2 interact via their transmembrane domains to establish a functional receptor (Müller et al., 2008). A role as co-receptor was also proposed for the Arabidopsis RLP TMM that negatively regulates three RLKs during the process of stomatal

patterning and differentiation. One of these RLKs is ERECTA that controls organ size and shape (Shpak et al., 2004; Torii, 2004) and was recently implicated in stomatal development, influencing plant transpiration efficiency (Masle et al., 2005). In addition, ERECTA was found to act in pathogen defense (Godiard et al., 2003; Llorente et al., 2005). Heterodimerization has been suggested for CLV2 and TMM, and since RLPs lack an obvious cytoplasmic signaling domain (Joosten and de Wit, 1999; Fritz-Laylin et al., 2005; Kruijt et al., 2005a; Wang et al., 2008), complex formation of RLPs with receptors containing cytoplasmic signaling domains seems likely to be required to activate an intracellular response. Heterodimer formation has been demonstrated to be an important signaling mechanism for members within the RLK family. The RLK BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) was found to act as co-receptor of different RLKs. These include on the one hand the hormone receptor BRASSINOSTEROID-INSENSITIVE1 (BRI1) that regulates brassinosteroid-dependent growth (Li et al., 2002; Nam and Li, 2002; Russinova et al., 2004), and on the other hand the PAMP receptor FLAGELLIN SENSITIVE 2 (FLS2) that mediates plant innate immunity upon perception of bacterial flagellin (Chinchilla et al., 2007; Heese et al., 2007). Furthermore, BAK1 was demonstrated to be required for cell death induced upon microbial infections, restriction of various bacterial, fungal and oomycete infections, and to regulate full responses to other PAMPs in addition to flagellin (Chinchilla et al., 2007; Heese et al., 2007; Kemmerling et al., 2007). Also, down-regulation of *BAK1* in tomato compromised resistance against *V. dahliae* by disturbing *Ve* signaling (Fradin et al., 2009). Therefore, BAK1 is likely to interact with additional innate immune receptors. Playing a role as co-receptor in different receptor complexes may explain why receptors like BAK1 and ERECTA function in processes as diverse as plant development and pathogen defense.

Binding and Recognition Specificities

RLPs perceive extracellular signals but how they interact remains unknown in most cases. Interaction may be direct, as was demonstrated for the LeEIX1 and LeEIX2 receptors with their ligand EIX. EIX was shown to interact with LeEIX1 and LeEIX2 in tobacco cell cultures and with LeEIX2 also in mammalian cells. Therefore, the interaction between EIX and LeEIX2 was suggested to be direct because binding was proven to be independent of other plant proteins (Ron and Avni, 2004). Furthermore, the interaction between Cf-4 and Avr4, was proposed to be direct, since Avr4 lacks other targets in the host (van Esse et al., 2007). The tomato Cf-4 resistance protein recognizes the chitin-binding effector molecule Avr4 that shields and protects hyphae of *C. fulvum* from the deleterious activity of host chitinases (Thomas et al., 1997; van den Burg et al., 2006; van Esse et al., 2007).

In most cases, demonstration of a direct interaction between receptor and ligand has failed, and therefore most interactions are thought to be indirect, complying with the guard hypothesis which assumes that the status of the host target of an effector is monitored by the resistance protein. In this way, Cf-2-mediated resistance against strains of *C. fulvum* secreting the cognate effector Avr2 is conferred by guarding the tomato Rcr3 protein, a secreted papain-like cysteine endoprotease (Krüger et al., 2002). It was shown that Avr2 is a cysteine protease inhibitor with high substrate affinity for the Rcr3 protease and several other extracellular cysteine proteases that are required for basal host defense (Rooney et al., 2005; van Esse et al., 2008).

In order to understand how signaling is activated by RLPs identification of domains important for recognition and signaling is required. Although for none of the Cf domains extracellular binding partners have been identified, extensive domain-swap and mutation analyses have been performed to reveal recognition specificities of the extracellular Cf domains. Several domain-swap experiments between Cf-2 and Cf-5, as well as between Cf-4 and Cf-9, demonstrated no role in recognition specificity for domain B, the mature N terminus, except for a ten amino acid deletion in the B domain that is required for Cf-4 function by at least some Cf-4/Cf-9 chimeras (van der Hoorn et al., 2001b; Wulff et al., 2001; Jones and Takemoto, 2004). The B domain is known to contain a number of conserved structural motifs, such as the CxWxGVxC motif in which the Cys residues are proposed to form cystine bridges (Jones et al., 1994; Jones and Jones, 1997; van der Hoorn et al., 2005). Fritz-Laylin et al (2005) found two structural variants in B domains of RLP proteins. One group of RLPs, known to be involved in development, contains a single pair of conserved Cys residues, while the other group includes RLPs characterized in defense pathways and contains two pairs of conserved Cys residues. The last group includes 54 rice and 27 Arabidopsis *RLP* genes, in addition to tomato and apple *RLP* genes such as *Cf-9*, *LeEIX1*, *Ve1*, *Vf1* and *Vf3* (Fritz-Laylin et al., 2005). Although the exact role for this B domain variation still remains unknown, substitution of Cys residues in the B domain of Cf-9 was shown to greatly attenuate Cf-9 function, thus demonstrating the importance of the B domain for Cf-9 function (van der Hoorn et al., 2005).

