INNATE IMMUNITY IN ARABIDOPSIS: MOLECULAR MECHANISMS OF *HOPA1* AND *AVRRPS4* - SPECIFIC DISEASE RESISTANCE SIGNALING PATHWAYS

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SANG HEE KIM

Dr. Walter Gassmann, Dissertation Supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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a candidate for the degree of doctor of philosophy

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Walter Gassmann

Professor Gary Stacey

Professor James Schoelz

Professor Melissa Mitchum

Professor Scott Peck

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ABSTRACT

Plants have evolved several layers of predetermined defenses, collectively called the innate immune system. Because of its effectiveness effector-triggered immunity (ETI) is a highly valuable agronomic trait. However, ETI has the potential to be highly deleterious to the host and needs to be tightly controlled. To understand the molecular basis for ETI, I used genetic approaches. Using a loss of resistance screen, I cloned the *hopA1*-specific *RPS6 (Resistance to Pseudomonas syringae 6)* resistance gene which is a member of TIR (Toll-Interleukin-1 Receptor)-NBS (Nucleotide Binding Site)-LRR (Leucine-Rich Repeat) class of resistance proteins. Using a gain of resistance screen, we cloned *SRFR1 (Suppressor of RPS4-RLD)*, which reactivates *avrRps4*- and *hopA1*-triggered immunity and encodes a pioneer TPR (Tetratricopeptide Repeat) protein conserved between plants and animals. Based on the genetic, molecular, biochemical, and phylogenic evidence, we propose that SRFR1 functions in a transcriptional repressor complex that balances plant immunity and

development. To date *RPS4 and RPS6* are the only Arabidopsis TIR-NBS-LRR resistance genes for which *P. syringae* effectors are known. Both pathways are negatively regulated by *SRFR1.* Functional characterization of *RPS6* and *SRFR1* will provide an important piece of the ETI puzzle.

Chapter I.

General Introduction

1. The plant immune response

Plants are exposed to a wide variety of potential pathogens and have developed a plethora of strategies aimed at protection from pathogen infections (Staskawicz et al., 1995). The plant immune response consists of at least two parts. One is PAMP (pathogen associated molecular pattern)-triggered immunity (PTI), and the other is effector-triggered immunity (ETI) (Ausubel, 2005; Chisholm et al., 2006; Jones and Dangl, 2006; Bittel and Robatzek, 2007). PTI is a first layer of the plant immune system where pattern recognition receptors (PRRs) of plants perceive PAMPs to elicit defense responses, including the activation of MAP (mitogen-activated protein) kinase cascades, production of reactive oxygen species (ROS) and nitric oxides, ion channel opening, an enhancement of preformed barriers, callose deposition, and activation of defense-related genes (Asai et al., 2002; Ausubel, 2005; Boller and Felix, 2009). PTI is conserved between plants and animals. Bacterial flagellin is recognized by the plasma membrane located FLS2 (FLAGELLIN SENSITIVE 2) in plants, and TLR5 (Toll-like receptor 5) in mammals to modulate defense responses (Ausubel, 2005; Boller and Felix, 2009). However, successful pathogens have created effectors to efficiently suppress PTI. Plants, in turn, also evolved a second layer of defense using resistance (R) proteins to monitor pathogen effectors, which is known as ETI (Chisholm et al., 2006; Grant et al., 2006; Jones and Dangl, 2006). Historically, ETI has been explained by the gene-for-gene hypothesis.

2. Effector triggered immunity (ETI)

2.1 Effector proteins of bacterial pathogens

A single plant pathogenic bacterium can secrete 20 to 50 effector proteins into cells of plants using type lll secretion systems (TTSS). The original function of type lll effectors is to enhance bacterial virulence by blocking PTI and eukaryotic cellular functions (Mudgett, 2005; Grant et al., 2006). For example, two effector proteins, AvrPto and AvrPtoB, from *P. syringae* tomato DC3000 interact with the kinase domains of PRRs including FLS2, EFR, and BAK1, suppressing the kinase activity of PRRs (Gohre et al., 2008; Shan et al., 2008; Xiang et al., 2008). However, effectors can trigger defense responses if they are recognized by specific host R proteins. The recognized effector protein monitored by a host R protein is named an avirulence (Avr) protein (Jones and Dangl, 2006).

2.2 Plant NB-LRR class of *R* genes

Over the last decade, functional *R* genes were cloned from both Arabidopsis and crop plants in the search for an explanation of how early R protein-mediated recognition leads to successful resistance. The first class of R proteins is the extracellular leucine-rich repeat (eLRR), which is further divided into RLK (receptor like kinase) and RLP (receptor like protein) according to the presence or absence of a kinase motif. Examples of RLK and RLP are rice Xa21 to *Xanthomonas oryzae* pv. oryzae containing AvrXa21, and tomato Cf proteins to *Cladosporium fulvum* carrying Avr proteins, respectively (Song et al., 1995; Dangl and Jones, 2001; Rivas and Thomas, 2005).

The second, but largest class of *R* genes cloned so far, encodes a family of cytosolic proteins containing a nucleotide binding site (NBS) domain, which is also known as the NB-ARC domain, and leucine-rich repeat (LRR) domains. The NBS-LRR domain is also found in the mammalian NOD immune receptor (Ausubel, 2005). There are approximately 150 *R* genes in Arabidopsis and 500 *R* genes in rice. The NB-ARC domain of R proteins has sequence homology to NB domains of apoptosis regulators such as Apoptotic protease activating factor-1 (Apaf-1) and cell death protein 4 (Ced4), suggesting the NB-ARC domain is involved in ATP binding and hydrolysis. The NB-ARC domains of two tomato R proteins, I-2 to *Fusarium oxysporum* and Mi-1 to root-knot nematodes and potato aphids, bind and hydrolyze ATP (Tameling et al., 2002; Tameling et al., 2006). The LRR domain is a common motif of 20–30 amino acids in length, represented in over 2,000 proteins from viruses to eukaryotes. Plant LRR motifs function in protein-protein interactions analogously to their function in animal systems, and they are responsible for recognition of upstream activators (Dangl and Jones, 2001; Belkhadir et al., 2004; Chisholm et al., 2006).

The NBS-LRR class of *R* genes can be grouped into two families, coiled-coil (CC)-NBS-LRR (CNL) and Toll-interleukin-1 receptor (TIR)-NBS-LRR (TNL), based on the N-terminal structure. The plant TIR domain has structural and functional similarity to the cytoplasmic domain of the *Drosophila* Toll and mammalian interleukin-1 transmembrane receptor. Similar functionality and organization of plant TIR-type R proteins and animal Toll-like receptors (TLRs) is thought to represent convergent evolution of the innate immunity pathway of

these kingdoms (Ausubel, 2005). In addition, NBS-LRR proteins with diverged structures of the N-terminal domain are present in rice, but NBS-LRR proteins containing a TIR domain are absent, suggesting a complex evolutionary history of NBS-LRR proteins in plants (Monosi et al., 2004). *RPM1* (Grant et al., 1995), *RPS2* (Bent et al., 1994), and *RPS5* (Warren et al., 1998) are CC-NBS-LRR *R* genes that recognize the *Pseudomonas syringae* type III effectors *avrRpm1, avrRpt2,* and *avrPphB*, respectively. *RPS4* (Gassmann et al., 1999) and *RPS6* (Kim et al., 2009) are TIR-NBS-LRR type *R* genes that monitor the *Pseudomonas syringae* effectors *avrRps4* and *hopA1*, respectively, to induce ETI. Another unique NBS-LRR *R* gene is *RRS1,* which mediates resistance to *Ralstonia solanacearum* carrying *popP2*. *RRS1* encodes a TIR-NBS-LRR class of R protein with a C-terminal NLS (nuclear localization signal) and a WRKY domain (Deslandes et al., 2002; Deslandes et al., 2003). The TIR and CC domains are thought to interact with the host target (guardee) or with downstream signaling partners (Belkhadir et al., 2004). In animal innate immunity, PAMPs are recognized by the LRR domains of TLRs and this recognition is delivered to cytoplasmic TIR domains (Barton and Medzhitov, 2002, 2003).

3. Modes of recognition in plant-pathogen interaction

One of the intriguing issues in the study of plant innate immunity is the mode of recognition between host R proteins and pathogen effector (Avr) proteins to elicit defense cascades. In the early days, researchers believed that R and effector

proteins interacted directly as implied by the gene-for-gene hypothesis. There are some reports in support of the direct interaction model (Tang et al., 1996; Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006). However, these studies are performed in heterologous systems, such as yeast two-hybrid and *in vitro* pull-down assays. Recently, another hypothesis has arisen where a plant R protein (guard) indirectly recognizes a corresponding effector (Avirulence) protein by monitoring effector perturbation of a host target (guardee), consistent with the "guard hypothesis" (Dangl and Jones, 2001). This scenario was substantiated by mounting evidence of R-guardee-Avr protein complex, such as AvrRpm1/AvrRpt2-RIN4-RPM1/RPS2 (Mackey et al., 2002; Axtell et al., 2003; Mackey et al., 2003), AvrPphB-PBS1-RPS5 (Shao et al., 2003), Avr2-Rcr3-Cf2 (Rooney et al., 2005), and P50-NRIP-N (Caplan et al., 2008b). The immediate downstream events after recognition of pathogen effectors by plant R proteins are still unknown and require further studies.

4. *R* gene-mediated plant defense signaling network

In response to pathogen effectors, R proteins elicit a cascade of defense responses including an oxidative burst, calcium and ion fluxes, induction of defense related genes, and the hypersensitive response (Hammond-Kosack and Parker, 2003; Belkhadir et al., 2004). Genetic screens have allowed the identification of signal transduction components in R protein-mediated resistance. In general, the signaling pathways of the two structurally different *R* gene families

require either a functional allele of *EDS1* (*Enhanced Disease Susceptibility1*) (Parker et al., 1996) or of *NDR1* (*Nonrace-specific Disease Resistance1*) (Century et al., 1995; Century et al., 1997). Plant innate immunity conferred by CNL *R* genes requires *NDR1* but not *EDS1*, whereas TNL *R* genes are dependent on *EDS1* and independent of *NDR1* (Parker et al., 1996; Century et al., 1997; Aarts et al., 1998). Although this simple dichotomy between *NDR1* and *EDS1* indicates structure-dependent R protein-mediated resistance signaling, there are some exceptions. Resistance mediated by the CC-NBS-LRR *HRT* gene, which specifies resistance to turnip crinkle virus (TCV), is *EDS1*-dependent but *NDR1*-independent (Chandra-Shekara et al., 2004). Some CC-NBS-LRR class *R* genes, such as *RPP7, RPP8,* and *RPP13* against powdery mildew, are partially dependent or independent of both *NDR1* and *EDS1* (McDowell et al., 2000; Bittner-Eddy and Beynon, 2001). The N-terminal CC or TIR domain is thought to function as an interacting determinant with downstream signaling components, based on the *NDR1* or *EDS1* requirement in different *R* gene-mediated resistance pathways, as well as the structural similarity of plant TIR-type R proteins and animal innate immunity Toll-like receptors (Belkhadir et al., 2004). Another component of R gene-mediated signaling, *PAD4* (*Phytoalexin Deficient 4*), was isolated from phytoalexin deficient (*pad*) mutants (Glazebrook et al., 1997). Both PAD4 and EDS1 have homology with eukaryotic lipases and physically interact with each other (Falk et al., 1999; Feys et al., 2001). A PAD4 and EDS1 complex is required for TIR-NBS-LRR type R protein signaling, which

acts upstream of the oxidative burst and programmed cell death (Wiermer et al., 2005).

RAR1 and SGT1 are co-chaperones of R proteins. They interact with each other and with diverse protein complexes (Austin et al., 2002; Azevedo et al., 2002; Muskett et al., 2002; Tornero et al., 2002; Shen et al., 2003). They play an important role in R protein-mediated resistance by affecting protein stability. However, there is no clear pattern between R protein structure and dependence on RAR1 and SGT1 (Shirasu and Schulze-Lefert, 2003). HSP90 (heat shock protein) also physically interacts with RAR1 and SGT1, and plays a chaperone function in the control of R protein stability (Hubert et al., 2003; Takahashi et al., 2003). The SGT1-HSP90 chaperone complex for maintenance of proper immune receptor folding is structurally and functionally conserved in eukaryotes, suggesting that both immune receptor and chaperone system operate similarly between plants and animals (Shirasu, 2009).

5. Localization of R proteins

Over the past decade, increasing evidence has revealed the subcellular localization of R proteins in plants. In animals, Toll-like receptors (TLRs) and NOD-like NBS-LRR receptors chiefly target the plasma membrane and cytoplasm to detect pathogen molecules, respectively (Kawai and Akira, 2006; Kanneganti et al., 2007). Bacterial effector proteins are injected into plant cells by the type III secretion system, and most bacterial effector-specific NBS-LRR class

R proteins are thought to be localized to the intracellular space (Martin et al., 2003; Chisholm et al., 2006). The Arabidopsis resistance protein RPM1 and its cognate effectors, AvrRpm1 and AvrB, are reported to associate with the plasma membrane on the cytoplasmic side (Boyes et al., 1998; Nimchuk et al., 2000). Cf proteins from tomato are detected in the plasma membrane to recognize their extracellular cognate Avr effectors (Piedras et al., 2000; Rivas and Thomas, 2005).

Other R proteins are localized to the nucleus or cytoplasm. The Arabidopsis RRS1-R protein, containing a classical nuclear localization signal (NLS) and a WRKY domain, recognizes the PopP2 effector from *Ralstonia solanacearum.* RRS1-R and PopP2 are co-localized to the nucleus (Deslandes et al., 2002; Deslandes et al., 2003). Nuclear localization of the MLA R protein in barley plays a pivotal role in conferring resistance against barley powdery mildew fungus containing the cognate effector protein, and this recognition leads to the interaction between MLA and WRKY transcription factors (TFs) in the nucleus (Bieri et al., 2004; Shen et al., 2007). RPS4 is distributed between endomembrane and nucleus, and its nuclear localization is required for AvrRps4-triggered immunity in Arabidopsis (Wirthmueller et al., 2007). In addition, tobacco N protein and its cognate pathogen protein, tobacco mosaic virus (TMV) p50, are distributed between the cytoplasm and nucleus. Nuclear localization of the N protein is required for its function (Burch-Smith et al., 2007).

Why do R protein immune receptors need to be localized to the nucleus? One of the possible functions of nuclear R proteins is an involvement in

transcriptional reprogramming. Arabidopsis RRS1-R may directly regulate the expression of defense related genes with its WRKY domain (Deslandes et al., 2002). In addition, the At4g12020 gene in Arabidopsis encodes a TNL class of R protein with a WRKY domain at the N-terminus and a MAPKKK (mitogen-activated protein kinase kinase kinase) domain at the C-terminus (Meyers et al., 2003). The physical interaction of barley MLA10 R protein and WRKY1 and WRKY2 TFs in the nucleus is induced by the fungal Avr10 effector protein, suggesting MLA R proteins, which themselves lack DNA binding domains, may control expression levels of defense genes through WRKY TFs (Shen et al., 2007). In addition to MLAs, there is some evidence that R proteins interact with transcriptional regulators. RPM1 (CNL) and RPP5 (TNL) R proteins in Arabidopsis bind to TIP49a, which in mammals interacts with the TATA binding protein and functions as a negative regulator of R protein-dependent resistance and a modulator of plant development (Holt et al., 2002). The tobacco N proteins were shown to associate with SPL (squamosa promoter-like) TFs through the LRR domain (Caplan et al., 2008a).

6. Alternative splicing and transcriptional regulation of *R* genes

Alternative splicing (AS) plays an important role in post-transcriptional regulation. The mechanism is well-characterized in animal systems, but little studied in plants. Approximately 60% of human genes and 22% of plant genes are reported to be alternatively spliced (Carninci et al., 2005; Nagasaki et al., 2005; Wang and

Brendel, 2006; Ner-Gaon et al., 2007; Barbazuk et al., 2008). Various forms of AS are known, including intron retention, alternative exon, and alternative donor or acceptor site. The dominant alternative splicing mechanism in humans is alternative exon recognition, and intron retention is rarely seen (Carninci et al., 2005). In contrast, intron retention accounts for over 56% of the AS of Arabidopsis. Intriguingly, stress- or stimulus- responsive genes predominate among genes showing transcripts with retained introns, suggesting that the importance of AS in plants is underestimated because expression levels of stress- or stimulus- responsive genes are low (Ner-Gaon et al., 2004; Wang and Brendel, 2006). When compared to the CC-NBS-LRR class, TIR-NBS-LRR type R genes more often produce AS products. This process can introduce premature stop codons in open reading frames, causing truncated proteins. These truncated proteins encoded by splice variants commonly are TIR-NBS (TN) proteins lacking most of the LRR domain (Whitham et al., 1994; Lawrence et al., 1995; Parker et al., 1997; Gassmann et al., 1999; Dinesh-Kumar and Baker, 2000; Borhan et al., 2004; Schornack et al., 2004). Alternative splicing of Arabidopsis *RPS4* and the tobacco *N* gene is indispensable for full *R*-mediated resistance (Dinesh-Kumar and Baker, 2000; Zhang and Gassmann, 2003). In contrast, truncated splicing products are not essential for flax *L6*-mediated resistance to rust (Ayliffe et al., 1999). Even though AS occurs predominantly in the TIR-NBS-LRR class of R genes, there are some reports of AS in the CC-NBS-LRR class, including barley *Mla6* and *Mla13*, maize *Rp1-D,* and tobacco *NRG1* (Halterman et al., 2001;

Halterman et al., 2003; Ayliffe et al., 2004; Halterman and Wise, 2004; Peart et al., 2005).

7. *hopA1* as a new avirulence gene for Arabidopsis

The TTSS (type lll secretion system) is found in Gram-negative bacterial pathogens including *Pseudomonas syringae,* and is encoded by *hrp* (*hypersensitive response* and *pathogenicity*) and *hrc* (*hrp* and *conserved*) genes in the Hrp pathogenicity island. Bacterial effectors are injected into plant cells using the TTSS, mainly to suppress host resistance (Collmer et al., 2000; Alfano and Collmer, 2004). However, pathogen effectors have the opposite effect when they are recognized by the host surveillance system (Jones and Dangl, 2006). Approximately 40 effectors are present in *P. syringae* tomato strain DC3000 (Lindeberg et al., 2006; Cunnac et al., 2009).

The bacterial effector gene *hopA1* (formerly *hrmA* and *hopPsyA*) is from *P. syringae* pv. syringae strain 61 (Pss61). The pHIR11 cosmid that contained *hopA1* and Pss61 type lll secretion system genes, when expressed in *P. fluorescens*, can induce a strong hypersensitive response (HR) in tobacco (Huang et al., 1988). Alfano and colleagues established that HopA1 is responsible for the pHIR11-dependent HR in tobacco and is required to be translocated into the plant cell for HR (Alfano et al., 1997). HopA1 directly binds to the molecular chaperone ShcA, which is required for secretion of HopA1 (van Dijk et al., 2002). In addition, most effectors from *P. s* pv. tomato strain DC3000

can suppress the pHIR11-dependent HR in tobacco (Jamir et al., 2004; Guo et al., 2009), suggesting that the pHIR11 is an effective tool to elucidate the function of the TTSS in bacterial pathogens.

In Arabidopsis, high inoculum (10^8cfu/ml) of *P. fluorescens* expressing *hopA1* gives an HR in Ws-0, but HR isn't detected in Col-0 and RLD. However, Col-0 and RLD show a non-HR resistant phenotype in response to *P. syringa*e pv. tomato (Pst) DC3000 expressing *hopA1* used at 10^6cfu/ml. Both the HR and resistance phenotype is *EDS1*-dependent and *NDR1*-independent (Gassmann, 2005). This resistance without HR is reminiscent of the *avrRps4*-triggered immunity of Arabidopsis governed by the *RPS4 R* gene (Gassmann et al., 1999). Together, these data indicate that *hopA1* is an avirulence gene for Arabidopsis which shows a resistance phenotype with no HR.