Domain-swap experiments with Cf proteins showed that recognition specificities mainly reside in the C1 LRR domain. The Cf-2 and Cf-5 proteins are very similar by sharing 90% sequence identity but they harbor different LRR copy numbers: Cf-2 having 38 and Cf-5 having 32 LRRs. Swapping N-terminal domains between Cf-2 and Cf-5 delimited recognition specificity for Avr2 and Avr5 to LRRs 4 to 27 in Cf-2 and LRRs 4 to 21 in Cf-5, respectively (Seear and Dixon, 2003). Furthermore, Cf-2/Cf-9 chimeras demonstrate that the N-terminus of Cf-2 including domains A, B and 34 LRRs, fused to the C-terminus of Cf-9, is functional to induce Avr2/Rcr3-mediated responses (Rivas et al., 2004). This demonstrates that recognition

specificity resides in the C1 LRR domain for both Cf-2 and Cf-5 proteins. In addition, domain-swap and mutation experiments for Cf-4 (25 LRRs) and Cf-9 (27 LRRs) showed that recognition specificity of Cf-4 and Cf-9 proteins depends on putative solvent-exposed amino acids in the C1 LRRs as well as on LRR copy number. Deletion of LRRs and introduction of point mutations in the C1 region of Cf-9 changed the specificity of Cf-9 to that of Cf-4, whereby ligand specificity of Cf-4 depends on three solvent exposed amino acid residues in LRR 11, 12 and 14 (and on the deletion in the B-domain). By contrast, ligand specificity of Cf-9 was displayed over a large number of LRRs from LRRs 10 to 18, where many mutations were found to attenuate Cf-9 function, except for mutations in three solvent-exposed amino acid residues in LRRs 12, 16 and 18 that abolished Cf-9 function (van der Hoorn et al., 2001b; Wulff et al., 2001). The loss of function caused by point mutations in LRR 12 and 18 was attributed to the introduction of new glycosylation sites, leading to (putative) glycosylation of solvent exposed N residues (van der Hoorn et al., 2005). Also the LRR copy number was demonstrated to play an important role in recognition specificity, as no Cf-4/Cf-9 chimeras that conferred Avr9 responsiveness contained fewer than 27 LRRs and no Cf4/Cf-9 chimeras with more than 25 LRRs conferred an Avr4 dependent HR (van der Hoorn et al., 2001b; Wulff et al., 2001). Interestingly, in Arabidopsis the LRR copy number of RLPs greatly varies, which could hint towards very diverse recognition specificities.

The variable C2 region connects the C1 LRRs with the more conserved C3 LRRs and is present in most of the tomato, apple, rice and Arabidopsis RLPs (Jones et al., 1994; Jones and Jones, 1997; Fritz-Laylin et al., 2005; Wang et al., 2008). Fritz-Laylin et al. (2005) noted a novel conserved Yx(6-8)KG motif in the C2 region of 33 rice and 37 Arabidopsis RLPs, of which the function is unknown. Although no information from RLP mutants or structure/function analysis of this domain is available, the C2 region has been shown to be important for signaling of the RLK BRI1 (Diévart and Clark, 2003), where the C2 region was found to bind brassinolide (Kinoshita et al., 2005). Also concerning the conserved C3, D, E and F domains information is limited. Only two EMS-induced loss-of-function alleles of *Cf-9* have been reported to be mutated in these domains: one has a single substitution in a solvent-exposed amino acid of LRR 24 in the C3 domain whereas the other has a substitution in a GXXXG motif of the transmembrane domain F (Wulff et al., 2004). The GXXXG motif is required for homo- or heterodimerization of other membrane proteins (Gerber and Shai, 2001; Curran and Engelman, 2003; Bennasroune et al., 2004). Like all functionally characterized RLPs, 80 rice and 55 Arabidopsis RLPs contain a (G/S/T)XXX(G/S/T) motif. The conservation of this motif across species may indicate a function in intra- or intermolecular interactions (Fritz-Laylin et al., 2005).