8. HopA1- and AvrRps4- triggered immunity in Arabidopsis

Comparative studies with the CNL class resistance genes *RPS2, RPM1* and *RPS5* and isogenic *P. syringae* strains expressing single corresponding avirulence genes have been particularly fruitful in dissecting specific and common resistance signaling components. However, the major TNL class is represented by a single known *P. syringae* resistance gene, *RPS4*. To increase the understanding of the TNL R gene-mediated resistance pathways, we used genetic approaches. Using a loss of resistance screen, we identified and cloned the *hopA1*-specific *RPS6 (Resistance to Pseudomonas syringae 6) R* gene, which encodes a TNL R protein. *RPS6* generates alternatively spliced transcripts.

Both RPS6 and HopA1 are localized to the nucleus and cytoplasm. In addition, nuclear localization of HopA1 may be necessary to induce the cell death phenotype in *N. benthamiana*.

Using a gain of resistance screen, we cloned *SRFR1 (Suppressor of RPS4-RLD)*, which reactivates *avrRps4*-triggered immunity and encodes a pioneer TPR (Tetratricopeptide Repeat) protein conserved between plants and animals. The SRFR1 TPR domain has significant sequence similarity to those of *Saccharomyces cerevisiae* Ssn6 and *Caenorhabditis elegans* OGT1, which function as transcriptional repressors. Transcript levels of defense related genes are induced in *srfr1* mutants. SRFR1 physically interacts with three members of the TCP (TEOSINTE BRANCHED1, CYCLOIDEA and PCF) transcription factor family, TCP8, TCP20 and TCP22, in the nucleus. Interestingly, mutations in *SRFR1* also enhanced *hopA1*-triggered immunity in *rps6* mutants, suggesting that SRFR1 may function as a general negative regulator in the TNL R protein-mediated resistance pathway.

Functional characterization of RPS6 and comparisons with RPS4 will contribute to a closer dissection of the TNL resistance pathway, which is regulated by the positive regulator EDS1 and negative regulator SRFR1 in Arabidopsis. My research to increase the understanding of plant innate immunity in the reference plant Arabidopsis can be applied to crop plants for durable pathogen resistance, which can reduce our reliance on chemical disease control and improve agricultural safety and crop yields.

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Chapter II.

Resistance to the *Pseudomonas syringae* effector HopA1 is governed by the TIR-NBS-LRR protein RPS6 and is enhanced by mutations in *SRFR1*

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Abstract

The *Pseudomonas syringae* - *Arabidopsis thaliana* (Arabidopsis) interaction is an extensively studied plant-pathogen system. Arabidopsis possesses approximately 150 putative resistance genes encoding nucleotide binding site (NBS) and leucine-rich repeat (LRR) domain-containing proteins. The majority of these belong to the Toll/Interleukin-1 receptor (TIR)-NBS-LRR class. Comparative studies with the coiled coil (CC)-NBS-LRR genes RPS2, RPM1 and RPS5 and isogenic *P. syringae* strains expressing single corresponding avirulence genes have been particularly fruitful in dissecting specific and common resistance signaling components. However, the major TIR-NBS-LRR class is represented by a single known *P. syringae* resistance gene, *RPS4*. We previously identified *hopA1* from *P. syringae* pv. syringae strain 61 as an avirulence gene that signals through *EDS1*, indicating that the corresponding resistance gene *RPS6* belongs to the TIR-NBS-LRR class. Here we report the identification of *RPS6* based on a forward-genetic screen and map-based cloning. Among resistance proteins of known function, the deduced amino acid sequence of *RPS6* shows highest similarity to the TIR-NBS-LRR resistance protein RAC1 that determines resistance to the oomycete pathogen *Albugo candida*. Similar to *RPS4* and other TIR-NBS-LRR genes, *RPS6* generates alternatively spliced transcripts, although the alternative transcript structures are *RPS6*-specific. We previously characterized SRFR1 as a negative regulator of *avrRps4*-triggered immunity. Interestingly, mutations in *SRFR1* also enhanced

HopA1-triggered immunity in *rps6* mutants. In conclusion, the cloning of *RPS6* and comparisons with *RPS4* will contribute to a closer dissection of the TIR-NBS-LRR resistance pathway in Arabidopsis.

Introduction

Effector-triggered immunity (ETI) is a potent defense response in plants that depends on the detection of the presence of pathogen effector proteins by host resistance (R) proteins (Chisholm et al., 2006; Jones and Dangl, 2006; Bent and Mackey, 2007). This branch of the plant innate immune system has been studied genetically for decades, ever since Flor formulated the gene-for-gene hypothesis (Flor, 1971). Understanding of ETI mechanisms was accelerated tremendously by the advent of molecular biology and the adoption of model plant-pathogen interactions. Among these, the *Pseudomonas syringae* - *Arabidopsis thaliana* (Arabidopsis) interaction has been an especially fruitful plant-pathogen system. The *P. syringae* species is divided into a large number of distinct pathovars based on the plant host from which a strain was originally isolated. This and the ease of molecular manipulation of this facultative bacterial plant pathogen has made *P. syringae* an important experimental system in the study of plant innate immunity, the effector complement of plant pathogens, and the evolution and genomics of host range (Fouts et al., 2002; Rohmer et al., 2004; Chang et al., 2005; Almeida et al., 2009). In particular, *P. syringae* pv. tomato strain DC3000 (DC3000) and *P. syringae* pv. maculicola strain 4326 (Psm4326) were found to be pathogenic on Arabidopsis (Dong et al., 1991; Whalen et al., 1991) and were used to characterize effector genes from other *P. syringae* pathovars and cognate Arabidopsis *R* genes.

Most *R* gene products identified in Arabidopsis and other plant species contain nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains (Meyers et al., 1999). Analysis of the Arabidopsis genome suggests that this plant has approximately 150 NBS-LRR genes. The majority of Arabidopsis NBS-LRR genes encode a protein with an N-terminal domain that shows sequence similarity with the cytosolic domain of the animal innate immune transmembrane receptors Toll and Interleukin-1 Receptor (TIR). A second class of NBS-LRR R proteins possesses an N-terminal coiled-coil (CC) domain (Martin et al., 2003; Meyers et al., 2003; Nimchuk et al., 2003). While the distinction is not absolute, genetically TIR-NBS-LRR (TNL) and CC-NBS-LRR (CNL) defense pathways can be separated based on the general requirement of TNL proteins for a functional *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) gene, whereas most characterized CNL proteins require *NONRACE SPECIFIC DISEASE RESISTANCE1* (*NDR1*) (Aarts et al., 1998).

The first Arabidopsis *R* genes to be cloned were the CNL genes *RPS2*, *RPM1* and *RPS5* (Bent et al., 1994; Grant et al., 1995; Warren et al., 1998). Isogenic DC3000 and Psm4326 strains with the different cognate avirulence genes were instrumental in determining common and specific downstream components of the CNL signaling pathway (Glazebrook, 2001; Martin et al., 2003; Belkhadir et al., 2004). In contrast, among the large TNL class of R proteins only RPS4 is known to recognize a *P. syringae* effector, namely the *P. syringae* pv. pisi effector AvrRps4 (Hinsch and Staskawicz, 1996; Gassmann et al., 1999). Significant insights have been gained by comparing *RPS4* to

Hyaloperonospora parasitica TNL *R* (*RPP*) genes (Austin et al., 2002; Muskett et al., 2002). However, a thorough dissection of the TNL signaling pathway would benefit from a second well-characterized *P. syringae*-TNL gene system.

Here we report the identification of *RPS6* using a forward genetic screen and map-based cloning. *RPS6* governs ETI to the effector gene *hopA1* (formerly *hrmA* and *hopPsyA*) from *P. syringae* pv. syringae strain 61 and requires *EDS1* (Gassmann, 2005). Among resistance proteins of known function, the deduced amino acid sequence of *RPS6* shows highest similarity to the TNL R protein RAC1 that determines resistance to the oomycete pathogen *Albugo candida*. Similar to *RPS4* and other TNL genes, *RPS6* generates alternatively spliced transcripts, although the alternative transcript structures are *RPS6*-specific. To illustrate the value of comparative studies between *RPS6* and *RPS4*, we tested the effects of mutations in *SRFR1*, a negative regulator of AvrRps4-triggered immunity (Kwon et al., 2009), on *rps6* mutants. Interestingly, mutations in *SRFR1* also enhanced HopA1-triggered immunity in *rps6* mutants, indicating a more general function of *SRFR1* in regulating ETI. The cloning of *RPS6* and comparisons with *RPS4* will contribute to a closer dissection of the TNL resistance pathway in Arabidopsis.

Results

Isolation of *hps* Mutants

We previously identified *hopA1* from *P. syringae* pv. syringae strain 61 (Huang et al., 1991; Alfano et al., 1997) as an avirulence gene for Arabidopsis. The *hopA1* gene is present on clone pHIR11 that encompasses the type three secretion system of this strain, but was not recognized as an avirulence gene for Arabidopsis because the reference accession Columbia-0 (Col-0) does not respond with a hypersensitive response to *hopA1* or *avrRps4* (Gassmann, 2005). A survey of 37 Arabidopsis accessions did not identify a naturally occurring *rps6* mutant (data not shown). We therefore proceeded by screening ethyl-methyl sulfonate (EMS) treated RLD pools (Kwon et al., 2004) for mutants susceptible to DC3000(*hopA1*) (see "Materials and Methods").

We screened approximately 25,000 M2 plants representing 1100 M1 plants from 67 different pools by dip-inoculation with DC3000(*hopA1*). Of 138 M2 individuals that were scored as susceptible after the first screen, we identified 13 M3 lines from distinct M2 pools that were chlorotic 5 days after dip-inoculation with virulent DC3000 and DC3000(*hopA1*). In a third screen, we syringe-infiltrated individual leaves of the same plant with DC3000 and DC3000(*hopA1*), respectively, to more closely examine segregation of the susceptible phenotype in the M3 generation. Because contaminants of *eds1-1*, a mutant in the Ws-0 accession that was used as a susceptible control during the screen, would show the same symptoms, we used cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993) specific for the *eds1-1* mutation and for the RLD accession to confirm that all mutants came from RLD pools (data not shown). We thus identified 4 mutants classified as *hopA1-susceptible* (*hps*) that

bred true and showed consistent chlorosis to DC3000(*hopA1*). Back-crossing *hps* mutants to RLD and to each other established that the mutations were recessive and fell into 3 complementation groups (Table II-I). In this study we focused on the *HPS1* locus, which was represented by two mutant alleles. As shown below, *HPS1* encodes an R protein, and for clarity we will refer to *HPS1* as *RPS6* henceforth in accordance with the Arabidopsis *R* gene nomenclature.

Map-Based Cloning of *RPS6*

The *rps6-1* and *rps6-2* mutants were susceptible to DC3000 and DC3000(*hopA1*) (Figure II-1). Direct sequencing of the *EDS1* gene eliminated the possibility that the *rps6* mutants represented *eds1* alleles (data not shown). We proceeded with a map-based cloning approach to identify *RPS6*. An out-cross of *rps6-1* to Col-0 established a mapping population in which the susceptible phenotype also segregated as a recessive trait (data not shown). Susceptible F2 plants were identified by syringe-inoculation with DC3000(*hopA1*). An initial set of 21 CAPS or microsatellite markers (Bell and Ecker, 1994) distributed over the 5 *Arabidopsis* chromosomes and analysis of chromosome break-points placed the *RPS6* locus on the bottom of chromosome 5 between the markers RPS4 and NGA129 (Table II-2). For fine scale mapping, approximately 300 *hopA1*-susceptible F2 plants were selected, and additional mapping markers were identified by testing SSLP and CAPS markers at bacterial artificial chromosome (BAC) ends (Table II-3). The *RPS6* locus was confined between markers MUGCAPS05 and MUGSSLP07, a 0.35 Mbp interval (Figure II-2A).

Table II-1. Genetic analysis of *hps* mutants

Cross	Resistant[a]	Susceptible[a]	χ^2
rps6-1 x RLD	71	26	0.168 ($P > 0.5$)[b]
RLD x *hps2-1*	36	13	0.061 ($P > 0.7$)[b]
rps6-2 x *rps6-1*	0	34	43.7 ($P < 0.001$)[c]
rps6-2 x *hps3-1*	17	15	0.127 ($P > 0.7$)[c]
rps6-2 x *hps2-1*	18	16	0.151 ($P > 0.5$)[c]
hps2-1 x *hps3-1*	22	16	0.042 ($P > 0.8$)[c]

[a] *hps* mutants were crossed to wild-type RLD and to each other. Plants from F2 populations were scored as resistant or susceptible 5 days after inoculation with DC3000(*hopA1*).

[b] χ^2 value for expected ratio of 3 resistant : 1 susceptible.

[c] χ^2 value for expected ratio of 9 resistant : 7 susceptible.

Table II-2. Recombination frequency between *rps6-1* and genetic markers on chromosome 5

Marker[a] (cM)	Recombinant chromosomes	Total number of chromosomes	Recombination frequency (%)
NGA225 (14.31)	27	92	29.3
NGA249 (23.72)	40	128	31.3
NGA139 (50.48)	30	92	32.6
RPS4 (90)	12	106	11.3
NGA129 (105.4)	15	108	13.9
M555 (132.6)	27	92	29.3

[a]Other CAPS or SSLP markers that displayed no linkage to *rps6-1* were NGA63, CIW12, NGA280, and ATPASE (chromosome 1); NGA1145, GPA1 and NGA168 (chromosome 2); NGA32, NGA162, GAPA, and NGA6 (chromosome 3) ; and NGA8, DET1.2, NGA1139 and NGA1107 (chromosome 4).

Table II-3. *RPS6* fine-mapping markers on the bottom of chromosome 5

Marker name	Start position (bp)	PCR primer sequences (5' → 3')	Primer position in bp (annotation unit)	Restr. enzyme	DNA fragment sizes in bp (Col-0 / RLD)
MUG SSLP05	18597126	CTCATTGAATTAATTTACTTTGTG CAACTAATTTTAGTGATTCGGA	83945-83968 84058-84079 (MRA19)	n.a	135/123
MUG SSLP06	18738437	ACTCCGGCGGTAGAGAATC TTTGTCCTCTGTTCGTTCGT	78886-78904 79011-79030 (MCL19)	n.a	145/135
MUG CAPS05	18752769	GAATCATGAGCATCTGGTCA GATCAGGCTAGACAAAGAGA	8708-8727 9278-9297 (MDE13)	*Hind*III	140+450 /590
MUG SSLP07	19109932	TCGCC TAATTCTCCT CAAAT GTAGTTG GTTTAACGGGAAAA	61206-61225 61344-61364 (MQD22)	n.a	159/145
MUG SSLP08	19167128	ACGTGGAGGAATACTGGAGC GATTGCAG TTGGGCTTAACA	31742-31761 31943-31962 (K14A3)	n.a	221/121
MUG CAPS06	19587134	ACCCAATCTTGGCCCAGAAT CAATGGCGTCTGTATCTGCA	2151-2170 2567-2586 (K23F3)	*Alu*I	436/ 268+168

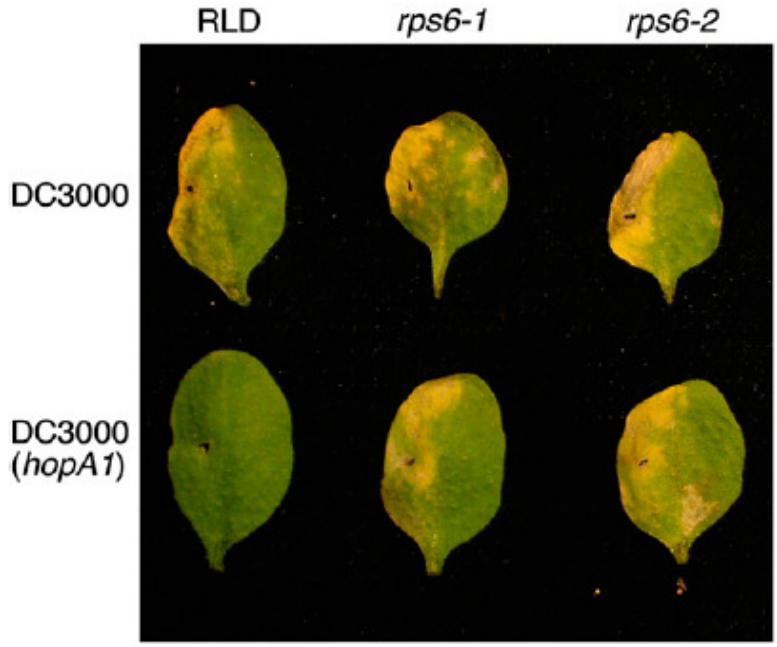

Figure II-1

Figure II-1. *rps6* mutants are susceptible to DC3000(*hopA1*). Disease symptoms of parental RLD (left), *rps6-1* (middle) and *rps6-2* (right) syringe-inoculated with DC3000 (top row) or DC3000(*hopA1*) (bottom row). Leaves infiltrated with DC3000 and DC3000(*hopA1*) came from the same plant and remained attached until disease symptoms (leaf chlorosis) were recorded 5 days after inoculation. Only the left halves of leaves were infiltrated.

Based on the physical location of putative Arabidopsis resistance genes (Meyers et al., 2003), 7 TNL genes on the two BAC clones MPL12 and K11I1 were identified within the genomic interval defined by MUGCAPS05 and MUGSSLP07 (Figure II-2A). Because *hopA1*-triggered immunity required *EDS1*, we speculated that the *hopA1*-specific *R* gene belongs to the TNL class. To test this directly, we cloned the Col-0 wild-type alleles of these 7 TNL genes from BAC clones K11I1 and MPL12 and tested for complementation of *rps6-1* in stable transgenic lines (see "Materials and Methods"). Among the 7 candidate genes, only the pSHK103 construct containing At5g46470 changed the *rps6* mutant susceptible phenotype to the parental *hopA1*-resistant phenotype (Figure II-2B). *In planta* bacterial growth curve assays were performed to quantify the level of resistance in complemented plants. The growth of virulent DC3000 was similar in all plants tested. In contrast, transgenic *rps6-1* lines in the T3 generation containing the At5g46470 gene were as resistant as parental RLD plants and had aproximately 100-fold lower bacterial growth than *rps6-1* mutant plants (Figure II-2C). Disease assays with these lines also showed complementation by At5g46470 (Figure II-2D). The lines shown in these figures were representative of a total of 5 out of 5 independent single-locus transgenic lines followed to the T3 generation. We sequenced the RLD wild-type and *rps6-1* and *rps6-2* mutant alleles of At5g46470 and identified a unique base change in each of the mutants (see below). Based on complementation of *rps6-1* by At5g46470 and the identification of mutations in this gene in the mutants, we concluded that we had cloned *RPS6*.

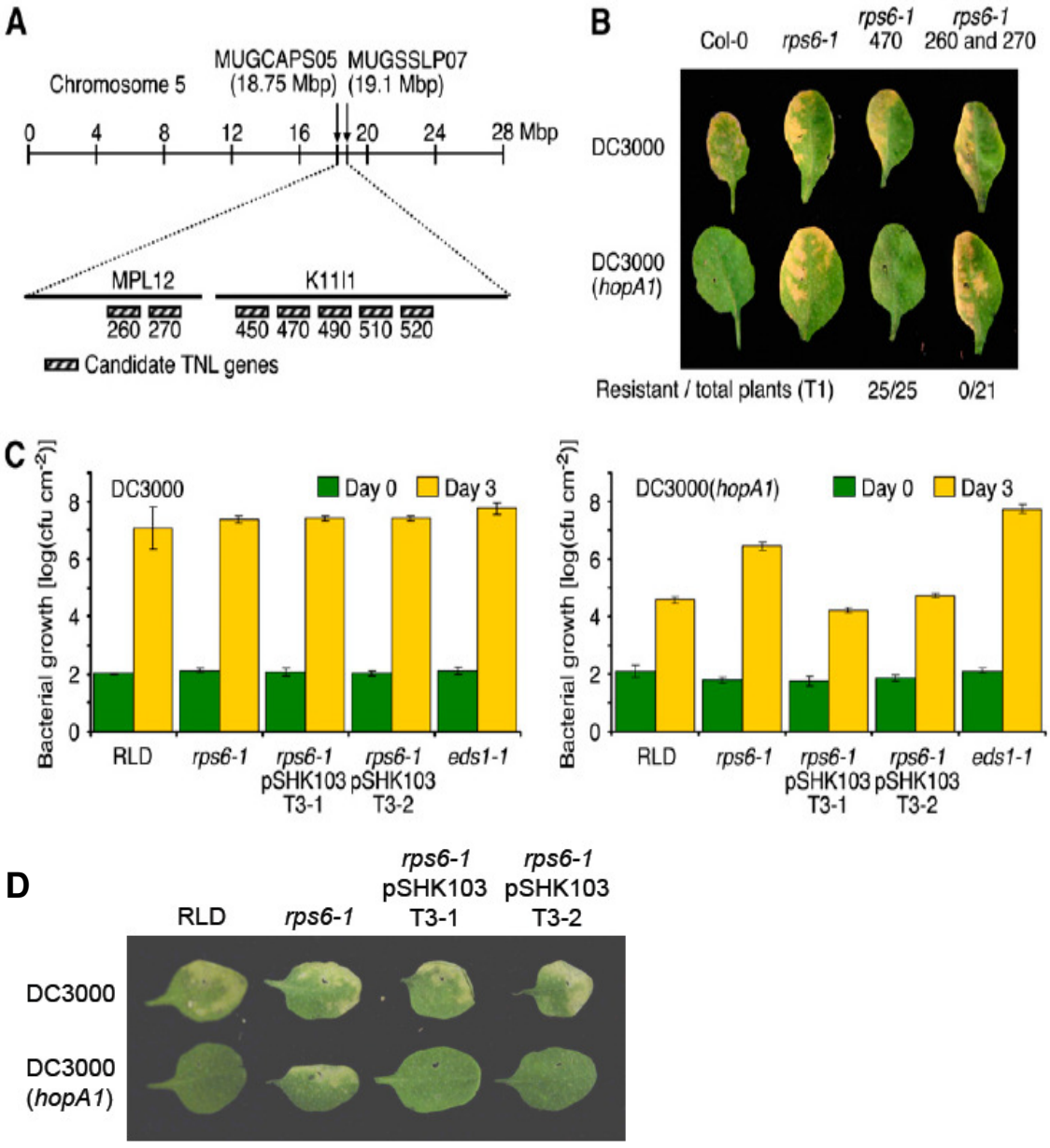
A
Chromosome 5
MUGCAPS05 (18.75 Mbp)
MUGSSLP07 (19.1 Mbp)
0 4 8 12 16 20 24 28 Mbp
MPL12
K11I1
260 270
450 470 490 510 520
Candidate TNL genes
B
Col-0
rps6-1
rps6-1 470
rps6-1 260 and 270
DC3000
DC3000 (hopA1)
Resistant / total plants (T1) 25/25 0/21
C
DC3000
DC3000(hopA1)
Day 0
Day 3
Bacterial growth [log(cfu cm-2)]
RLD
rps6-1
rps6-1 pSHK103 T3-1
rps6-1 pSHK103 T3-2
eds1-1
D
RLD
rps6-1
rps6-1 pSHK103 T3-1
rps6-1 pSHK103 T3-2
DC3000
DC3000 (hopA1)

Figure II-2

Figure II-2. Map-based cloning of *RPS6*. **(A)** Schematic diagram of the *RPS6* locus on chromosome 5. Two genetic markers confining *RPS6*, MUGCAPS05 and MUSSLP05, are indicated by arrows (top). Seven candidate TNL genes located on BAC clones MPL12 and K11I1 within the *RPS6* locus are indicated by hatched bars (bottom). The numbers 260, 270, 450, 470, 490, 510 and 520 represent At5g46260, At5g46270, At5g46450, At5g46470, At5g46490, At5g46510 and At5g46520, respectively. **(B)** Representative complementation assays of *rps6-1* transformed with BAC subclones containing TNLs At5g46470 (470) or At5g46260 and At5g46270 (260 and 270). T1 plants were inoculated with DC3000 (top) or DC3000(*hopA1*) (bottom) at a density of 10^6 colony forming units (cfu)/mL. The number of resistant T1 plants are indicated below the figure. Disease symptoms were recorded 5 days after inoculation. **(C)** *In planta* bacterial growth of DC3000 (left) and DC3000(*hopA1*) (right) on day 0 (green bars) and day 3 (yellow bars) after inoculation of indicated plant lines with bacteria at 5×10^4 cfu/mL. Values represent averages of cfu/cm^2 leaf tissue from triplicate samples, and error bars denote standard deviation. This experiment was repeated twice with similar results. **(D)** Complementation of *rps6-1* by At5g46470. Disease symptoms of parental RLD, *rps6-1* and two transgenic *rps6-1* lines containing pSHK103 syringeinoculated with DC3000 (top row) or DC3000(*hopA1*) (bottom row) at a bacterial density of 10^6 cfu/mL. Leaves infiltrated with DC3000 and DC3000(*hopA1*) came from the same plant and remained attached until disease symptoms (leaf chlorosis) were recorded 5 days after inoculation. Only the upper halves of leaves were infiltrated.

RPS6 Encodes a TNL Protein

Gene models for *RPS6* were inconsistent between TAIR7 and MIPS, and included an extensive intron-containing 3'-untranslated region. We experimentally verified the *RPS6* gene model for Col-0 and RLD using reverse transcription PCR and 3'-Rapid Amplification of cDNA Ends (RACE). This showed that the *RPS6* transcript is approximately 5.8 kb long and contains 9 exons (Figure II-3A), indicating that neither gene model in the databases at the time was correct. Compared to the updated gene model for At5g46470 in the TAIR8 release, most 3'-RACE products provided evidence for poly-adenylation occurring in exon 9. One product from Col-0 contained a poly-A tail in exon 8, and one RLD product a poly-A tail within what is annotated as intron 9 in TAIR8. We found no experimental evidence in multiple 3'-RACE products from either Col-0 or RLD for intron 10 and exon 11. Based on data in the Genevestigator database (https://www.genevestigator.ethz.ch/at/), the Bio-Array Resource for Arabidopsis Functional Genomics (http://bar.utoronto.ca/) and the AtGenExpress Visualization Tool (http://jsp.weigelworld.org/expviz/expviz.jsp), *RPS6* expression is found at very low levels throughout the plant at all stages and is not strongly regulated by various treatments, including pathogens.

RPS6 encodes a protein of 1127 amino acids (Figure II-3B) that falls into the TNL-F clade of R proteins (Meyers et al., 2003). Based on amino acid sequence comparisons using the BLAST algorithm (Altschul et al., 1997), among resistance proteins of known function RPS6 shows highest similarity to the Arabidopsis TNL protein RAC1 that determines resistance to the oomycete

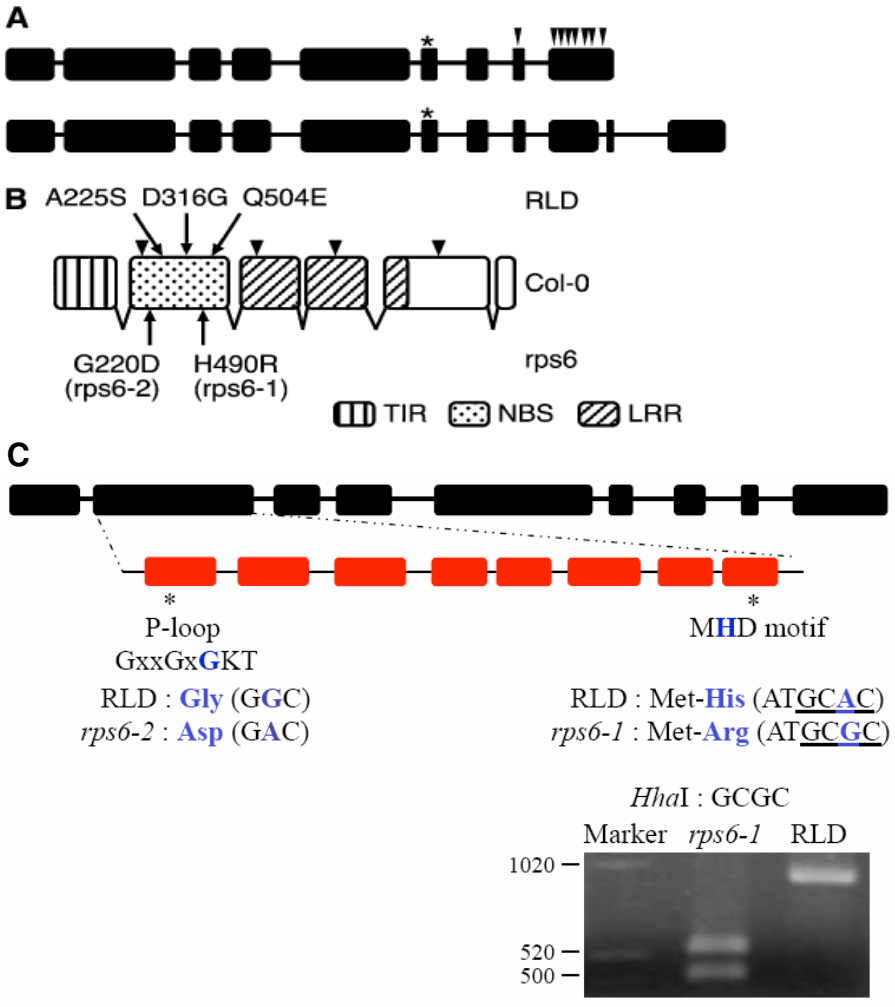
A
B A225S D316G Q504E
RLD
Col-0
rps6
G220D
(rps6-2)
H490R
(rps6-1)
TIR NBS LRR
C
P-loop
GxxGxGKT
RLD : Gly (GGC)
rps6-2 : Asp (GAC)
MHD motif
RLD : Met-His (ATGCAC)
rps6-1 : Met-Arg (ATGCGC)
HhaI : GCGC
Marker
rps6-1
RLD
1020
520
500

Figure II-3

Figure II-3. *RPS6* encodes a member of the TNL class of R proteins. **(A)** *RPS6* gene model as experimentally verified by reverse transcription PCR and 3'-RACE (top) compared with the TAIR8 gene model (bottom). Exons are indicated by black boxes, introns by lines, and stop codons by asterisks. Positions of poly-adenylation sites as determined by 3'-RACE products from RLD and Col-0 are indicated by arrowheads. **(B)** Schematic diagram of the RPS6-Col protein. Amino acid and silent nucleotide polymorphisms in RLD are shown by arrows and arrowheads, respectively, above the diagram. Both *rps6-1* and *rps6-2* contain missense mutations in *RPS6-RLD*. Amino acid substitutions in the predicted rps6 mutant proteins are indicated by arrows below the diagram. **(C)** Point mutations in *rps6-1* and *rps6-2.* (top) Gene model for *RPS6* (Black). (middle) DNA and deduced amino acid sequence of *RPS6* in NBS domain. Red boxes indicate NB-ARC motifs. Mutated bases and their corresponding amino acids are indicated in blue. (bottom) *rps6-1* mutation visualized by CAPS marker. PCR products were digested with *Hha*I. Approximate band sizes are indicated in bp. Primers used were 5′- GTGGGAGTAGAATCATTGTG -3′ and 5′- AGCAGAGCAAACTTGAGAAG -3′.

pathogen *Albugo candida* (Borhan et al., 2004). Sequencing the RLD wild-type allele identified several silent and amino acid polymorphisms between the two functional *RPS6* alleles from Col-0 and RLD (Figure II-3B). The *rps6-1* allele has an A to G transition at base number 1563 (numbering according to the TAIR8 genomic sequence annotation), which changes a highly conserved His within the hxhHD motif in the NBS (Takken et al., 2006) to Arg at amino acid position 490. The base pair change in *rps6-1* can be visualized by a CAPS marker (Figure II-3C). In *rps6-2*, a G to T transversion at base number 753 changes Gly at position 220 within the highly conserved Walker A or P-loop motif (GxxxxGKS/T) to Asp.

RPS6 is Alternatively Spliced

In the course of verifying the *RPS6* gene model, we obtained evidence for alternative splicing. Reverse transcription PCR with primers flanking intron 1 and intron 2, respectively, produced more than one band (Figure II-4, A and B). We cloned and sequenced the resulting PCR products and found that apart from the strong band representing the regular transcript with introns 1 and 2 spliced out, the upper bands represent transcripts with retained introns (Figure II-4, B and C). Control reactions without reverse transcriptase showed that these bands did not arise from genomic DNA contamination (Figure II-4B). In addition, the shorter PCR product with primers flanking intron 2 represented transcripts in which a cryptic intron in exon 2 was spliced out (Figure II-4, B and C). Because of in-frame stop codons in introns 1 and 2, and a frame shift with splicing of the cryptic intron, all three alternative transcripts encode severely truncated TIR-only or TN

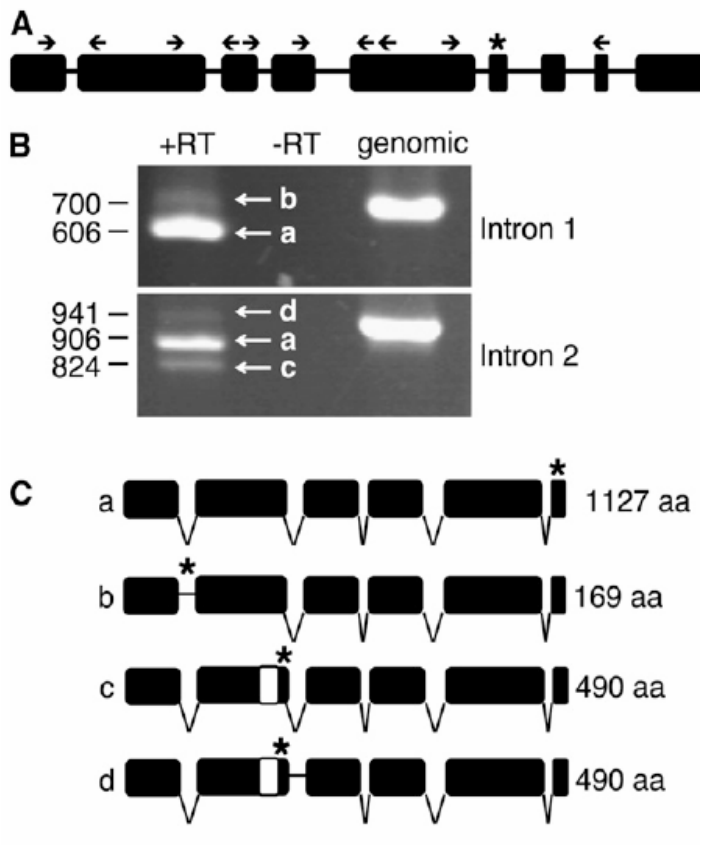
A
B
+RT
-RT
genomic
700
606
b
a
Intron 1
941
906
824
d
a
c
Intron 2
C
a
1127 aa
b
169 aa
c
490 aa
d
490 aa

Figure II-4

Figure II-4. Alternative splicing of *RPS6*. **(A)** Schematic diagram of the *RPS6* gene structure. PCR primer pairs flanking introns are indicated by black arrows above the diagram. **(B)** PCR with primers flanking introns 1 (top) and 2 (bottom) yield multiple products, indicated by white arrows. Reverse transcription prior to PCR was performed with total RNA from RLD and an oligo-dT primer in the presence (+RT) or absence (-RT) of reverse transcriptase. In the right lane, genomic DNA was used as a template for direct size comparison. Numbers to the left denote product sizes in base pairs as determined by sequencing. **(C)** Schematic diagrams of *RPS6* transcripts produced by alternative splicing based on sequencing of PCR products shown in B. Retained introns are shown as thick lines, and spliced introns are indicated by diagonal lines. The cryptic intron within exon 2 is shown as a white box. Asterisks indicate predicted stop codons of full-length and truncated *RPS6* open reading frames. The amino acid lengths of the corresponding predicted RPS6 proteins are shown to the right of the diagrams.

proteins. No other sections of the *RPS6* transcript provided evidence for alternative splicing.

HopA1 Distribution in *P. syringae* Strains

The original predicted HopA1 (HrmA) amino acid sequence from *P. syringae* pv. syringae strain 61 was replaced when pHIR11 was reannotated (Ramos et al., 2007), yet unflagged database entries for the outdated HopA1 sequence still exist in GenBank and the *P. syringae* Hop database (http://www.pseudomonas-syringae.org). We therefore sequenced *hopA1* from pML123-based plasmid pLN92 that we used in our disease assays (van Dijk et al., 2002; Gassmann, 2005). This plasmid contains *shcA-hopA1* from pHIR11, the original source of *hopA1* sequence (Heu and Hutcheson, 1993; Alfano et al., 1997). Sequencing independently verified that the genomic and predicted amino acid sequence of *hopA1* on pLN92 is identical to the updated sequence (GenBank accession number AAF71481.2), and is also identical to the reported *hopA1* sequence of *P. syringae* pv. syringae strain 226 (Deng et al., 2003). Protein sequence database searches using the BLAST algorithm (Altschul et al., 1997) identified several predicted HopA1 proteins in other *P. syringae* pathovars and strains, including pathovars tomato, viridiflava, syringae, atrofaciens and morsprunorum.

Interestingly, DC3000 contains HopA1, and genome sequencing of *P. syringae* pv. tomato strain T1 provides evidence for a *hopA1* pseudogene (Almeida et al., 2009). This gene would encode a virtually identical HopA1 protein as in DC3000 except for a premature stop codon at amino acid position

55. We aligned the HopA1 sequence from *P. syringae* pv. syringae strain 61 and DC3000 to determine conserved and diverged amino acids that may be the basis for virulence of DC3000 on Arabidopsis. The two amino acid sequences are 57% identical, and diverged amino acids are distributed throughout the proteins (Figure II-5). Secondary structure and hydrophobicity analyses also did not identify striking differences between the proteins. We conclude that in-depth structure-function analysis will be required to identify the critical changes in $HopA1_{DC3000}$ that prevent RPS6 activation.

Mutations in *SRFR1* Enhance HopA1-Triggered Resistance

SRFR1 is a negative regulator of ETI identified by a suppressor screen for *avrRps4*-specific resistance enhancement in the naturally *rps4*-mutant accession RLD (Kwon et al., 2004). A major open question regarding *srfr1*-mediated resistance is whether mutations in *SRFR1* also enhance resistance to effector genes other than *avrRps4*. Because RLD is resistant to bacteria expressing other known bacterial effector genes, we first addressed this question by crossing *srfr1* mutants to mutants in the corresponding *R* genes *RPS2* and *RPM1*, with negative results (data not shown). These tested *R* genes differ from *RPS4* in that they encode CNL proteins and do not require *EDS1* for function, whereas *srfr1*-mediated resistance requires *EDS1* (Kwon et al., 2009). Although several *RPP* genes that confer resistance to *H. parasitica* encode TNL proteins and require *EDS1*, RLD is an accession which shows resistance to all of the commonly used laboratory isolates of *H. parasitica* (John M. McDowell, personal communication).