Localization and Endocytosis

RLPs are receptors for extracellular signals that are predicted to be anchored in the plasma membrane. This was indeed demonstrated for AtRLP30 in Arabidopsis; transgenic plants expressing C-terminal YFP tagged AtRLP30 showed a clear plasma membrane localization (Wang et al., 2008), similar to other known cell surface receptors as the FLS2 (Robatzek et al., 2006) and the AtPEP1 receptor (PEPR1; Yamaguchi et al., 2006). In case of Cf-9, studies into subcellular localization delivered diverse results. The Hcr9 proteins contain a putative endoplasmatic reticulum (ER)-retention signal (KKRY; Fig. 1), which could cause retrieval of membrane proteins from the Golgi to the ER. ER localization was indeed shown for Cf-9 upon overexpression in tobacco and Arabidopsis (Benghezal et al., 2000). However, experiments by Piedras et al. (2000) showed that over-expressed Cf-9 resides in the plasma membrane in tobacco. Moreover, the ER retention signal was found not to be required for Cf-9 function (van der Hoorn et al., 2001c; Wilson et al., 2005). Additional experiments with an antibody against Cf-9 were in agreement with the PM localization for Cf-9 (unpublished results from Heese-Peck & Jones, in Rivas and Thomas, 2005). It has been shown for other membrane proteins that overexpression can result in mislocalization to membranes of other compartments (Reaves and Banting, 1994; Leyman et al., 2000; Volker et al., 2001; Lisenbee et al., 2003; Sickmann et al., 2003). Therefore, different expression levels may be responsible for the different subcellular localizations of Cf-9 when expressed from heterologous promoters.

Several RLPs were found to contain a mammalian YXXΦ endocytosis motif, where Φ represents a bulky, hydrophobic amino acid. In recent years evidence became available that the YXXΦ endocytosis motif also stimulates receptor-mediated endocytosis in plants, in order to regulate signaling activity at the cell surface by receptor down-regulation or to start signaling after internalization (Lam et al., 2007; Müller et al., 2007; Robinson et al., 2008). Triggering signaling after internalization appears to be a common mechanism, as it has been reported for several RLKs such as ACR4 and BRI1 receptors (Gifford et al., 2005; Geldner et al., 2007). The tomato LeEIX2 protein contains a YXXΦ motif within the short cytoplasmic domain (Fig. 1), and this motif was shown to be necessary for HR induction in tobacco (Ron and Avni, 2004). Furthermore, EIX was found to be transported to the cytoplasm after binding the plasma membrane (Hanania et al., 1999). This suggests that EIX is internalized to induce signaling by endocytosis of LeEIX. Like LeEIX, also the Ve2 protein harbors a YXXΦ motif. Ruthard et al. (2007) performed several mutation and localization studies on Ve2, but since Ve2 failed to provide resistance in tomato (Fradin et al., 2009), care should be taken to draw conclusions from these studies. The RLP resistance protein Ve1 that also contains an endocytosis signature (E/DXXXLΦ; Kawchuk et al., 2001; Fig. 1) may prove to be a better candidate

for further investigations. In contrast to LeEIX2 and Ve1, all Hcr2 and Hcr9 proteins harbor the YXXΦ endocytosis motif within the transmembrane domain (Jones et al., 1994), where the functionality of such a motif has not been investigated yet. Nevertheless, a function for the YXXΦ endocytosis motif of Cf-9 may be found in the results of a yeast two-hybrid screen (Laurent et al., 2000). In this screen the vesicle-associated protein VAP27 was identified to interact with the EFG domains of Cf-9 (Fig. 1). VAP27 is most likely localized in the plasma membrane and may be involved in membrane trafficking, and thus VAP27 might play a role in endocytosis of the Cf-9 receptor complex. The YXXΦ motif is also present in the apple Vf proteins, both in the transmembrane domain and in the cytoplasmic tail (Fig. 1; Vinatzer et al., 2001). In Arabidopsis and rice, a small number of 9 and 20 RLPs, respectively, were found to harbor an endocytosis motif (Fritz-Laylin et al., 2005), demonstrating representation of the endocytosis motifs in RLPs across several species. Although endocytosis is a well conserved mechanism that may play a role in RLP signaling, the importance of this mechanism remains to be demonstrated.