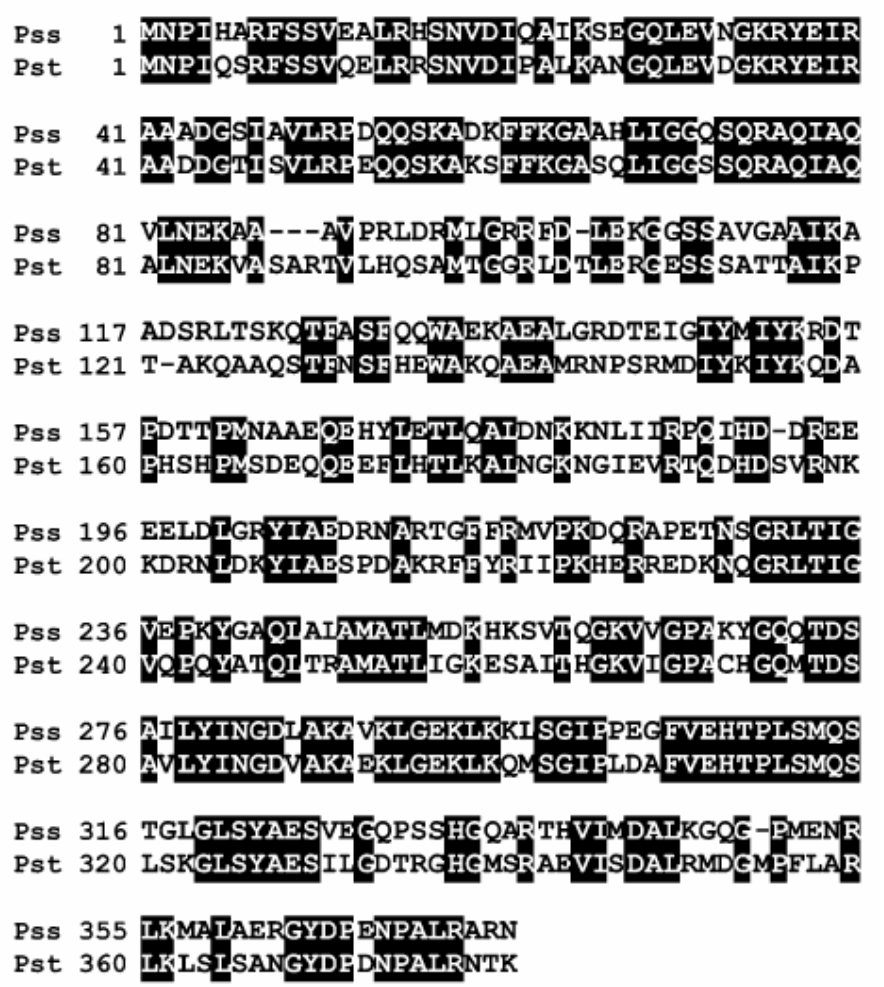
Pss 1 MNPIHARFSSVEALRHSNVDIQAIKSEGQLEVNGKRYEIR
Pst 1 MNPIQSRFSSVQELRRSNVDIPALKANGQLEVDGKRYEIR
Pss 41 AAADGSIAVLRPDQQSKADKFFKGAAHLIGGQSQRAQIAQ
Pst 41 AADDGTISVLRPEQQSKAKSFFKGASQLIGGSSQRAQIAQ
Pss 81 VLNEKAA---AVPRLDRMLGRRFD-LEKCGSSAVGAAIKA
Pst 81 ALNEKVASARTVLHQSAMTGGRLDTLERCESSSATTAIKP
Pss 117 ADSRLTSKQTPASEQQWAEKAEALGRDTEIGIYMIYKRDT
Pst 121 T-AKQAAQSTFNSFHEWAKQAEAMRNPSRMDIYKIYKQDA
Pss 157 PDTTPMNAAEQEHYLETLQALDNKKNLIDRPQIHD-DREE
Pst 160 PHSHPMSDEQQEEFLHTLKAINGKNGIEVRTQDHDSVRNK
Pss 196 EELDLGRYIAEDRNARTGFFRMVPKDQRAPETNSGRLTIG
Pst 200 KDRNLDKYIAESPDAKREFYRIIPKHERREDKNQGRLTIG
Pss 236 VEPKYGAQLAIAMATLMDKHKSVTQGKVVGPAKYGQQTDS
Pst 240 VQFQYATQLTRAMATLIGKESAITHGKVIGPACHGQMTDS
Pss 276 AILYINGDLAKAVKLGEKLKKLSGIPPEGFVEHTPLSMQS
Pst 280 AVLYINGDVAKAEKLGEKLKQMSGIPLDAFVEHTPLSMQS
Pss 316 TGLGLSYAESVEGQPSSHGQARTHVIMDALKGQG-PMENR
Pst 320 LSKGLSYAESILGDTRGHGMSRAEVISDALRMDGMPFLAR
Pss 355 LKMALAERGYDPENPALRARN
Pst 360 LKLSLSANGYDPDNPALRNTK

Figure II-5

Figure II-5. Protein sequence alignment of HopA1 from *P. syringae* pv. syringae strain 61 (Pss, top) and *P. syringae* pv. tomato strain DC3000 (Pst, bottom). Sequences were aligned with ClustalW using the Gonnet 250 amino acid weight matrix. Identical amino acid residues are shaded black.

The molecular characterization of RLD *rps6* mutants therefore enabled us to test the spectrum of *srfr1*-mediated resistance in a uniform genetic background with an effector gene that signals through the *EDS1* pathway.

We crossed *srfr1-1* to *rps6-1* and identified double-homozygous mutant plants with allele-specific CAPS markers. *In vivo* bacterial growth assays showed that mutations in *SRFR1* did enhance HopA1-triggered resistance in *rps6-1* (Figure II-6). While virulent DC3000 grew to comparable high levels in all plant lines, DC3000(*hopA1*) growth was restricted to levels approximately 1000-fold lower in resistant RLD and *srfr1-1*, both of which have a functional *RPS6* gene. Similar to Figure II-2C, *rps6-1* was not fully susceptible compared to growth of virulent DC3000. In *srfr1-1 rps6-1* double mutants, growth of DC3000(*hopA1*) was consistently restricted to levels 10-fold less compared to *rps6-1*. While the apparent effect of mutations in *SRFR1* on *avrRps4*-triggered resistance in the *rps4* mutant RLD can be larger (50- to 100-fold lower DC3000(*avrRps4*) levels on *srfr1-1* than on RLD) (Kwon et al., 2004), the main difference is the full susceptibility of RLD to DC3000(*avrRps4*) compared to the partial susceptibility of *rps6-1* to DC3000(*hopA1*). In both cases, mutations in *SRFR1* led to a 100-fold reduction in pathogen growth with the effector gene present compared to full susceptibility as measured with virulent DC3000 (Figure II-6) (Kwon et al., 2004). Resistance to DC3000(*avrRps4*) in *srfr1* mutants was proposed to depend on a second *EDS1*-dependent *R* gene (Kwon et al., 2004). To test whether this second *R* gene is *RPS6* we measured growth of DC3000(*avrRps4*) in *srfr1-1 rps6-1* double mutants. However, growth of DC3000(*avrRps4*) in *srfr1-1 rps6-1*

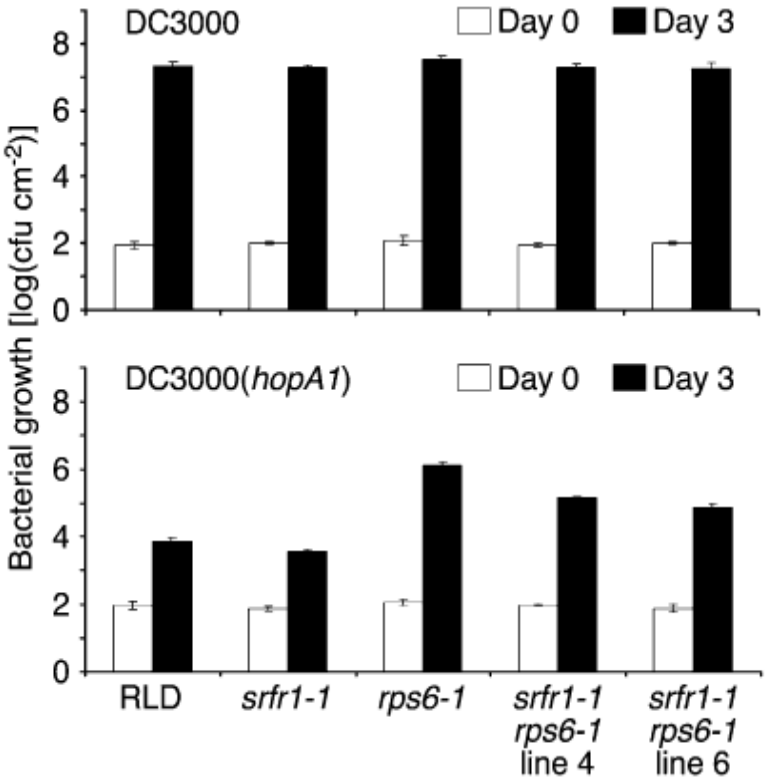
DC3000
Day 0
Day 3
DC3000(hopA1)
Day 0
Day 3
Bacterial growth [log(cfu cm-2)]
0
2
4
6
8
RLD
srfr1-1
rps6-1
srfr1-1 rps6-1 line 4
srfr1-1 rps6-1 line 6

Figure II-6

Figure II-6. Mutations in *SRFR1* enhance *hopA1*-triggered immunity in *rps6* mutants. *In planta* bacterial growth was measured in RLD, *srfr1-1*, *rps6-1* and two independent F3 *srfr1-1 rps6-1* double mutants on day 0 (white columns) and day 3 (black columns) after inoculation with DC3000 (top) and DC3000(*hopA1*) (bottom). Plants were inoculated with a bacterial suspension at a density of 5×10^4 cfu/mL. Values represent averages of cfu/cm^2 leaf tissue from triplicate samples, and error bars denote standard deviation. This experiment was repeated three times with similar results.

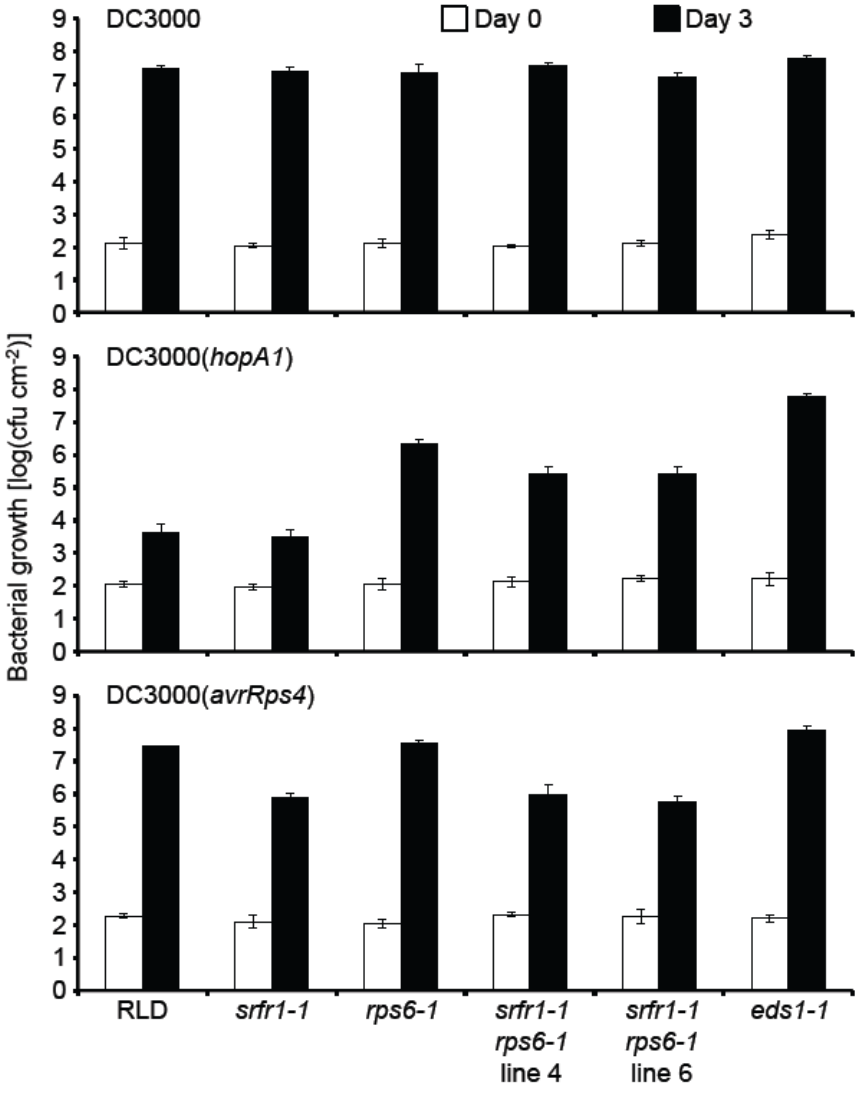
DC3000
Day 0
Day 3
DC3000(*hopA1*)
DC3000(*avrRps4*)
Bacterial growth [log(cfu cm-2)]
0
1
2
3
4
5
6
7
8
9
RLD
srfr1-1
rps6-1
srfr1-1 rps6-1 line 4
srfr1-1 rps6-1 line 6
eds1-1

Figure II-7

Figure II-7. Mutations in *RPS6* do not affect *srfr1*-mediated resistance to DC3000(*avrRps4*). *In planta* bacterial growth was measured in RLD, *srfr1-1*, *rps6-1*, two independent F3 *srfr1-1 rps6-1* double mutants and *eds1-1* on day 0 (white columns) and day 3 (black columns) after inoculation with DC3000 (top), DC3000(*hopA1*) (middle) and DC3000(*avrRps4*). Plants were inoculated with a bacterial suspension at a density of 5×10^4 cfu/mL. Values represent averages of cfu/cm^2 leaf tissue from triplicate samples, and error bars denote standard deviation. This experiment was repeated once with similar results.

double mutants did not increase compared to growth in the *srfr1-1* single mutant (Figure II-7). Conversely, *RPS4* cannot be providing partial resistance to DC3000(*hopA1*) in the *rps6-1* single or the *srfr1-1 rps6-1* double mutants because all these lines in the RLD background lack functional *RPS4*.

Discussion

Here we report the cloning of *RPS6*, a TNL protein-encoding gene that governs resistance to *P. syringae* strains expressing HopA1 from *P. syringae* pv. syringae strain 61. While many *R* genes have been cloned from many plant species, *RPS6* is only the second TNL gene that interacts with a known *P. syringae* effector. The importance of using isogenic strains both on the plant and pathogen side is illustrated by many studies comparing CNL *R* gene signaling pathways. Similar comparisons are now possible between *RPS4* and *RPS6*.

Characterization of *RPS6*

We isolated *RPS6* using a forward genetic screen and map-based cloning. The *rps6-1* allele had a missense mutation in the NBS domain that changes a highly conserved His within the hxhHD motif to Arg. Interestingly, a change of the equivalent His to Ala in the flax TNL protein L6 leads to constitutive activation of the R protein (Howles et al., 2005), while rps6-1 is largely non-functional. The hxhHD motif is proposed to bind the β-phosphate of ATP (Takken et al., 2006).

Introduction of a stable positive charge in this motif as in rps6-1 may lead to tight ATP binding and prevention of nucleotide turn-over and activation, or it may destabilize the protein. In the *rps6-2* allele, a missense mutation changes Gly at position 220 within the highly conserved Walker A or P-loop motif (GxxxxGKS/T) to Asp. Mutations in the P-loop usually reduce ATP binding by the NBS (Takken et al., 2006), and in the specific cases of Rx and N prevent association of the N- and C-terminus (Moffett et al., 2002) and oligomerization (Mestre and Baulcombe, 2006), respectively.

RPS6 is found in a cluster of seven *R* genes on the bottom of chromosome 5. The closest sequence similarity to a known R protein exists with the oomycete resistance protein RAC1 on chromosome 1 from the Arabidopsis accession Ksk-1 (Borhan et al., 2004). In Col-0, the closest relative of RPS6 among known R proteins is the oomycete R protein RPP4. It is a common observation that no correlation exists between R protein structures and pathogen type. This finding is highlighted by recent observations that oomycete effectors delivered by bacterial pathogens are as potent in restricting bacterial colonization as bacterial effectors when the cognate *R* gene is present, and also as potent in promoting bacterial colonization when the *R* gene is absent (Sohn et al., 2007; Rentel et al., 2008). The ease with which bacterial isogenic strains can be generated is therefore an advantage to study specific and shared principles of plant ETI signaling in response to any pathogen.

Alternative Splicing of *R* Genes

A hallmark of TNL genes from many plant species is that they generate more than one transcript by alternative splicing. While the mechanism of alternative splicing varies, these alternative transcripts usually encode TN proteins (Jordan et al., 2002; Gassmann, 2008). A first indication that these alternative transcripts provide a crucial function came from the observation that intronless cDNAs of the tobacco *N* gene were non-functional (Dinesh-Kumar and Baker, 2000). Similarly, intronless *Arabidopsis RPS4* transgenes, despite being expressed, failed to complement an *rps4* mutant line (Zhang and Gassmann, 2003). Combining full-length and truncated cDNAs that mimic the prevalent *RPS4* transcripts provided resistance however, demonstrating directly that a combination of *RPS4* transcripts is required for function. Both *N* and *RPS4* alternative transcript levels are temporally regulated after stimulus perception (Dinesh-Kumar and Baker, 2000; Zhang and Gassmann, 2007), suggesting that alternative splicing is fine-tuned to optimally regulate the plant innate immune response. The Arabidopsis genome also contains transcriptionally active TIR-only and TN protein-encoding genes that do not seem to have arisen by deletion or degeneration of the LRR-encoding region, but the function of these genes remains unknown (Meyers et al., 2002; Tan et al., 2007).

While *RPS4* and *RPS6* share the feature of alternative splicing, the transcript structures differ. The predominant *RPS4* alternative transcripts contain intron 2 or 3, or a combination of a spliced intron 2 and of an adjacent cryptic intron in exon 3 (Zhang and Gassmann, 2007). We did not detect retention of intron 3 in *RPS6*, but instead detected transcripts in which a cryptic intron in exon

2 is spliced, with or without retention of the adjacent intron 2. In addition, *RPS6* resembles *RAC1* in that intron 1 is retained in some alternative transcripts (Borhan et al., 2004). All three major *RPS6* alternative transcripts have premature stop codons and encode truncated TIR-only or TN proteins. The predicted TN protein lacks 78 C-terminal amino acids in the NBS domain encoded by exon 2, while the frame-shift adds 40 new amino acids between the 5'-splice junction of the cryptic intron and the premature stop codon. Both truncated proteins are possibly potent inducers of plant defense, as was shown for TIR and TN proteins of RPS4 and RPP1A (Zhang et al., 2004; Zhang and Gassmann, 2007; Swiderski et al., 2009). In addition to alternative splicing that changes the open reading frame of *RPS6*, the unusually long and intron-containing 3'-UTR of *RPS6* may function in regulating mRNA stability and RPS6 accumulation.

SRFR1 **Regulates HopA1-triggered Responses**

SRFR1 was identified in a suppressor screen for RLD mutants with enhanced resistance to DC3000(*avrRps4*). Because *srfr1* mutants were fully susceptible to virulent DC3000 and did not show evidence of constitutive defense activation, *SRFR1* was proposed to function as a negative regulator of ETI. However, beyond *avrRps4* the spectrum of resistance specificities regulated by *SRFR1* remained unclear. As a first indication of the value of comparing *RPS4* and *RPS6*, we show here that in *srfr1-1 rps6-1* double mutants resistance to DC3000(*hopA1*) is enhanced to a comparable degree as resistance in *srfr1 rps4*

mutants to DC3000(*avrRps4*). This suggests a more general role of *SRFR1* in regulating Arabidopsis resistance responses. To date this more general role is limited to the *EDS1* pathway, since in a first analysis resistance to DC3000 expressing avirulence genes that in the wild-type signal through *EDS1*-independent CNL proteins was not affected by mutations in *SRFR1*. *EDS1* encodes a lipase-like protein, but to date no enzymatic function has been described (Wiermer et al., 2005). In addition, the exact mechanistic function of EDS1 in regulating R protein-mediated responses is unclear. The rigorous genetic analysis presented here showing that *SRFR1* affects two separate *EDS1*-dependent resistance specificities, together with the observation that *srfr1*-mediated resistance itself is *EDS1*-dependent, warrant closer inspection of the relationship between *EDS1* and *SRFR1*.