Downstream Signaling

After perception at the plant cell surface, extracellular signals such as those from pathogen elicitors need to be relayed to intracellular responses. Two putative downstream signaling proteins were suggested to interact with Cf-9, both of which were identified in a yeast two-hybrid screen. In addition to VAP27 (described in the previous paragraph), the cytoplasmically localized Cf-9-interacting thio-redoxin (CITRX) was found to interact with the cytoplasmic domain of Cf-9 (Fig. 1; Rivas et al., 2004). CITRX is unrelated to earlier described thioredoxins and acts as a negative regulator of cell death mediated by Cf-9 and possibly Cf-4 but not Cf-2 (Rivas et al., 2004). Screening of rapidly elicited genes during the Cf-9/Avr9 interaction identified a Avr9/Cf-9-induced kinase 1 (ACIK1) that is required for Cf-9/Avr9- and Cf-4/Avr4-mediated HR but not for the HR mediated by other R/Avr systems such as Pto/AvrPto, Rx/*Potato virus* X, or N/*Tobacco mosaic virus* (Rowland et al., 2005). Recently, results of a yeast three-hybrid screen showed that CITRX might act as adapter recruiting the ACIK1 kinase to the cytoplasmic domain of Cf-9 upon elicitation by the Avr9 peptide (Fig. 1). It has been shown that the catalytic activities of both CITRX and ACIK1 are not required for their interaction (Nekrasov et al., 2006). Furthermore, CITRX was shown to induce kinase activity of mitogen-activated protein kinases (MAPKs), while it represses the kinase activity of calcium-dependent protein kinases (CDPKs) within 30 minutes after elicitation with Avr9 (Rivas et al., 2004). These results support earlier findings that protein phosphorylation plays an important role in early signaling events of the Cf-9/Avr9 interaction, as was shown for MAPKs, such

as wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK; Romeis et al., 1999), a CDPK (NtCDPK2; Romeis et al., 2000; 2001) and a plasma membrane-localized syntaxin implicated in ABA response and secretion (NtSyp121; Heese et al., 2005). NtSyp121 is only phosphorylated in presence of Cf-9 and Avr9 but not in presence of the PAMP elicitor flg22, and is known to play a role in fusion of incoming transport vesicles with a target membrane throughout the endomembrane system. Also in the Cf-4/Avr4 interaction phosphorylation events have been reported. In Cf-4-mediated resistance at least three tomato MAP kinases, LeMPK1, LeMPK2, and LeMPK3, are simultaneously activated, as was shown after temperature-dependent induction of defense responses and HR in seedlings expressing Cf4 and Avr4 (Stulemeijer et al., 2007). These LeMPKs show different phosphorylation specificities, indicating different downstream roles for the LeMPKs. Interestingly, VIGS of the genes encoding the individual kinases showed that LeMPK2 and LeMPK3 were required for Cf-4/Avr4-induced HR, while LeMPK1 and LeMPK3 compromised Cf-4-mediated resistance. This suggests that LeMPK1, LeMPK2, and LeMPK3 have overlapping but also different roles with regard to HR and resistance (Stulemeijer et al., 2007).

The Cf-4/Avr4 expressing seedlings were also used to perform a cDNA-AFLP screening, in which several hundreds of differentially expressed genes were identified (Gabriëls et al., 2006). In four cases, silencing by VIGS clearly compromised Cf-4/Avr4-induced HR. One of these four genes required for Cf-4/Avr4-induced HR encodes a CC-NB-LRR type R protein analogue, designated NRC1 (NB-LRR required for HR associated cell death; Gabriëls et al., 2006). Silencing of NRC1 in tomato not only affected the Cf-4/Avr4-induced HR but also compromised Cf-4-mediated resistance to *C. fulvum*, where NRC1 was found to act downstream of Cf-4 and upstream of a MAP kinase pathway. In addition, NRC1 seems to be required for HR induced by other R/Avr systems, including Cf-9/Avr9, LeEIX2/EIX, Pto/AvrPto and Rx/*Potato virus* X (Gabriels et al., 2007), and Ve1-mediated resistance (Fradin et al., 2009).

Not only phosphorylation but also ubiquitination and sumoylation, two other types of post-translational modification known to regulate protein function in plant defense (Zeng et al., 2006; Miura et al., 2007) have been implicated in RLP signaling. In tobacco, two putative ubiquitin ligase components were shown to be essential for generation of HR for the Cf-9/Avr9 and Cf-4/Avr4 gene pairs (Rowland et al., 2005). One of these components, the putative U-box E3 ubiquitin ligase CPMG1, was shown to be required for full resistance of tomato to *C. fulvum* (González-Lamothe et al., 2006), proposing that ubiquitination may play a role in Cf-9-mediated resistance. Sumoylation might play a role in the LeEIX2-mediated response, since the EIX elicitor was demonstrated to interact with the tomato cytoplasmatic small ubiquitin related modifier protein (SUMO) in a yeast two hybrid system. In addition,

SUMO was shown to suppress or enhance the EIX-induced ethylene biosynthesis and HR when overexpressed in sense or antisense direction, respectively (Hanania et al., 1999). The internalization of EIX may occur through binding of LeEIX2 and subsequent receptor-mediated endocytosis, thus allowing the receptor and/or EIX to interact with cytoplasmic proteins such as SUMO (Ron and Avni, 2004).