In conclusion, we have cloned *RPS6*, a TNL protein-encoding gene, and show the utility of a second *P. syringae* resistance specificity that signals through the *EDS1* pathway. Together with the demonstrated ability to transfer oomycete effectors to DC3000 (Sohn et al., 2007; Rentel et al., 2008), the isolation of RPS6 will contribute to the evaluation of novel TNL pathway components and the characterization of TNL protein-mediated resistance in general. In addition, while *hopA1* does not belong to the highly conserved set of effectors found in all *P. syringae* pathovars, it is fairly widely distributed. The observation that $hopA1_{Pss61}$ triggers ETI in tobacco and several other *Nicotiana* species (Alfano et al., 1997) and in all Arabidopsis accessions tested to date (Gassmann, 2005), together with the finding that *hopA1* in *P. syringae* pv. tomato T1 is a pseudogene (Almeida et

al., 2009), suggests that *hopA1* may contribute to host range specificity. The virulence function of HopA1 is currently unknown. The evolution of *hopA1* and whether $HopA1_{Pss61}$ and $HopA1_{DC3000}$ have different virulence targets in Arabidopsis warrant further study.

Materials and Methods

Bacterial Strains, Disease Assays and Screens

Pseudomonas syringae pv. tomato strain DC3000 containing the empty vector pML123 (Labes et al., 1990), or expressing *hopA1* from *P. syringae* pv. syringae strain 61 on plasmid pLN92 (van Dijk et al., 2002) were grown as described previously (Gassmann, 2005). DC3000 *shcA-hopA1* was cloned by PCR using genomic DC3000 DNA as template and the primers 5'-CCTGGTAATACCCGCAAATC-3' and 5'-ACTTCACGCCAGCCAAAGGA-3'. The PCR product was subcloned into vector pGEM-T Easy (Promega) and verified by sequencing. Flanking pGEM-T Easy *Eco*RI sites were used to clone the insert into pML123 to generate vector pSHK104, and correct orientation of the insert was verified by restriction digest. Vector pSHK104 was mobilized into DC3000 by triparental mating as described (Gassmann, 2005).

For disease assays, Arabidopsis plants were grown in a Conviron GR48 walk-in (dip-inoculations) or an E-7/2 reach-in (syringe-infiltrations) growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) under an

8 h light/16 h dark cycle at 24°C, 70% relative humidity and a light intensity of 90-140 μmol photons m^{-2} s^{-1}. For dip-inoculations, rosettes of 3-4 week old plants were dipped in a bacterial suspension of 4×10^8 colony-forming units (cfu)/mL in 10 mM $MgCl_2$ and 0.01% of the surfactant Silwet L77. Disease symptoms (chlorosis) were scored 5 days post inoculation on a scale of 0 to 5 (0 being no symptoms and 5 being extreme chlorosis). For disease assays by syringe infiltration, leaves of 5-week old plants were infiltrated with a bacterial suspension of 1×10^6 cfu/mL in 10 mM $MgCl_2$ using a 1 ml needless syringe. *In planta* bacterial growth assays were performed by syringe infiltration as described (Gassmann, 2005). Briefly, leaves of 4-week old plants were infiltrated with bacterial suspensions of 5×10^4 cfu/mL. Leaf discs with a total area of 0.5 cm^2 per sample were ground in 10 mM $MgCl_2$, and solutions were plated in serial dilutions on selective medium in triplicate at the indicated time points.

In the primary screen to identify *hps* mutants, approximately 25,000 M2 plants representing about 1100 M1 plants from 67 different pools were dip-inoculated with DC3000 (*hopA1*) and scored for disease symptoms 5 days after inoculation. Each flat of approximately 300 M2 plants contained a pot of RLD and *eds1-1* as resistant (scores of 0–1) and susceptible controls (scores of 4–5), respectively. M2 plants that scored above 3 were propagated to the M3 generation. Putative mutants from the same M2 pool were considered siblings. In the secondary screen, M3 plants were dip-inoculated with DC3000 and DC3000(*hopA1*), and the genotypes of susceptible mutants were confirmed as not being *eds1-1* using an allele-specific CAPS marker. In the tertiary screen,

putative mutants for *hopA1*-specific susceptibility were confirmed by syringe-inoculation of individual leaves of the same M3 plant with DC3000 and DC3000(*hopA1*).

Mapping and cloning of *RPS6*

Crosses were performed by removing stamens from recipient flowers before anther dehiscence and transferring pollen from donor plants to recipient stigmas. For mapping, the *rps6-1* (*hps1-1*) mutant was crossed to Col-0, and susceptible F2 plants from self-pollinated F1 plants were selected after syringe inoculating with DC3000(*hopA1*). F3 progenies from susceptible F2 plants were tested with DC3000 and DC3000(*hopA1*) to confirm their F2 susceptible phenotypes. Map-based cloning was performed as described previously (Gassmann et al., 1999; Kwon et al., 2009). Briefly, genomic DNA from approximately 300 susceptible F2 plants was isolated and analyzed using SSLP (Bell and Ecker, 1994) and CAPS (Konieczny and Ausubel, 1993) markers. Linkage between *RPS6* and genetic markers was determined by calculating the recombination frequency and the deviation from random segregation using the chi-square test with one degree of freedom.

The BAC clones K11I1 and MPL12 were obtained from the Arabidopsis Biological Resources Center (ABRC). To generate subclones containing At5g46260, At5g46270, At5g46450, At5g46470, At5g46490, At5g46510 and At5g46520, the BAC clones were partially digested with *Hind*III, and DNA was subcloned into the cosmid binary vector pCLD04541 (Bancroft et al., 1997). In

the case of the *RPS6*-specific genomic subclone pSHK103, a 10 kb band from a partial digest of BAC K11I1 with *Hind*III encompassed At5g46470 including 3.1 kb upstream of the start codon and 3 kb downstream of the stop codon. Subclones were transferred to *A. tumefaciens* strain GV3101 and transformed into the *rps6-1* mutant by floral dip (Clough and Bent, 1998). Plants were screened on half-strength Murashige and Skoog medium (Invitrogen) containing 50 µg/mL kanamycin.

Wild-type RLD and mutant allele *RPS6* sequences were analyzed using Sequencher software (Gene Codes Corporation), and HopA1 amino acid sequences were aligned using the MegAlign software in the Lasergene package (DNAStar).

Reverse Transcription PCR and RACE

Total RNA was isolated using TRIzol reagent (Invitrogen) and treated with Turbo DNase (Ambion) to prevent genomic DNA contamination according to the manufacturer's instructions. Single-strand cDNA was synthesized by reverse transcription using 2 µg of total RNA, an oligo(dT)15 primer and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). To test splicing variation, PCR was performed using a primer pair flanking introns (5'-TGAGGAAACAGACCGGAGAC-3' and 5'-CAAGATGTGCTAGATGCTTTAGCGG-3' for intron 1; 5'-GTGGGAGTAGAATCATTGTG-3' and 5'-AGCAGAGCAAACTTGAGAAG-3' for intron 2; 5'-GCTTCGACTATTTGCCCTCTAGACT-3' and 5'-

AACAACCCAAGTTTTGTGGAAGTCC-3' for introns 3 and 4; 5'-ACAGCGGACATTCCCTCTAA-3' and 5'-GGGACTCTATCTCCCTACCT-3' for intron 4; 5'- AATTTACTATGTGTATCCCC-3' and 5'-GATGGATGTCTCTGTTTCTG-3' for introns 5-7). To identify the *RPS6* cDNA 3'-end, the 3'-RACE system from Invitrogen was used according to the manufacturer's instructions. PCR products were ligated into the pGEM-T Easy vector (Promega) for sequencing.

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Chapter III

Characterization of HopA1-triggered RPS6-mediated resistance in Arabidopsis

Abstract

Plant resistance (*R*) genes recognize cognate pathogen avirulence (*avr*) determinants to implement the innate immune response called effector triggered immunity (ETI). To identify molecular and genetic interactions between plants and pathogens, the model system *Pseudomonas syringae* - *Arabidopsis thaliana* is extensively studied. Although, Arabidopsis possesses approximately 150 putative resistance genes encoding nucleotide binding site (NBS) and leucine-rich repeat (LRR) domain-containing proteins, few are characterized because most of their corresponding avirulence genes are unknown. Previously, we reported that *hopA1* from *Pseudomonas syringae* pv. *syringae* strain 61 was identified as an *avr* gene for Arabidopsis, and *hopA1* signaling is *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY1*) dependent. Using a forward genetic screen approach, we cloned a *hopA1*-specific TIR-NBS-LRR class disease resistance gene, *RPS6*. *RPS6* produces alternative transcripts. Mutations in *SRFR1*, a negative regulator of *avrRps4*-triggered immunity, enhanced *hopA1*-triggered immunity in *rps6* mutants. Here, we show the localization of RPS6 and HopA1, and provide a preliminary characterization of the HopA1 protein. Both RPS6 and HopA1 are localized to the nucleus and cytoplasm. In addition, nuclear localization of HopA1 is necessary to induce the cell death phenotype in *Nicotiana benthamiana*. In summary, the localization of plant R protein and its cognate pathogen effector protein may be an important mechanism to determine plant immunity.

Introduction

Plants are exposed to a broad range of pathogens (Staskawicz et al., 1995). They have developed a plethora of strategies aimed at blocking infection by potential pathogens and have evolved several layers of predetermined defenses, collectively called the innate immune system. Generally, pathogen recognition and defense responses occur either in response to widely distributed pathogen-associated molecular patterns (PAMPs), or to very specific virulence effectors. Recognition of PAMPs via specific receptors, basal defense, is sufficient to stop the growth of nonpathogenic microbes. Pathogenic microbes suppress basal plant defenses by deploying effector proteins. Plants in turn have evolved an effector detection system to activate so-called effector-triggered immunity (ETI) (Chisholm et al., 2006; da Cunha et al., 2006; Jones and Dangl, 2006). One form of ETI is the hypersensitive response (HR), during which cells immediately surrounding the site of infection rapidly die (Goodman and Novacky, 1994; Greenberg and Yao, 2004). In addition there is a series of defense-related reactions such as an oxidative burst, cell wall reinforcements, and the accumulation of pathogenesis-related proteins (Hammond-Kosack and Jones, 1997; Jones and Dangl, 2006). Historically, ETI has been explained by the gene-for-gene hypothesis, where plant resistance is governed in part by the genetic interaction between plant disease resistance (*R*) genes and cognate avirulence (*avr*) genes of pathogen. The recognition is thought to initiate a cascade of defense responses that ultimately lead to thwarting pathogen colonization and

multiplication (Flor, 1971). ETI is a highly valuable agronomic trait due to its effectiveness. However, ETI needs to be controlled both positively and negatively, because excessive ETI is detrimental to the host (McDowell and Simon, 2006).

Over 40 resistance genes have been cloned (Chisholm et al., 2006). The largest class of *R* genes encodes a family of cytosolic proteins possessing a nucleotide binding (NB)-ARC domain, which is also called the NBS domain, and a leucine-rich repeat (LRR) domain (Meyers et al., 1999). Arabidopsis and rice have approximately 150 and 400 NBS-LRR genes, respectively (Meyers et al., 2002). The NBS-LRR class of *R* genes can be grouped into two families, coiled-coil (CC)-NBS-LRR (CNL) and Toll-interleukin-1 receptor (TIR)-NBS-LRR (TNL), based on the N-terminal structure. To date, five Arabidopsis resistance genes have been cloned for which *avr* genes from *Pseudomonas syringae* are known. *RPM1* (Grant et al., 1995), *RPS2* (Bent et al., 1994), and *RPS5* (Warren et al., 1998) are CNL-type *R* genes that recognize the *P. syringae* pathovar type III effectors *AvrRpm1, AvrRpt2,* and *AvrPphB*, respectively. *RPS4* and *RPS6* are TNL-type *R* genes that encode resistance to *P. syringae* effectors *avrRps4,* and *hopA1*, respectively (Hinsch and Staskawicz, 1996; Gassmann et al., 1999; Gassmann, 2005; Kim et al., 2009). Though there are some exceptions, plant innate immunity conferred by CNL *R* genes require *NDR1* (*NONRACE SPECIFIC DISEASE RESISTANCE1*) but not *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY1*), whereas TNL *R* genes are dependent on *EDS1* and independent of *NDR1* (Century et al., 1995; Parker et al., 1996; Century et al., 1997; Aarts et al., 1998; Falk et al., 1999).

There is growing number of reports of subcellular localization of R proteins in recent years. Bacterial effector proteins are injected into plant cells by the type III secretion system and bacterial effector-specific NBS-LRR class R proteins are thought to be localized to the intracellular space (Martin et al., 2003; Chisholm et al., 2006). The Arabidopsis resistance protein RPM1 and its cognate effectors, AvrRpm1 and AvrB, have been reported to associate with the plasma membrane on the cytoplasmic side (Boyes et al., 1998; Nimchuk et al., 2000). Cf proteins from tomato were detected in the plasma membrane where they recognize their cognate extracellular Avr effectors (Piedras et al., 2000; Rivas and Thomas, 2005). Other reports demonstrate that R proteins are localized to the nucleus or cytoplasm. Arabidopsis RRS1-R protein, contains a classical nuclear localization signal (NLS) and a WRKY domain, and recognizes the PopP2 effector from *Ralstonia solanacearum.* RRS1-R and PopP2 are co-localized to the nucleus (Deslandes et al., 2003). Nuclear localization of the MLA R proteins in barley plays a pivotal role in conferring resistance against the fungal powdery mildew pathogen. The recognition of the Avr10 effector protein leads to the interaction between MLA10 and WRKY transcription factors in the nucleus (Bieri et al., 2004; Shen et al., 2007). RPS4 is distributed between endomembranes and nuclei and its nuclear localization is required for AvrRps4-triggered immunity in Arabidopsis (Wirthmueller et al., 2007). In addition, the tobacco N protein and its cognate pathogen protein, the tobacco mosaic virus (TMV) p50 protein are distributed between cytoplasm and nucleus. Nuclear localization of N is required for its function (Burch-Smith et al., 2007).

The bacterial effector gene *hopA1* (formerly *hrmA* and *hopPsyA*) originates from *P. syringae* pv. syringae strain 61. The pHIR11 cosmid, containing *hopA1* and *P. syringae* type lll secretion system genes, can induce a strong hypersensitive response (HR) on tobacco (Huang et al., 1988). HopA1 directly binds to the molecular chaperone ShcA, which is required for secretion of HopA1 *(van Dijk et al., 2002)*. In Arabidopsis, high inoculum (10^8cfu/ml) of *P. fluorescens* expressing *hopA1* gives an HR in Ws-0, but HR isn't detected in Col-0 or RLD. However, Col-0 and RLD show a non-HR resistant phenotype in response to *P. syringa*e pv. tomato (*Pst*) DC3000 expressing *hopA1* at 10^6cfu/ml. Both the HR and resistance phenotype is *EDS1*-dependent and *NDR1*-independent (Gassmann, 2005). This resistance without HR is reminiscent of the *avrRps4*-triggerrd immunity of Arabidopsis governed by the *RPS4 R* gene (Gassmann et al., 1999). Together, these data indicate that *hopA1* is an avirulence gene for Arabidopsis which shows a resistance phenotype with no HR. In previous studies, we identified the *hopA1*-specific *RPS6* disease resistance gene that was cloned using a forward genetic screen and map-based cloning (Kim et al., 2009). *RPS6* encodes a TIR-NBS-LRR class R protein and generates alternatively spliced transcripts, like *RPS4* and other TNL genes. In addition, HopA1-triggered immunity is positively controlled by EDS1 and negatively regulated by SRFR1. Here, we report the localization of RPS6 and HopA1 proteins and characterize the HopA1 effector protein.

Results

Localization of RPS6

To elucidate where RPS6 localizes inside the plant cell, we used the *Agrobacterium*-mediated transient expression system in *Nicotiana benthamiana* leaf cells. Prediction of protein subcellular locations indicated that RPS6 may localize to the nucleus and cytoplasm (http://www.genome.jp/SIT/plocdir/) (Park and Kanehisa, 2003), albeit without a canonical nuclear localization signal. Genomic *RPS6* from Col-0 was fused in frame with green fluorescent protein (GFP) and yellow fluorescent protein (YFP) at the N-terminus under control of the strong CaMV 35S promoter to generate 35S-*GFP-gRPS6* and 35S-*YFP-gRPS6.* These constructs and control GFP alone were transiently expressed in *Nicotiana benthamiana* leaf cells and observed under the confocal microscope. As shown in Figure lll-1A, the control GFP alone was uniformly distributed in the nucleus and cytoplasm. Interestingly, GFP-RPS6 and YFP-RPS6 fusion proteins were detected in the nucleus, perinuclear region and cytoplasm, reminiscent of the pattern of SRFR1 protein localization (Kwon et al., 2009). The expression of GFP-RPS6 and YFP-RPS6 was confirmed by western blot analysis (Figure lll-1B) suggesting the localization of RPS6 was based on full-length protein expression. We transformed a *rps6-1* mutant that contains the mis-sense mutation, which changes a highly conserved His within the hxhHD motif in the NBS to Arg with *35S-GFP-gRPS6* (Kim et al., 2009). However, we failed to obtain homozygous stable transgenic lines. We speculate that constitutively

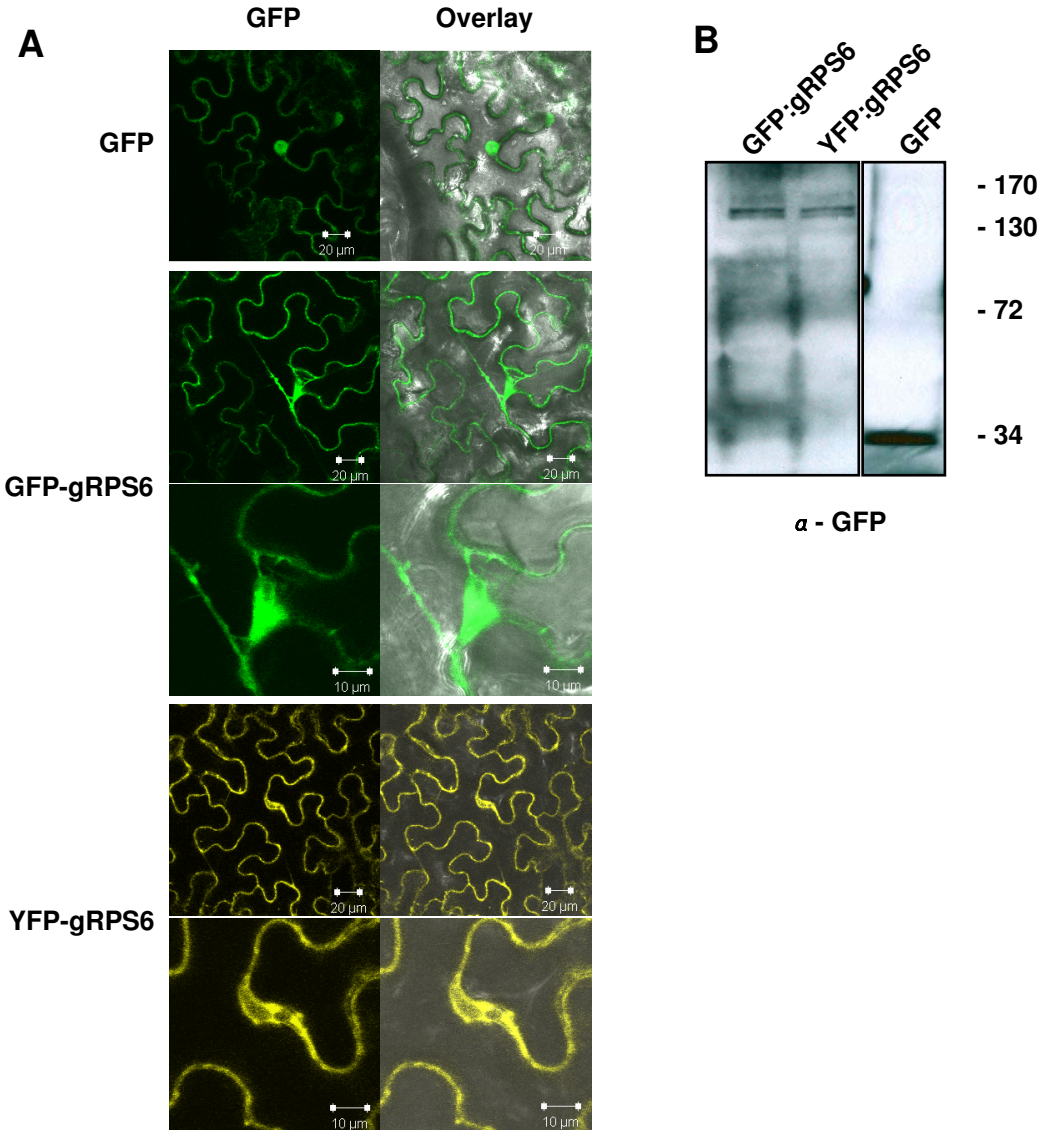
A
GFP
Overlay
GFP
GFP-gRPS6
YFP-gRPS6
20 µm
10 µm
B
GFP:gRPS6
YFP:gRPS6
GFP
- 170
- 130
- 72
- 34
α - GFP

Figure III-1

Figure III-1. RPS6 protein localizes to the nucleus and cytoplasm. **(A)** *GFP, GFP-gRPS6,* and *YFP-gRPS6* fusion constructs (from top to bottom) were transiently expressed in *Nicothiana benthamiana* leaves using Agrobacterium. GFP-gRPS6 (middle), and YFP-gRPS6 (bottom) are detected in the nucleus, perinuclear region and cytoplasm. GFP alone (top) is used for comparison. Cells expressing fusion proteins were analyzed 3 days after infiltration under GFP fluorescence (left) and GFP/bright field overlay (right). **(B)** Detection of RPS6 protein in *N. benthamiana* by western blot. Expression of GFP-gRPS6 and YFP-gRPS6 in samples shown in (A) was confirmed by western blot with anti-GFP antibody. Total protein was extracted from three leaf discs at 3 days post inoculation.

expressed RPS6 is possibly lethal, a common observation with R proteins. Therefore, we constructed a genomic *RPS6* clone that was under the control of 554 bp of *RPS6* native promoter, and that was N-terminally fused in frame to a GFP tag (denoted *pRPS6-GFP-gRPS6*). This construct was transformed into the *rps6-1* mutant to isolate stable transgenic lines. *In planta* bacterial growth assays were performed to quantify the level of resistance. The transgenic *rps6-1* line containing the *pRPS6-GFP-gRPS6* was as resistant as wild-type RLD and had approximately 50 to 100-fold lower DC3000(*hopA1*) growth than *rps6-1* mutant plants (Figure lll-2), supporting the complementation of an *rps6-1* mutant using a *RPS6* genomic construct (pSHK103) (Kim et al., 2009). The growth of virulent DC3000 was similar in all plants tested, suggesting this construct does not provide a constitutive defense response (Figure lll-2). However, we could not detect the GFP fluorescence signal in transgenic plants.

Localization of the bacterial effector HopA1

HopA1 is found in both avirulent *Pseudomonas syringae* p.v. syringae stain 61 (denoted $HopA1_{Pss61}$) and virulent *P. s.* p.v. tomato strain DC3000 (denoted $HopA1_{DC3000}$). The amino acid sequences of $HopA1_{Pss61}$ and $HopA1_{DC3000}$ are 57% identical, and diverged amino acids are distributed throughout the proteins (Kim et al., 2009). In order to investigate the subcellular localization of the $HopA1_{Pss61}$ effector protein, we fused a *GFP* tag to the N- and C- terminus of HopA1 driven by the 35S promoter (denoted *GFP-hopA1*$_{Pss61}$ and *hopA1*$_{Pss61}$-*GFP*). *GFP* control alone, *GFP-hopA1*$_{Pss61}$, and *hopA1*$_{Pss61}$-*GFP* were transiently

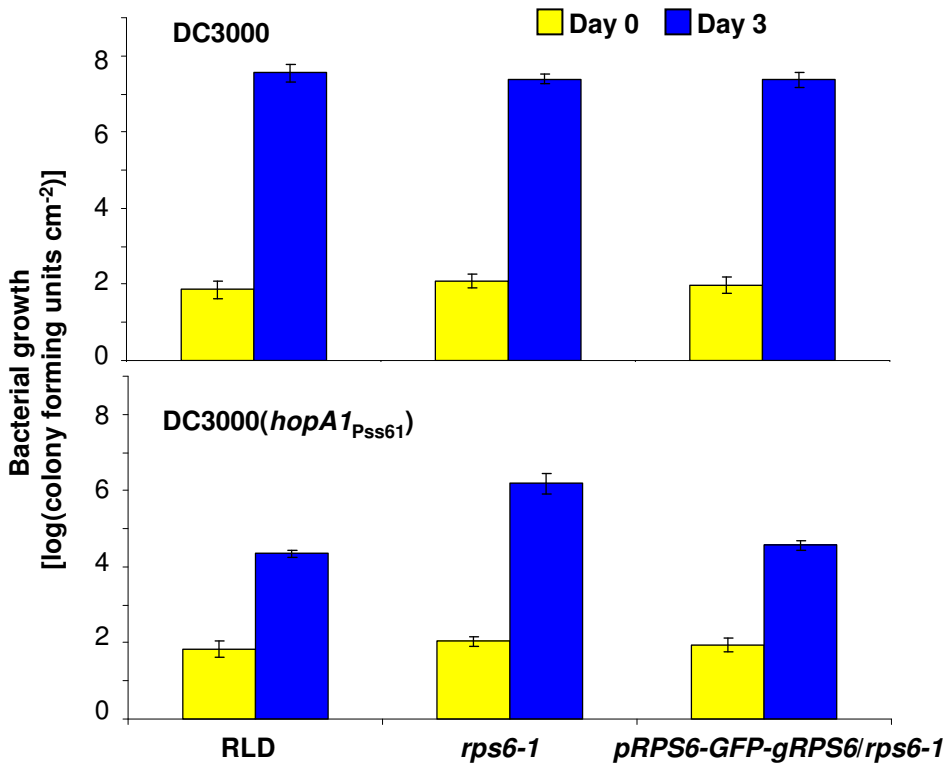
DC3000
Day 0
Day 3
Bacterial growth
[log(colony forming units cm-2)]
0
2
4
6
8
DC3000(hopA1Pss61)
RLD
rps6-1
pRPS6-GFP-gRPS6/rps6-1

Figure III-2

Figure III-2. Complementation of *rps6-1* with a genomic clone of *RPS6* tagged with GFP. *In planta* bacterial growth was measured in RLD, *rps6-1* and a *rps6-1 pRPS6-GFP-gRPS6* transgenic line in the T3 generation (from left to right) on day 0 (yellow columns) and day 3 (blue columns) after inoculation with DC3000 (top) and DC3000(*hopA1*$_{Pss61}$) (bottom). Plants were inoculated with a bacterial suspension at a density of $5x10^4$ cfu/mL. Values represent averages of cfu/cm^2 leaf tissue from triplicate samples, and error bars denote standard deviation.

expressed in *N. benthamiana* leaves using agroinfiltration. GFP alone was distributed throughout the nucleus and cytoplasm (Figure III-3A). GFP-HopA1$_{Pss61,}$ GFP at the N-terminus of HopA1$_{Pss61,}$ was localized to the nucleus and cytoplasm. Intriguingly, GFP-HopA1$_{Pss61}$ was localized not only in the nucleoplasm but also the nucleolus (Figure III-3B). Similarly, GFP-HopA1$_{DC3000}$ was targeted to the nucleus, nucleolus and cytoplasm (Figure III-3C). Surprisingly, we detected HopA1$_{Pss61}$-GFP only in the cytoplasm (Figure III-3D) and HopA1$_{DC3000}$-GFP was also a cytoplasmic protein (Figure III-3E). Taken together, these results demonstrated that RPS6 R protein and its cognate HopA1 effector protein are localized to both nucleus and cytoplasm.

HopA1-dependent cell death in *N. benthamiana*

The pHIR11 cosmid that contained *hopA1* and Pss61 type III secretion system genes, when expressed in *P. fluorescens*, can induce a strong hypersensitive response (HR) in tobacco (Huang et al., 1988; Alfano et al., 1997). To test whether HopA1 alone can elicit cell death in *N. benthamiana*, agrobacteria expressing the *GFP*-tagged *hopA1* constructs, *GFP-hopA1*$_{Pss61}$*, hopA1*$_{Pss61}$*-GFP, GFP-hopA1*$_{DC3000}$, *hopA1*$_{DC3000}$*-GFP* and *GFP* alone, were infiltrated into *N. benthamiana* leaves at a density of $3x10^8$ cfu/ml. Interestingly, only *GFP-hopA1*$_{Pss61}$ was capable of inducing HR-like cell death 2 days after infiltration (Figure III-4A). *N. benthamiana* leaves inoculated with *hopA1*$_{Pss61}$*-GFP, GFP-hopA1*$_{DC3000}$ or *hopA1* $_{DC3000}$*-GFP* did not exhibit the cell death response. The

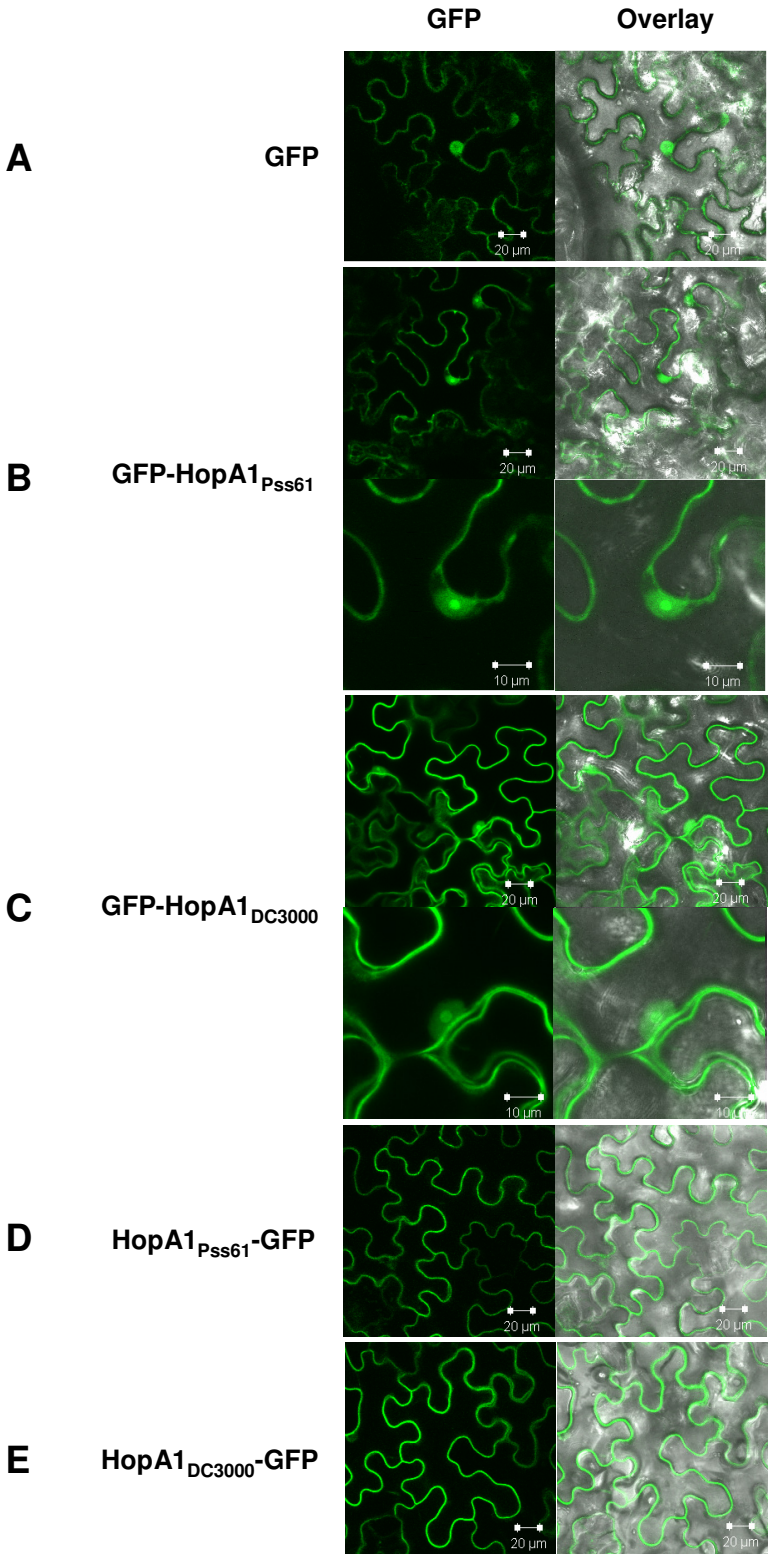
GFP
Overlay
A
GFP
B
GFP-HopA1Pss61
C
GFP-HopA1DC3000
D
HopA1Pss61-GFP
E
HopA1DC3000-GFP
20 µm
10 µm

Figure III-3

Figure III-3. HopA1 protein localizes to the nucleus and cytoplasm. **(A)** *GFP, GFP-hopA1*$_{Pss61}$*, GFP-hopA1*$_{DC3000}$*, hopA1*$_{Pss61}$*-GFP* and *hopA1*$_{DC3000}$*-GFP* fusion constructs (from top to bottom) were transiently expressed in *N. benthamiana* leaves using Agrobacterium. GFP-HopA1$_{Pss61}$ **(B)** and GFP-HopA1$_{DC3000}$ **(C)** are detected in the nucleus and cytoplasm. However, HopA1$_{Pss61}$-GFP **(D)** and HopA1$_{DC3000}$-GFP **(E)** are cytoplasmic. GFP alone **(A)** is used for comparison. Cells expressing fusion proteins were analyzed 3 days after infiltration under GFP fluorescence (left) and GFP/bright field overlay (right).

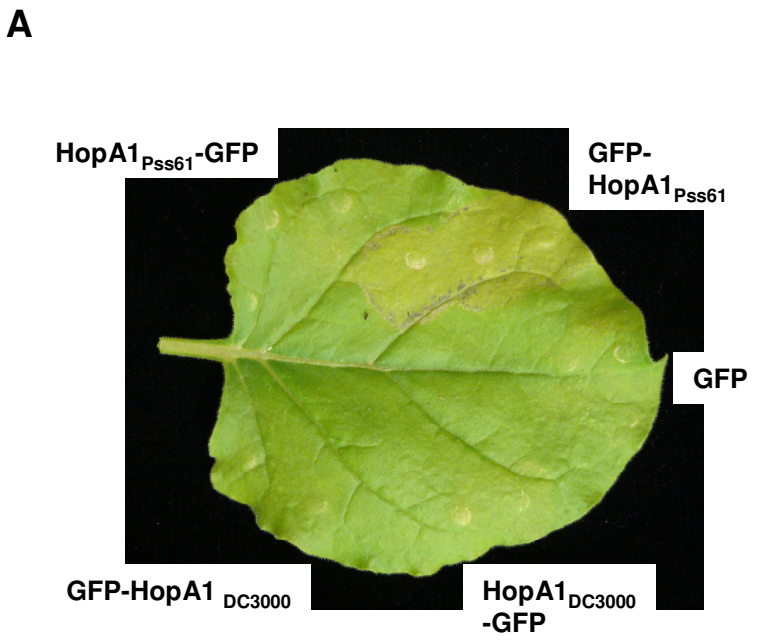
A
HopA1Pss61-GFP
GFP-
HopA1Pss61
GFP
GFP-HopA1 DC3000
HopA1DC3000
-GFP
B
GFP
GFP-
HopA1Pss61
HopA1Pss61
-GFP
GFP-
HopA1DC3000
HopA1DC3000
-GFP
130 -
95 -
72 -
55 -
34 -
α - GFP
C
Myc-
HopA1Pss61
unprocessed
processed
- 95
- 72
- 55
- 34
- 25
α - Myc

Figure III-4

Figure III-4. GFP-HopA1$_{Pss61}$ induces cell death in *Nicothiana benthamiana*. **(A)** *GFP, GFP-hopA1*$_{Pss61}$*, GFP-hopA1*$_{DC3000}$*, hopA1*$_{Pss61}$*-GFP* and *hopA1*$_{DC3000}$*-GFP* fusion constructs were transiently expressed in *N. benthamiana* leaves using Agrobacterium adjusted to an OD_{600} of 0.3. Only GFP-HopA1$_{Pss61}$ induced cell death (top right), whereas GFP alone (right), GFP-HopA1$_{DC3000}$ (bottom right), HopA1$_{Pss61}$-GFP (top left) and HopA1$_{DC3000}$-GFP (bottom left) did not cause cell death. Phenotypes were recorded at 2 days post inoculation. **(B)** Detection of HopA1 protein in *N. benthamiana* by western blot. Expression of GFP-HopA1$_{Pss61}$, GFP-HopA1$_{DC3000}$, HopA1$_{Pss61}$-GFP and HopA1$_{DC3000}$-GFP (Figure III-3 and Figure III-4A) were confirmed by western blot with anti-GFP antibody. Total protein was extracted from three leaf discs at 3 days post inoculation. **(C)** Western blot analysis of Myc-HopA1$_{Pss61}$ protein in *N. benthamiana* with horseradish peroxidase (HRP)-conjugated HA. Myc-HopA1$_{Pss61}$ was transiently expressed in *N. benthamiana.* Total protein was extracted from three leaf discs at 3 days post inoculation. Upper and lower arrows indicate the unprocessed and putative processed Myc-HopA1$_{Pss61}$ proteins, respectively.

expression of GFP-HopA1$_{Pss61}$, HopA1$_{Pss61}$-GFP, GFP-HopA1$_{DC3000}$, and HopA1$_{DC3000}$-GFP were confirmed by western blot analysis using anti-GFP antibody (Figure III-4B). In addition, when *Myc-hopA1$_{Pss61}$* was expressed in *N. benthamiana* cells, proteins with two different sizes were detected using anti-Myc antibody, (Figure III-4C). We speculate the smaller protein could be a processed form of HopA1. The molecular weight of processed Myc-HopA1 was approximately 30 kDa. In summary, our results suggest that nuclear localization of HopA1$_{Pss61}$ may be necessary to elicit cell death in *N. benthamiana*., and that HopA1 may be processed *in planta*.

HopA1$_{DC3000}$ does not boost a bacterial susceptibility in plants

To exclude the possibility that the differential Arabidopsis response to *hopA1*$_{DC3000}$ (no resistance) and *hopA1*$_{Pss61}$ (resistance) is based on different expression levels of genomic *hopA1*$_{DC3000}$ and vector-borne *hopA1*$_{Pss61}$, we cloned *shcA-hopA1* from DC3000 into pML123 and introduced it into DC3000. Disease assays showed that DC3000 expressing *hopA1*$_{DC3000}$ from vector pML123 also did not trigger resistance (Figure III-5A). To elucidate whether *hopA1*$_{DC3000}$ increases the bacterial virulence, we quantified the growth of bacteria in plants using DC3000(empty vector) and DC3000(*hopA1*$_{DC3000}$). As shown in figure III-5B, bacterial proliferation of DC3000(*hopA1*$_{DC3000}$) in RLD and *rps6-1* was equivalent to that of DC3000(empty vector), suggesting that vector-borne *hopA1*$_{DC3000}$ does not boost susceptibility in plants.