Ve-mediated resistance to *Verticillium* requires a tomato homologue of the Arabidopsis EDS1 gene, identified through reverse genetics (Hu et al., 2005). This tomato EDS1 homologue is also required for basal defense against virulent pathogens, and it was found to act upstream of SA accumulation and PR-gene induction upon pathogen challenge. In Arabidopsis, EDS1 is required for resistance mediated by several TIR-NB-LRR resistance genes (Parker et al., 1996; Aarts et al., 1998; Hu et al., 2005), but whether the Arabidopsis EDS1 also plays a role in basal defense responses in Arabidopsis mediated by *AtRLP52* and *AtRLP30* is not known.

Until now, in Arabidopsis only the recently identified *AtRLP52* and *AtRLP30* (and possibly *AtRLP18*) have been implicated in plant defense, but downstream signaling has not been investigated yet. Until now, information about AtRLPs is mainly restricted to transcript induction data such as microarray and RT-PCR data that might give an indication in what kind of processes and downstream signaling pathways RLPs might be involved. For instance, *AtRLP52* and *AtRLP30* were found to be upregulated by elicitors. While *AtRLP52* has been shown to be induced by chitin (Ramonell et al., 2005), *AtRLP30* has been found to be induced by various PAMPs. One of them is the flagellin peptide flg22, which was also shown to induce PRRs such as the RLKs FLS2 and EF-TU receptor (EFR; Zipfel et al., 2004; 2006). Although information about Arabidopsis RLP signaling is limited, signaling processes described for tomato RLPs, such as post-translational modifications, might also be involved in RLP signaling of other plants like Arabidopsis. Nevertheless, further investigations are necessary to identify components and pathways important in Arabidopsis RLP signaling.

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SUMMARY

Plants are under continuous attack of microbial plant pathogens. Since plants are sessile and cannot escape to more favorable environments they need an effective defense system to withstand attackers. Plant innate immunity can be divided into two inducible defense systems. First, the PAMP-triggered immunity (PTI) is activated upon recognition of pathogen-associated molecular patterns (PAMPs) of invading pathogens. Some pathogen strains can overcome PTI, utilizing effector molecules that interfere with, or suppress, PTI. In turn, some plant genotypes have developed resistance (R) proteins to detect the presence of pathogen effector molecules and activate effector-triggered immunity (ETI). Several receptor-like proteins (RLPs) have been implicated in plant innate immunity and where shown to mediate microbial perception, mostly as pathogen R proteins in tomato and apple (**chapter 1**). At the start of this research project, only two Arabidopsis *RLP* genes, *CLV2* and *TMM*, that are both involved in developmental processes, were functionally characterized.

In **chapter 2**, we identified 57 *AtRLP* genes in the Arabidopsis genome. A genome-wide collection of T-DNA insertion mutants for the 57 *AtRLP* genes was assembled and functionally analyzed for alterations in plant growth and development, and sensitivity to various stress responses, including susceptibility towards pathogens. From this analysis several new developmental phenotypes were identified for T-DNA insertion mutants in the *CLV2* and *TMM* genes. In addition, we found that mutations in *TMM* displayed altered sensitivity to abcisic acid (ABA) a phytohormone that is also found to regulate stomatal aperture. This suggests that ABA sensitivity might be a crucial factor in regulation of stomatal distribution by *TMM*. Another altered phenotype upon ABA treatment was identified for T-DNA insertion mutants of *AtRLP41*, which displayed enhanced sensitivity to exogenous application of ABA but no abnormalities in stomatal patterning. *AtRLP41* appeared to be highly induced during plant senescence, a process that can be induced by ABA, suggesting that *AtRLP41* may be involved in ABA-induced senescence. It was expected that the vast majority of the *AtRLP* genes would function as disease resistance genes based on sequence comparison and bioinformatic analyses. Despite extensive disease assays with many different pathogens, only *AtRLP30* and *AtRLP18* were found to be involved pathogen resistance, since corresponding T-DNA insertion mutants were found to display enhanced susceptibility towards the non-adapted bacterial bean pathogen *Pseudomonas syringae* pv. *phaseolicola*. *AtRLP30* and *AtRLP18* mutants affect Arabidopsis non-host resistance and thus are suggested to play a role in basal defense. Lack of identification of more novel phenotypes was thought to be due to functional redundancy. This led us to undertake an RNA interference (RNAi) strategy to target the expression of