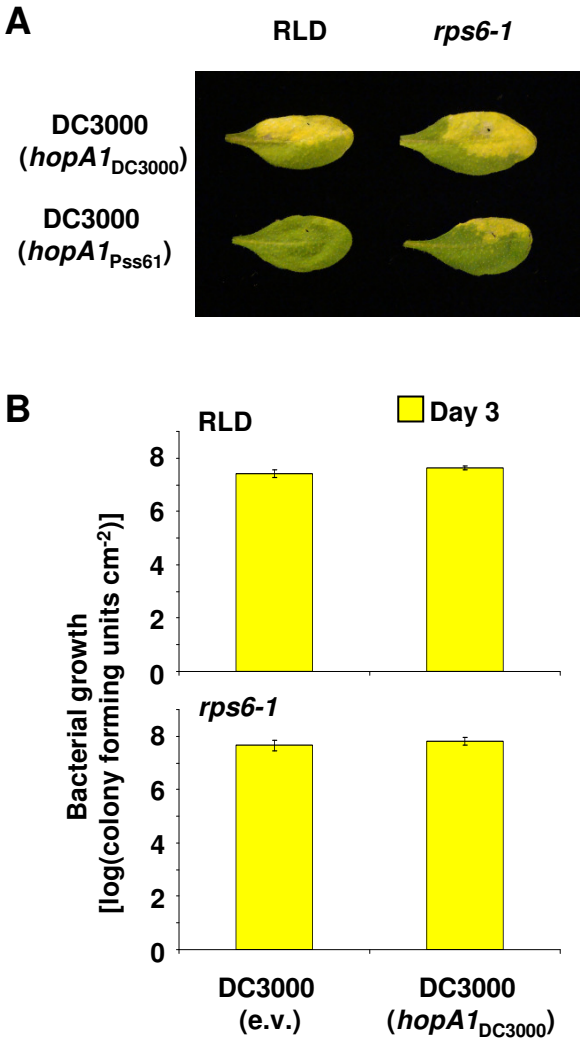
A
RLD
rps6-1
DC3000
(hopA1DC3000)
DC3000
(hopA1Pss61)
B
RLD
Day 3
Bacterial growth
[log(colony forming units cm-2)]
8
6
4
2
0
rps6-1
8
6
4
2
0
DC3000
(e.v.)
DC3000
(hopA1DC3000)

Figure III-5

Figure III-5. HopA1$_{DC3000}$ does not boost bacterial virulence in plants **(A)** DC3000 expressing *hopA1*$_{DC3000}$ from vector pML123 does not trigger resistance in Arabidopsis. Individual leaves of RLD (left) and *rps6-1* (right) from the same plant were infiltrated with DC3000(*hopA1*$_{DC3000}$) (top) and DC3000(*hopA1*$_{Pss61}$) (bottom) at a bacterial density of 10^6 cfu/ml. Disease symptoms (leaf chlorosis) were recorded 5 days after inoculation. Only the upper halves of leaves were infiltrated. Results are representative for 5 out of 5 RLD and 6 out of 6 *rps6-1* plants. **(B)** *In planta* bacterial growth was measured in RLD (top) and *rps6-1* (bottom) on day 3 after inoculation with DC3000 expressing empty vector (left) and *hopA1*$_{DC3000}$ (right). Plants were inoculated with a bacterial suspension at a density of $5x10^4$ cfu/mL. Values represent averages of cfu/cm^2 leaf tissue from triplicate samples, and error bars denote standard deviation.

The NRPS domain is not an avirulence determinant in HopA1$_{Pss61}$

Based on computational analyses of the predicted protein sequence, we found that HopA1 shows sequence similarity to a nonribosomal peptide synthase (NRPS), at the N- terminus (amino acid residues 5-12; http://pfam.sanger.ac.uk) (Finn et al., 2008). NRPS is required for biosynthesis of the phytotoxin albicidin, which is produced by the sugarcane leaf scald pathogen *Xanthomonas albilineans* (Huang et al., 2001; Royer et al., 2004). We hypothesized that the NRPS motif may be an avirulence determinant of HopA1$_{Pss61}$ because HopA1$_{Pss61}$ contained the NRPS domain, whereas the NRPS domain in HopA1$_{DC3000}$ had amino acid changes at conserved residues (data not shown). We constructed NRPS mutants, denoted H5Q, E12Q and H5QE12Q, from *hopA1*$_{Pss61}$ and generated DC3000(H5Q*hopA1*$_{Pss61}$), DC3000(E12Q*hopA1*$_{Pss61}$) and DC3000(H5QE12Q*hopA1*$_{Pss61}$). However, the growth of DC3000(H5Q*hopA1*$_{Pss61}$), DC3000(E12Q*hopA1*$_{Pss61}$) and DC3000(H5QE12Q*hopA1*$_{Pss61}$) in RLD wild-type plants did not increase compared to the growth of DC3000(*hopA1*$_{Pss61}$) in RLD (Figure lll-6). Therefore, we concluded that the NRPS domain is not an avirulence determinant in HopA1$_{Pss61}$.

Discussion

Effector triggered immunity (ETI) is mediated by genetic interactions between plant resistance (*R*) genes and pathogen avirulence (*avr*) genes, and is highly

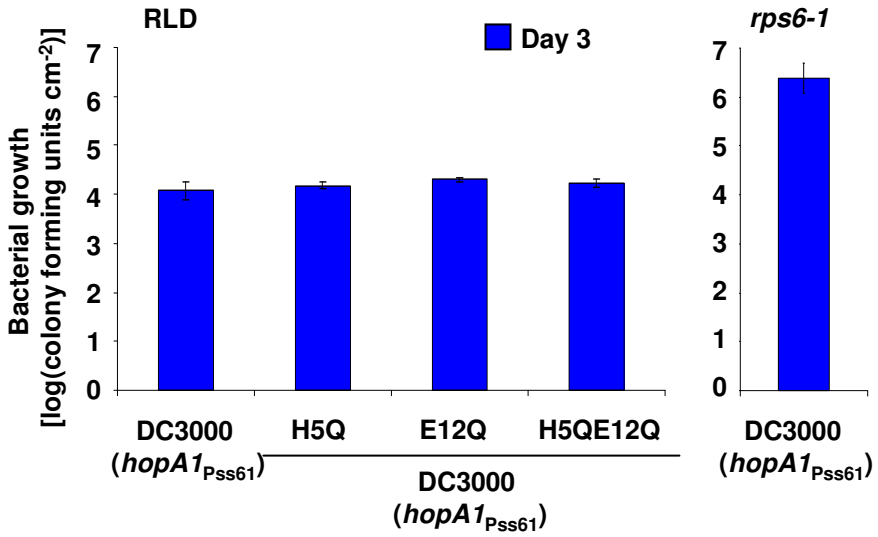
RLD
Day 3
rps6-1
Bacterial growth
[log(colony forming units cm-2)]
7
6
5
4
3
2
1
0
DC3000
(hopA1Pss61)
H5Q
E12Q
H5QE12Q
DC3000
(hopA1Pss61)
DC3000
(hopA1Pss61)

Figure III-6

Figure III-6. The NRPS domain is not an avirulence determinant in $HopA1_{Pss61.}$ *In planta* bacterial growth was measured in RLD (left) and *rps6-1* (right) on day 3 after inoculation with DC3000 expressing $HopA1_{Pss61}$ and mutants of the $HopA1_{Pss61}$ NRPS domain (denoted H5Q, E12Q and H5QE12Q). Plants were inoculated with a bacterial suspension at a density of $5x10^4$ cfu/mL. Values represent averages of cfu/cm^2 leaf tissue from triplicate samples, and error bars denote standard deviation.

effective in protecting plants from pathogens. Although over the past 10 years many *R* genes have been identified, the mechanism of how R proteins induce resistance upon perception of cognate Avr proteins is still unclear. Recently, we identified *RPS6* encoding a TIR-NBS-LRR (TNL) protein, using a loss of resistance screen and a positional cloning approach. RPS6 specifically recognizes a bacterial effector gene HopA1 (formerly *hrmA and hopPsyA*) from *P. syringae* pv. syringae strain 61 to trigger an immune response (Gassmann, 2005; Kim et al., 2009). To date, *RPS4* (Gassmann et al., 1999) and *RPS6* are the only Arabidopsis TIR-NBS-LRR *R* genes for which bacterial *avr* genes are known, both of which are *EDS1*-dependent. *RPS6* allows a direct comparison with *RPS4* to dissect the *EDS1*-dependent signaling pathway in Arabidopsis.

RPS6 is alternatively spliced, producing three alternative transcripts encoding truncated TIR-only or TIR-NBS proteins (Kim et al., 2009). When compared to other TNL *R* genes including *RPS4*, which are known to produce alternative transcripts, the structure of alternative transcripts from *RPS6* is unique. In addition, *RPS6* enables genetic tools to test the range of the effector-spectrum in *srfr1*-mediated resistance in the RLD background. SRFR1 is a negative regulator in AvrRps4-triggered immunity (Kwon et al., 2009). Surprisingly, mutations in *SRFR1* also increase HopA1-triggered immunity, demonstrating a possible role of SRFR1 as a general negative regulator in TNL R protein-mediated immunity (Kim et al., 2009).

In this paper, to gain a better understanding of RPS6 function, and to further compare RPS6 and RPS4, we tested the subcellular localization of RPS6.

The GFP- and YFP-tagged genomic *RPS6* constructs controlled by the CaMV 35S promoter (*35S-GFP-gRPS6* and *35S-YFP-gRPS6*) were overexpressed in *rps6-1* mutant plants for complementation and detection of fluorescent signals. However, we could not generate homozygous *RPS6* overexpressing plants. This may have been caused by an inappropriate activation of the RPS6 protein. Alternatively, *35S-GFP-gRPS6* and *35S-YFP-gRPS6* constructs were transiently expressed in *Nicotiana benthamiana* leaf cells using *Agrobacterium.* Interestingly, RPS6 was found to be localized to the nucleus, perinuclear region, and cytoplasm (Figure lll-1). Recently, we showed that SRFR1 is also distributed to the nucleus, perinuclear region, and cytoplasm (Kwon et al., 2009). Together with the finding that SRFR1 suppresses HopA1-triggered immunity (Kim et al., 2009), this raises the possibility that SRFR1 may directly or indirectly interact with RPS6 to control defense responses.

So far, there are seven examples elucidating R protein localization. The first report was the Arabidopsis RPM1 protein, detected in the plasma membrane, which is in accordance with its corresponding effector proteins, AvrRpm1 and AvrB (Boyes et al., 1998; Nimchuk et al., 2000). Two other R proteins were found to be localized to the plasma membrane: the tomato Cf proteins, with their cognate Avr proteins (Piedras et al., 2000; Rivas and Thomas, 2005), and Arabidopsis RPS2, with the AvrRpt2 effector protein (Axtell et al., 2003). These three cases of membrane localization were determined by biochemical assays. In addition to biochemical fractionation, localization of R protein tagged with fluorescent protein can be visualized by confocal microscopy, as shown in the

nuclear localization of RRS1, a TNL-WRKY R protein in Arabidopsis (Deslandes et al., 2003). More recently, there were three convincing reports of R protein distribution in the nucleus and cytoplasm, such as tobacco N (Burch-Smith et al., 2007), barley MLA (Shen et al., 2007), and Arabidopsis RPS4 (Wirthmueller et al., 2007), performed using biochemical fractionation assays and confocal fluorescence microscopy. All three examples suggest that nuclear localization of these R proteins plays a pivotal role in ETI, demonstrating the importance of localization experiments of R proteins. Furthermore, the localization of the TNL proteins, N and RPS4, and now also RPS6, suggest that TNL proteins are nuclear and cytoplasmic.

The HopA1$_{Pss61}$ effector, which is recognized by RPS6, was also localized to the nucleus and cytoplasm when GFP was fused to the N-terminus (GFP-HopA1$_{Pss61}$) (Figure lll-3B). GFP-HopA1$_{Pss61}$ induces cell death in *N. benthamiana* leaves (Figure lll-4A). Interestingly, HopA1$_{Pss61}$-GFP (GFP fused to the C-terminus of HopA1$_{Pss61}$) was found in the cytoplasm and failed to trigger cell death for unknown reasons (Figures lll-3D and lll-4A). We speculate that the C-terminally tagged GFP may cause improper protein folding or occlude a nuclear localization signal (NLS) of HopA1$_{Pss61}$. Localization of GFP-HopA1$_{DC3000}$ and HopA1$_{DC3000}$-GFP were similar to that of GFP-HopA1$_{Pss61}$ and HopA1$_{Pss61}$-GFP, respectively; however, they did not elicit cell death. Taken together, the nuclear localization of HopA1$_{Pss61}$ may be necessary to induce cell death in *N. benthamiana*. Again, it is likely that a nuclear pool of RPS6 and HopA1$_{Pss61}$ may play an important role in ETI, as was proposed for N and RPS4 (Burch-Smith et

al., 2007; Wirthmueller et al., 2007). Also, we can not exclude the possibility that RPS6 and HopA1$_{Pss61}$ may interact in the nucleus. The nucleolus is not only involved in the biogenesis of ribosomal RNA, but also implicated in the control of disease, regulation of cell cycle, and as a storage site (Carmo-Fonseca et al., 2000). Recently, Fuhrman and coworkers showed that the NOL-6 nucleolar protein in *Caenorhabditis elegans* suppressed innate immunity against bacteria pathogens by inhibiting the transcriptional activity of the tumor suppressor p53 (Fuhrman et al., 2009). Both GFP-HopA1$_{Pss61}$ and GFP-HopA1$_{DC3000}$ localized to the nucleolus, suggesting that both HopA1 proteins may interact with host target(s) in the nucleolus to enhance bacterial virulence, whereas only HopA1$_{Pss61}$ is monitored by RPS6 to trigger an immune response. It would be interesting to further study whether HopA1 interacts with RPS6 *in planta*, or further identify the host target protein(s), which would allow us to elucidate the molecular mechanism of HopA1-triggered immunity.

The *hopA1* gene is found in *Pss*61 and *Pst* DC3000, and is a pseudogene in *Pst* T1 (Almeida et al., 2009; Kim et al., 2009). *hopA1*$_{Pss61}$ is avirulent to all Arabidopsis accessions tested to date (Gassmann, 2005) and induces HR in tobacco and several other *Nicotiana* species (Alfano et al., 1997). We used DC3000 expressing empty vector pML123, and *hopA1*$_{Pss61}$ in vector pML123 as virulent and avirulent bacteria, respectively, arguing that the vector pML123-borne *hopA1*$_{Pss61}$ might induce resistance in Arabidopsis. However, DC3000 expressing *hopA1*$_{DC3000}$ in vector pML123 was virulent to wild-type RLD, suggesting that vector pML123 and *hopA1*$_{D3000}$ does not induce ETI (Figure III-

5A). Also, DC3000 expressing *hopA1*$_{DC3000}$ was as virulent as DC3000 expressing empty vector in RLD and *rps6-1*, indicating that *hopA1*$_{DC3000}$ does not induce effector-triggered susceptibility (ETS) (Figure III-5B). Based on the detection of a small sized Myc-HopA1 protein (Figure III-4C), HopA1 is likely to be processed, producing approximately a 10 kDa protein. Sohn and colleagues revealed that processing of AvrRps4 occurs at amino acids 133GG134. The processed AvrRps4 is required for virulence function, but not for avirulence function of AvrRps4 (Sohn et al., 2009). Two GG motifs, 70GG71 and 105GG106, in HopA1 could be candidate recognition sites for processing. Protein sequencing of the small size band is required to verify HopA1 processing. As shown in the example of AvrRps4, which promotes bacterial virulence in Arabidopsis, and *N. benthamiana*, and suppresses PTI (Sohn et al., 2009), HopA1$_{Pss61}$ may compromise PTI in the absence of RPS6, albeit the virulence function of HopA1 is unknown.

In summary, the localization of the RPS6 immune receptor and the cognate HopA1$_{Pss61}$ effector is nuclear and cytoplasmic, and nuclear localization of HopA1$_{Pss61}$ may be required to elicit cell death. Further experiments are required to determine whether RPS6 and HopA1$_{Pss61}$ interact directly or indirectly, how SRFR1 serves to regulate RPS6 and HopA1$_{Pss61}$, and what the host target(s) of HopA1$_{Pss61}$ and HopA1$_{DC3000}$ are, to elucidate the mechanism of HopA1-triggered immunity.

Materials and Methods

Growth, plasmid construction and mating of bacteria

Pseudomonas syringae pv tomato (*Pst*) strain DC3000 containing the empty vector pML123 (DC3000), or expressing the plasmid pLN92 (DC3000(*hopA1*$_{Pss61}$)) were grown on Difco Pseudomonas Agar F (Becton, Dickinson and Company, Sparks, MD, USA) as described previously (Gassmann, 2005). The *hopA1* gene from *Pst* DC3000 was amplified by polymerase chain reaction (PCR) with *hopA1*$_{DC3000}$ specific primers containing *Eco*RI and was subcloned into the pML123 expression vector using *Eco*RI restriction. To generate *hopA1*$_{Pss61}$ mutants, overlapping PCR was performed using pLN92 as template with *hopA1*$_{Pss61}$ specific primers to produce H5Q, E12Q and H5QE12Q, which were subcloned into the pML123 expression vector using *Eco*RI restriction to generate H5Q*hopA1*$_{Pss61}$, E12Q*hopA1*$_{Pss61}$ and H5QE12Q*hopA1*$_{Pss61}$. These constructs were mobilized into recipient DC3000 by triparental mating using the helper plasmid pRK2013 to produce DC3000(*hopA1*$_{DC3000}$), DC3000(H5Q*hopA1*$_{Pss61}$), DC3000(E12Q*hopA1*$_{Pss61}$) and DC3000(H5QE12Q*hopA1*$_{Pss61}$).

Plant growth and disease assays

Arabidopsis plants used in this study were grown in an E-7/2 reach-in growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) under an 8 h light/16 h dark cycle at 24°C, 70% relative humidity and a light intensity of 90-

140 μmol photons m^{-2} s^{-1}. Disease assays and *in planta* bacterial growth assay were performed by syringe infiltration as described (Gassmann, 2005). For disease assays, 4 to 5-week old Arabidopsis leaves were infiltrated with a bacterial suspension of 1×10^6 colony-forming units (cfu)/mL in 10 mM $MgCl_2$ using a 1 ml needless syringe. For *in planta* bacterial growth assays, bacterial suspensions of 5×10^4 cfu/ml were infiltrated into leaves of 4 to 5-week old plants. A total of 0.5 cm^2 of leaf discs was ground in 10 mM $MgCl_2$ and plated in serial dilution on selective medium in triplicate at the indicated time points.

Transient expression constructs

To generate epitope tagged *RPS6* constructs, genomic *RPS6* DNA including introns was amplified by PCR from Col-0. *In vitro* BP clonase recombination reactions were carried out to insert the PCR products into the pDONR201 entry vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). LR reactions were performed to recombine the entry clones into GATEWAY compatible destination vectors following the manufacturer's protocols (Invitrogen, Carlsbad, CA, USA). Using BP and LR reactions, we constructed *GFP-gRPS6* and *YFP-gRPS6* under the control of the CaMV 35S promoter (denoted *35S-GFP-gRPS6* and *35S-YFP-gRPS6*). To produce *GFP*-tagged genomic *RPS6* with its native promoter (*pRPS6-GFP-gRPS6*), 554 bp of the *RPS6* promoter were amplified with primers containing *Pme*I and *Kpn*I and cloned into *35S-gGFP-RPS6* in place of the 35S promoter using *Pme*I and *Kpn*I. For epitope-tagged *hopA1* constructs, the coding region of *hopA1* was amplified

by PCR from *hopA1*$_{Pss61}$ and *hopA1*$_{DC3000}$ plasmids. Using GATEWAY BP and LR reactions (Invitrogen, Carlsbad, CA, USA), we produced *Myc-hopA* $_{Pss61}$, *GFP-hopA1*$_{Pss61}$, *hopA1*$_{Pss61}$*-GFP, GFP-hopA1*$_{DC3000}$, *hopA1*$_{DC3000}$*-GFP*.

Agrobacterium-mediated transient expression

RPS6 and *hopA1* constructs were mobilized into the *Agrobacterium tumefaciens* strain C58C1 containing the virulence plasmid pCH32. After overnight culture in LB media, agrobacteria cells were pelleted and resuspended in 10 mM $MgCl_2$ with 100 µM acetosyringone (Sigma-Aldrich, St. Louis, Mo, USA) adjusted to an OD_{600} of 0.3. The Agrobacterium was incubated for 2 hr at room temperature and infiltrated into 4 to 5-week-old *N. benthamiana* leaves with a 1-ml needleless syringe. Tissues were collected three days after infiltration for western blot, HR assay and confocal microscopy. *N. benthamiana* plants were grown in an E-7/2 reach-in growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) under a 16 h light/8 h dark cycle at 25°C, 70% relative humidity.