multiple *AtRLP* genes simultaneously (**chapter 3**). The *AtRLP* RNAi lines were functionally analyzed in a similar fashion as the *AtRLP* T-DNA insertion lines. Although novel phenotypes were not discovered in this analysis, we were able to show that RNAi-mediated gene silencing can be used as a mechanism to investigate the function of RLP receptors. RNAi lines for a construct predicted to target *AtRLP41* amongst other *AtRLP* genes also displayed enhanced ABA sensitivity similar to the *AtRLP41* knock-out lines. Nevertheless, from this analysis the question remains whether novel phenotypes for *AtRLP* T-DNA insertion lines were obscured by functional redundancy.

In the past decade, many biological processes in plants, such as regulation of gene expression and viral defense, were shown to be regulated by RNA silencing. This conserved mechanism has recently been shown to play a role in defense against bacterial plant pathogens. The work described in **chapter 4** implicates a role for RNA silencing in fungal defense, since several components of RNA silencing pathways were found to affect *Verticillium* defense. No altered defense in mutants of RNA silencing components was found towards other fungal pathogens, including *Alternaria brassicicola*, *Botrytis cinerea* and *Plectosphaerella cucumerina*, but also the vascular pathogen *Fusarium oxysporum*. Since the observed differences in *Verticillium* susceptibility cannot be explained by notable differences in root architecture or expression of downstream signaling components, it is proposed that gene silencing affects regulation of *Verticillium*-specific defense responses.

In **chapter 5,** obtained results of this thesis are discussed with respect to recent developments in the RLP research.

SAMENVATTING

Planten staan doorlopend bloot aan microbiële belagers. Aangezien planten zich niet kunnen verplaatsen om aan bedreigingen te ontkomen, zijn ze volledig aangewezen op de effectiviteit van hun afweer. In de aangeboren afweer van planten kunnen twee induceerbare mechanismen onderscheiden worden. De zogenaamde PAMP-geactiveerde afweer ("PAMP-triggered immunity", PTI) wordt aangeschakeld wanneer pathogeen-geassocieerde moleculaire patronen (PAMPs) van microbiële belagers herkend worden. Bepaalde stammen van microbiële belagers zijn in staat om PTI te inactiveren met behulp van effector moleculen die PTI onderdrukken. Echter, bepaalde waardplanten hebben vervolgens resistentie-eiwitten ontwikkeld die erop gericht zijn om de aanwezigheid of de activiteit van microbiële effectoren te herkennen, waarop effector-geactiveerde afweer ("effector-triggered immunity", ETI) wordt aangeschakeld. Verschillende receptor-achtige eiwitten ("receptor-like proteins", RLPs) die een rol in de aangeboren afweer van planten spelen zijn geïdentificeerd, met name in appel en tomaat. Deze RLPs zijn als resistentie-eiwitten betrokken in de specifieke herkenning van microblële belagers (**hoofdstuk 1**). Bij de start van dit onderzoeksproject waren slechts twee RLPs functioneel gekarakteriseerd in de modelplant Arabidopsis; *CLV2* en *TMM* die beiden een rol spelen in plantontwikkeling.

In **hoofdstuk 2** zijn 57 *RLP* genen geïdentificeerd in de genoomsequentie van Arabidopsis (*AtRLPs*). Een collectie van T-DNA insertielijnen voor de 57 *AtRLP* genen is samengesteld, en vervolgens is deze mutanten collectie functioneel gekarakteriseerd met betrekking tot groei en ontwikkeling van de plant en gevoeligheid voor verschillende stress factoren, inclusief microbiële belagers. Deze karakterisering heeft een aantal nieuwe ontwikkelings-fenotypes opgeleverd voor *CLV2* en *TMM*. Daarnaast bleken mutaties in *TMM* te leiden tot veranderde gevoeligheid voor het plantenhormoon abscisinezuur ("abcisic acid", ABA) dat de opening van huidmondjes reguleert. Mogelijk is abscisinezuur-gevoeligheid belangrijk voor de voor de verdeling van huidmondjes, een proces dat gereguleerd wordt door TMM. Lijnen met T-DNA inserties in *AtRLP41* vertoonden een verhoogde gevoeligheid voor ABA, hoewel de verdeling van huidmondjes in deze mutant normaal was. Tijdens veroudering, een proces dat door ABA geactiveerd kan worden, wordt *AtRLP41* sterk geactiveerd, wat suggereert dat *AtRLP41* betrokken is in ABA-geactiveerde veroudering. Bij aanvang van dit project namen we aan dat de meeste *AtRLP* genen een rol bij de afweer zouden spelen. Maar hoewel we alle T-DNA insertielijnen met veel verschillende microbiële belagers hebben geïnoculeerd kon slechts voor twee genen, *AtRLP18* en *AtRLP30*, een rol bij de afweer aangetoond worden. Inserties in deze genen leidden tot gevoeligheid voor *Pseudomonas syringae* pv. *phaseolicola*, een

bacterie die normaal gesproken wel boon maar niet Arabidopsis infecteert. Dat voor geen van de andere *AtRLP* genen een rol bij heeft mogelijk te maken met functionele redundantie. Om dit te ondervangen werd met behulp van RNA interferentie (RNAi) de expressie van meerdere *AtRLP* genen gelijktijdig onderdrukt (**hoofdstuk 3**). *AtRLP* RNAi lijnen werden functioneel gekarakteriseerd op dezelfde wijze als de T-DNA insertielijnen. Deze aanpak leverde geen nieuwe fenotypes op, maar toonde wel aan dat het onderdrukken van de expressie van *AtRLP* genen met behulp van RNAi mogelijk is. RNAi lijnen die, naast andere *AtRLP* genen, ook de expressie van *AtRLP41* zou moeten onderdrukken vertoonden, net als de *AtRLP41* T-DNA insertielijn, verhoogde gevoeligheid voor ABA.

RNAi speelt een rol in verscheidene biologische processen, zoals regulering van genexpressie en virale afweer. Recent is aangetoond dat RNAi een rol speelt in afweer tegen bacteriële belagers. **Hoofdstuk 4** beschrijft een rol voor RNAi in afweer tegen schimmels, aangezien mutanten in verschillende componenten die een rol spelen bij RNAi een veranderde vatbaarheid voor de vaatbundel-schimmel *Verticillium* vertoonden. De vatbaarheid voor andere schimmels zoals *Alternaria brassicicola*, *Botrytis cinerea* en *Plectosphaerella cucumerina*, maar ook de vaatbundel-schimmel *Fusarium oxysporum*, was ongewijzigd in deze mutanten. De veranderde vatbaarheid voor de vaatbundel-schimmel *Verticillium* kon niet verklaard worden door veranderingen in wortelmorfologie of veranderde activatie van bekende afweergenen in de verschillende mutanten, waaruit afgeleid wordt dan RNAi specifiek *Verticillium*-afweer beinvloedt.

Hoofdstuk 5 betreft een algemene discussie waarbij alle verkregen resultaten nog eens besproken worden in het licht van recente ontwikkelingen in het onderzoek aan RLPs.

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It is done; the thesis is completed, fulfilling me with pleasure! I am looking back to a turbulent time gathering intensive experiences that for sure enriched my life. However, the accomplishment of this thesis would not have been possible without collaboration and support of people I want to thank at this point.

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A PhD thesis is not only about working on scientific research questions but also working together in a group and socializing with people.

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Ursula

CURRICULUM VITAE

Ursula Ellendorff was born on March 19th, 1978 in Gütersloh, Germany. After she obtained her graduation diploma at the Gymnasium Nepomucenum Rietberg in 1997, she performed a social voluntary year at the institution of Von Bodelschwingh in Bethel (Bielefeld, Germany), caring for people with a mental and physical handicap. Starting her study in biology at the Westfälische Wilhelms University of Münster, Germany in 1998, her main focus was directed to microbiology and, as subsidiary subjects, zoology and biochemistry. In 2003, she completed her university education with her thesis entitled: *"Molecular characterization of signal chain components of the phytopathogenic fungus Botrytis cinerea"* and with the diploma in biology. From 2004 to 2008, she conducted her PhD research at the laboratory of Phytopathology, Wageningen University, The Netherlands, investigating roles of Arabidopsis receptor-like proteins in pathogen defense. Since October 2008, she is working as Phytopathology researcher at the seed production company Rijk Zwaan in De Lier, The Netherlands.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Ursula Ellendorff
Date: 11 March 2009
Group: Laboratory of Phytopathology, Wageningen University

1) Start-up phase	date
► **First presentation of your project**	
defense signalin	24 Aug 2004
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
Amsterdam	03 Jun 2004
Nijmegen	02 Jun 2005
Wageningen	19 Sep 2006
Wageningen	13 Sep 2007
► **EPS theme symposia**	
EPS Theme2 Sypmosium: Interactions between plants and biotic agents (Leiden)	23 Jun 2005
EPS Symposium on Intracellular Signalling (Amsterdam)	02 Feb 2006
EPS Theme2 Sypmosium &WCS day: Interactions between plants and biotic agents (Amsterdam)	02 Feb 2007
► **NWO Lunteren days and other National Platforms**	
The ALW-NWO meeting in Lunteren	05-06 Apr 2004
The ALW-NWO meeting in Lunteren	04-05 Apr 2005
ALW Platform Molecular Genetics Annual Meeting in Lunteren	13-14 Oct 2005
The ALW-NWO meeting in Lunteren	03-04 Apr 2006
ALW Platform Molecular Genetics Annual Meeting in Lunteren	05-06 Oct 2006
The ALW-NWO meeting in Lunteren	02-03 Apr 2007
The ALW-NWO meeting in Lunteren	07-08 Apr 2008
► **Seminars (series), workshops and symposia**	
CBSG Workshop: Intellectual Property Rights: the basics	03 Oct 2004
Minisymposium: Investigating the Genetics of Natural Variations	26 Nov 2004
Minisymposium: Fungal Pathogenicity to Plants and Humans	08 Jul 2005
Seminar of Prof. Dr. Sophien Kamoun "Reprogramming the host: The effector secretome of Phytophthora infestans"	05 Oct 2005
Seminar of Dr. James Correl "Applied plant pathology in the genomics era"	20 Apr 2006
Seminar of Prof. Nicholas Talbot "Investigating the molecular genetics of plant infection by the rice blast fungus Magnaporhte grisea"	03 May 2006
Symposium: Chemische gewasbescherming tegen plantenpathogenen	29 Jun 2006
Seminar of Philip Zimmerman on Genvestigator	26 Sep 2006
	06 Nov 2007
CBSG Summit	06-07 Feb 2007
Bio Career Event 2007	31 May 2007
Seminar of Prof. Andrew Bent: Active site discovery in LRR domains-FLS2/flagellin perception and other examples	18 Jun 2007
Joint CBS-PRI-Phyto Symposium	22 Jun 2007
Seminar of Prof. Aad Termorshuizen "Magical & other Mushrooms	05 Sep 2007
Plasterk Symposium "The small RNA Revolution"	08 Sep 2007
Seminar of Prof. Scott Poethig "Regulation of phase change in plants by miRNAs and trans-acting siRNAs"	24 Sep 2007
Seminar of Dr. Andre Drenth "Challenges to control plant diseases in tropical tree crops"	08 Oct 2007
CBSG Summit	17-18 Mar 2008
► **Seminar plus**	
Seminar plus of Prof. Scott Poethig "Regulation of phase change in plants by miRNAs and trans-acting siRNAs"	24 Sep 2007
► **International symposia and congresses**	
Symposium: NON-SPECIFIC AND SPECIFIC INNATE AND ACQUIRED PLANT RESISTANCE Budapest, Hungary	31 Aug- 03 Sep 2006
XIII IS-MPMI Congress in Sorrento, Italy	21-27 Jul 2007
► **Presentations**	
Poster presentation at the Symposia in Budapest 2006	02 Sep 2006
Oral presentation at ALW Platform Molecular Genetics Annual Meeting in Lunteren 2006	05 Oct 2006
Oral presentation at the ALW-NWO meeting in Lunteren	07-08 Apr 2008
► **IAB interview**	18 Sep 2006
► **Excursions**	
Subtotal Scientific Exposure	*16.7 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
EPS-Summerschool: Environmental Signaling: Arabidopsis as a model	22-24 Aug 2005
Systems Biology course: Principles of -omics data analysis	07-10 Nov 2005
EPS-Summerschool: Signaling in Plant Development and Defense:towards Systems Biology	19-21 Jun 2006
EPS-Summerschool: Environmental Signaling: Arabidopsis as a model	27-29 Aug 2007
► **Journal club**	
Member of literature discussion group 'Phytopathology'	2004-2008
► **Individual research training**	
Subtotal In-Depth Studies	*6.9 credits**

4) Personal development	date
► **Skill training courses**	
Academic writing	Apr-Jun 2005
Teaching and Supervising Theses Students	14-15 Jun 2005
Scientific writing	Oct-Nov 2005
Career Perspectives	Mar-May 2008
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*5.4 credits**

TOTAL NUMBER OF CREDIT POINTS*	**30,5**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

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Cover: Silhouettes of a 3-4 week-old Arabidopsis plant.

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