Western blot analysis

N. benthamiana tissues expressing the protein of interest were ground in 100 µl of 8M urea buffer to extract total protein as described previously (Kwon et al., 2009). After adding 5X loading dye to samples, the mixtures were boiled for 5 min. Cell debris was pelleted at 13,200 rpm for 5 min and the collected supernatant was used for immunoblotting. 40 µl of protein samples were separated on a 8–10% SDS-polyacrylamide gel and were transferred onto

immune-blot PVDF membrane (Bio-Rad, Hercules, CA, USA). Immunodetection was performed as described previously (Moffet et al., 2002). Myc tag was detected with 1:2000 diluted HRP-conjugated Myc antibody (A-14) (Santa Cruz biotechnology, Santa Cruz, CA, USA). GFP and YFP tags were detected with 1:5000 diluted rabbit anti-GFP primary antibodies (Invitrogen, Carlsbad, CA, USA) and 1:5000 diluted goat anti-rabbit HRP-conjugated secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA). Detected proteins were visualized with an ECL Plus chemiluminescent kit (GE Healthcare, Buckinghamshire, UK).

Confocal fluorescence microscopy

Plant tissues for live imaging were observed with a Zeiss LSM 510 META NLO two-photon point-scanning confocal system mounted on an Axiovert 200M inverted microscope with a 40·/1.2 C-Apochromat water immersion objective (Carl Zeiss, Thornwood, New York, USA). The GFP and YFP fluorescence were excited by a 488-nm laser and a 514-nm argon laser, respectively. Samples were detected using a 500- to 550-nm band-pass emission filter.

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Chapter IV.

SRFR1, a suppressor of effector-triggered immunity, encodes a conserved tetratricopeptide repeat protein with similarity to transcriptional repressors

Reprinted with permission from: Soon Il Kwon†, Sang Hee Kim†, Saikat Bhattacharjee, Jae-Jong Noh and Walter Gassmann (2009) *SRFR1*, a suppressor of effector-triggered immunity, encodes a conserved tetratricopeptide repeat protein with similarity to transcriptional repressors. Plant Journal 57, 109-119. "Copyrighted by Wiley-Blackwell" †These authors contributed equally to this work.

Abstract

Effector-triggered immunity provides plants with strong protection from pathogens. However, this response has the potential to be highly deleterious to the host and needs to be tightly controlled. The molecular mechanisms in the plant that regulate the balance between resistance activation and suppression are not fully understood. Previously, we identified *Arabidopsis suppressor of rps4-RLD 1* (*srfr1*) mutants with enhanced resistance to the bacterial effector AvrRps4. These mutants were recessive and retained full susceptibility to virulent bacteria, suggesting that *SRFR1* functions as a negative regulator and that AvrRps4-triggered immunity was specifically enhanced in the mutants. Consistent with this, we show here that the response to flagellin, an elicitor of basal resistance, is unaltered in *srfr1-1*. In contrast, resistance to AvrRps4 in *srfr1-1* requires *EDS1*, a central regulator of effector-triggered immunity via multiple resistance genes. *SRFR1* is a single-copy gene encoding a pioneer tetratricopeptide repeat protein conserved between plants and animals. The SRFR1 tetratricopeptide repeat domain shows sequence similarity to those of transcriptional repressors in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. Indeed, a sub-pool of SRFR1 transiently expressed in *Nicotiana benthamiana* leaf cells localizes to the nucleus. Identification of SRFR1 may therefore provide insight into the regulation of the transcriptional reprogramming that is activated by effector-triggered immunity.

Introduction

Plant resistance to microbial pathogens in large part is mediated by highly specific resistance (*R*) genes that elicit effective defense responses upon perception of pathogen avirulence genes. These avirulence genes encode effector proteins whose original function is to promote pathogen fitness (Abramovitch *et al*., 2006; Grant *et al*., 2006). Plant R proteins evolved to directly or indirectly detect the presence or activity of these effector proteins, and this resistance mechanism has therefore been named effector-triggered immunity (ETI) (Chisholm *et al*., 2006; Jones and Dangl, 2006). One outcome of ETI can be localized programmed plant cell death (Goodman and Novacky, 1994; Greenberg and Yao, 2004), and plants with constitutively activated defenses usually display stunted growth and reduced seed set. Therefore, regulation of the ETI response must balance the capacity for rapid responses to pathogens and prevention of host cell damage (McDowell and Simon, 2006). However, the mechanisms regulating R protein and defense response activation are not fully understood.

R genes and the proteins they encode are regulated on multiple levels to achieve minimal side effects and yet optimal responses to pathogens. Expression of several *R* genes such as rice *Xa1* (Yoshimura *et al*., 1998), barley *Mla6* and *Mla13* (Halterman *et al*., 2003), tobacco *N* (Levy *et al*., 2004), and *Arabidopsis RPW8* (Xiao *et al*., 2003), *HRT* (Chandra-Shekara *et al*., 2004) and *RPS4* (Zhang and Gassmann, 2007) is induced during the resistance response,

as is alternative splicing of *N*, *Mla13* and *RPS4* (Dinesh-Kumar and Baker, 2000; Halterman *et al.*, 2003; Zhang and Gassmann, 2007). R protein stability and activity are regulated by co-chaperone complexes (Shirasu and Schulze-Lefert, 2003; Schulze-Lefert, 2004). In addition, R proteins can be kept in an inactive state by the proteins they guard (Belkhadir *et al.*, 2004).

To identify additional components that may negatively regulate AvrRps4-triggered immunity, we used a gain-of-resistance screen using EMS-mutagenized populations of the *avrRps4*-susceptible *Arabidopsis* accession RLD. This accession possesses a natural missense mutation in the *avrRps4*-cognate *R* gene *RPS4* (Hinsch and Staskawicz, 1996; Gassmann *et al.*, 1999; Zhang and Gassmann, 2003). We identified two *suppressor of rps4-RLD* (*srfr*) mutants, *srfr1* and *srfr3*, that were in separate complementation groups (Kwon *et al.*, 2004). Resistance in both mutants was specific to *Pseudomonas syringae* pv. tomato strain DC3000 expressing *avrRps4* [DC3000(*avrRps4*)]; they were fully susceptible to virulent DC3000. The resistance phenotype in both mutants was recessive, suggesting that the wild-type gene functions as a negative regulator in an AvrRps4-triggered immune response. Here we report the cloning of *SRFR1* and further characterize *srfr1*-mediated resistance to AvrRps4.

Results

Isolation of *SRFR1*

We cloned *SRFR1* using a combination of fine-mapping, shotgun complementation, and sequencing of candidate genes. A mapping population was generated by crossing *srfr1* and *srfr3* to the SAIL *RPS4* T-DNA knock-out line *rps4-1* (Kwon *et al.*, 2004). Both *srfr1* and *srfr3* were mapped to an interval of approximately 193 kb on the bottom of chromosome 4 that was fully covered by two bacterial artificial chromosomes (BACs), F6G17 and F19F18. We proceeded by generating cosmid subclones using HindIII partial digests of the two BACs for complementation testing. For F19F18, this yielded a series of overlapping clones that covered almost the whole BAC, while coverage for F6G17 was incomplete. Of over 20 cosmid clones tested, none converted *srfr1* or *srfr3* plants to the wild-type *avrRps4*-susceptible phenotype. However, sequencing candidate genes in this region identified additional cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993) (Table IV-1) that allowed us to delimit the *srfr3* position to approximately 107 kb between genes At4g37390 (IAA-amido synthase) and At4g37640 (calcium ATPase).

Together with genes eliminated by non-complementing cosmid clones, this allowed us to focus on a small number of remaining candidate genes. Within this locus we sequenced 8 genes in both the *srfr1* and *srfr3* mutant, thus enabling us to distinguish between natural polymorphisms between RLD and the published Col-0 sequence, and induced mutations. We identified premature stop codons in gene At4g37460 in both the *srfr1* and *srfr3* mutant (see below). Because of this physical evidence we renamed *srfr1* as *srfr1-1*, and *srfr3* as *srfr1-2*.

Table VI-1 *SRFR1* fine-mapping markers on the bottom of chromosome 4

Marker name	Start position (bp)	PCR primer sequences (5' → 3')	Primer position in bp (annotation unit)	Restr. enzyme	DNA fragment sizes in bp (Col-0 / RLD)
MUGSSLP01	17230390	GTCGATGTGATCTATAACAT GTGCTTGATTAGTTGAGTAT	59323-59342 59487-59468 (AP22)	n. a.	165 / 150
MUGSSLP02	17352128	TCCTCAACTGTTTTTCTCAGACAT CCACAATCTGATTCGTTGAGTAAT	181061-181084 181874-181851 (AP22)	n. a.	814 / 250
MUGCAPS01	17428923	ATCCGTTGATGATCTTCGCGAT TTGATGGTCTTGCGTTTGGCAT	257856-257877 258488-258467 (AP22)	HaeII	400+230 / 630
MUGSSLP03	17543486	GAAACAGCAGAAAAGACCTAT GAACGCTATGAAGCTCTCTAT	372419-372439 372544-372524 (AP22)	n. a.	126 / 110
MUGCAPS02	17579489	GCTTATTGTCGACTGTACCT CTGAGCTGCAGATACATACA	30657-30676 31363-31344 (F6G17)	NlaIII	700 / 400+300
MUGCAPS03	17651694	CAAGCATCCGGTTTTATCTG ATGAGATTCGGTTTCTACTG	22049-22068 22491-22472 (F19F18)	HincII	310+110+20 / 310+130
MUGCAPS04	17686073	AGTCATATCCTGAAGAGCTT CCAATTTATCTCTGGTGAGT	56428-56447 56822-56803 (F19F18)	Tsp509I	210+180 / 210+130+50
MUGSSLP04	17743592	CATTAATTGCATCGAAAATGTG ATCATAACGAACAGAACTGGAT	42328-42349 42448-42427 (T28I19)	n. a.	121 / 110

A 13.6 kb KpnI subclone of bacterial artificial chromosome (BAC) F6G17 that encompasses At4g37460 complemented both mutants (Figure IV-1A). Apart from At4g37460, this 13.6 kb clone also contains two small flanking genes, At4g37450 and At4g37470. No mutations were found in these genes in *srfr1-1* and *srfr1-2* plants. In addition, a smaller clone of 9 kb that only contains At4g37460 also complemented both mutants (Figure IV-1B). The complementation observed in crosses between *srfr1-1* and *srfr1-2* (Kwon *et al.*, 2004) is therefore a case of interallelic complementation, suggesting that SRFR1 functions as a multimer. Based on the map position, the identification of base pair changes consistent with EMS-induced mutations, and complementation of both mutants with a wild-type copy of At4g37460, we concluded that we had cloned *SRFR1*.

SRFR1 **encodes a TPR protein**

Annotation of *SRFR1* in the Arabidopsis Information Resource database (TAIR; http://www.arabidopsis.org) indicates that it is a 7.4 kb single-copy gene of complex structure (Figure IV-2A). The updated TAIR8 gene model with 22 introns and 23 exons was independently verified by RT-PCR and 3'-RACE (rapid amplification of cDNA ends). In *srfr1-1*, a C to T transition at base number 3764 (numbering according to the full-length genomic sequence in TAIR) introduced a premature stop codon at amino acid position 522 (Gln522*), and in *srfr1-2* a G to A transition at position 5430 a premature stop codon at amino acid position 701 (Trp701*). These mutations were independently verified by developing CAPS

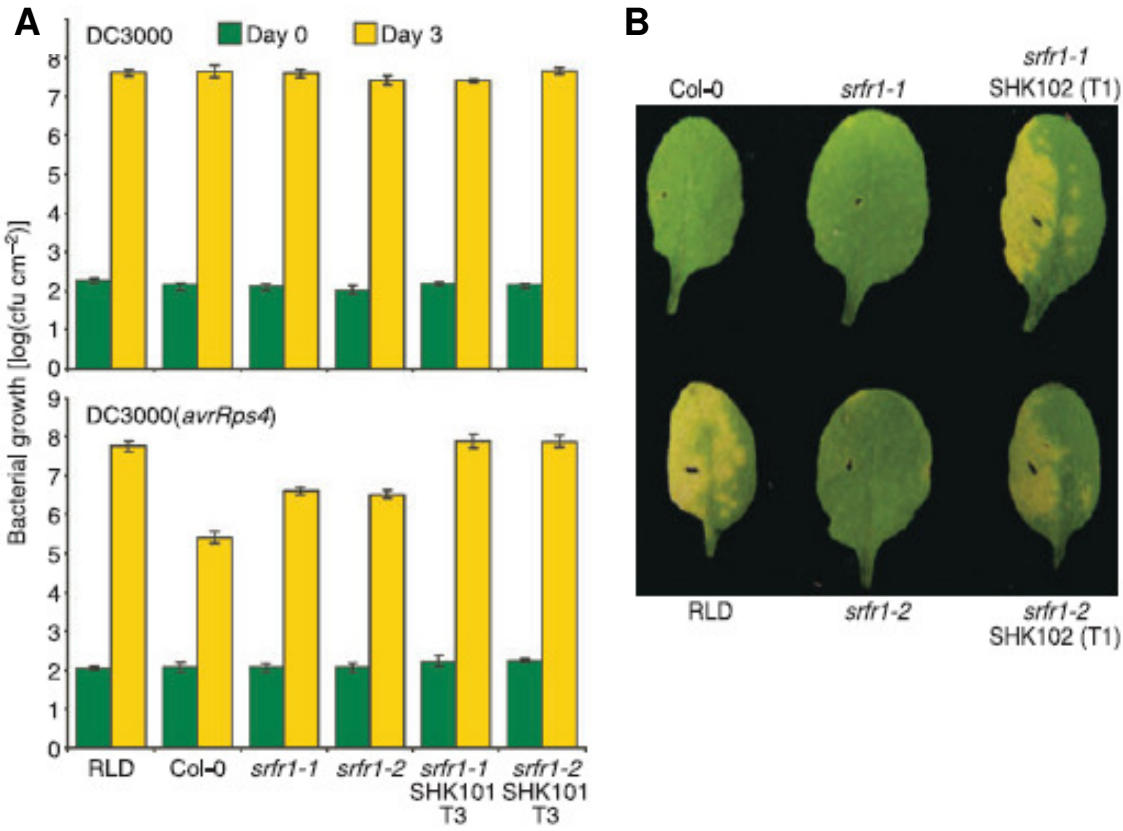
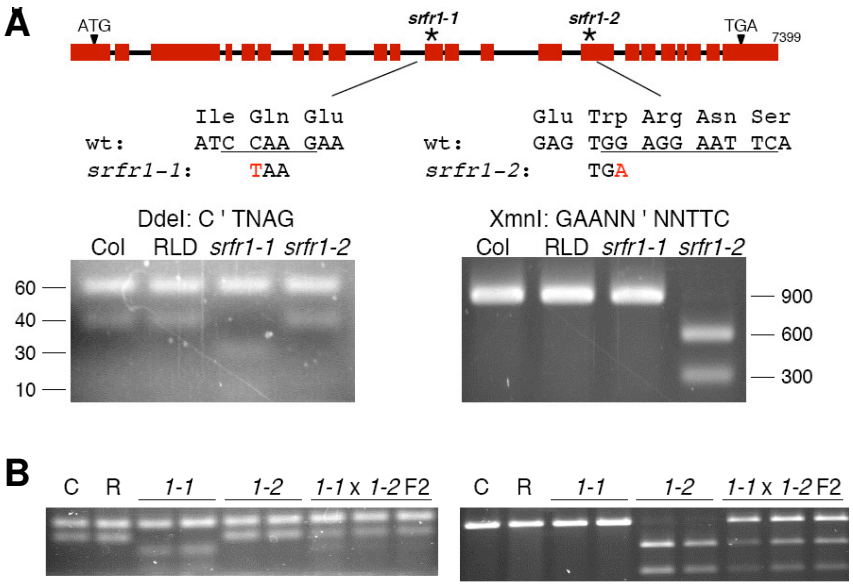

A
DC3000
Day 0
Day 3
Bacterial growth [log(cfu cm−2)]
DC3000(avrRps4)
RLD
Col-0
srfr1-1
srfr1-2
srfr1-1 SHK101 T3
srfr1-2 SHK101 T3
B
Col-0
srfr1-1
srfr1-1 SHK102 (T1)
RLD
srfr1-2
srfr1-2 SHK102 (T1)

Figure IV-1

Figure IV-1. Complementation of *srfr1-1* and *srfr1-2* with a genomic clone of At4g37460. **(A)** *In planta* bacterial growth of virulent DC3000 (top) and DC3000(*avrRps4*) (bottom) on day 0 (green bars) and day 3 (yellow bars) after inoculation of indicated plant lines with bacteria. Values represent averages of colony-forming units (cfu) per cm^2 leaf tissue from triplicate samples, and error bars denote standard deviation. This experiment was repeated once with similar results. For each *srfr1* allele, two additional independent homozygous lines transformed with pSHK101 showed complementation in disease assays in the T3 generation similar to the line chosen for *in planta* bacterial growth assays. **(B)** Complementation of *srfr1-1* and *srfr1-2* with genomic clone pSHK102 containing At4g37460 exclusively. Individual leaves of plants of the indicated lines were infiltrated with a suspension of DC3000(*avrRps4*) at a density of 10^6 cfu/ml. Symptoms were recorded 5 days after inoculation. Complementation as depicted in the image was observed in 15 out of 15 *srfr1-1* and 10 out of 10 *srfr1-2* independent transgenic T1 plants.



C

```
   1 MATATATSER FELAKHCSSR NWSKAIRVLD SLLAKESSIL DICNRAFCYN
  51 QLELHKHVIK DCDKALLLEP FAIQAFILKG RALLALGRKQ EAVLVLEQGY
 101 KSALQQTADV KQLLELEELL KDARREIDGI LKSHATESPQ ETPAYHSEKS
 151 DEKSDKLDNH ESGASSNGNS HESSSELGEQ SKIVSFSKVA SKASKQSDGN
 201 SDLCNGSVYK EKENGKCGSQ INGYYESCKP CNGSDLHDNL AESSDRFGEL
 251 SINGNKISIK SSKMSHKAEA RCGISDESRK NKKYTIARIS GTHSISVDFR
 301 LSRGIAQVNE GNYTKAISIF DKVLKEEPTY PEALIGRGTA YAFQRELESA
 351 IADFTKAIQS NPAASEAWKR RGQARAALGE YVEAVEDLTK ALVFEPNSPD
 401 VLHERGIVNF KSKDFTAAVK DLSICLKQEK DNKSAYTYLG LAFASLGEYK
 451 KAEEAHLKSI QLDSNYLEAW LHLAQFYQEL ADHCKALECI EQVLQVDNRV
 501 WKAYHLRGLV FHGLGEHRKA IQELSIGLSI ENTIECLYLR GSCYHAVGEY
 551 RDAVKDYDAT VDVELDAVEK FVLQCLAFYQ KELALYTASK VSSEFLCFDI
 601 DGDIDPMFKE YWCKRLHPKN VCEKVYRQPP LRESLKKGKL KKQDLAITKQ
 651 KANILRFADL IGKRIQYDCP GFLPNKRQHR MAGLAVIEIA QKVSKAWRIE
 701 WRNSTKGTTK NGKKNRRRER TNILSQNRGG AGCSSSSFSE TSTGYASLED
 751 RSSGRSILSW QDVYSPAVRW RQISEPCDPV VWVNKLSEEF NSGFGSHTPM
 801 VLGQAKVVRY FPNYERTLTL AKSIIKDKLS VRSKKDKVID LSKDEKIEKI
 851 MRAETCDELH NIVGEDFWVA TWCDSTGSEG KRLEGTRITC IQKPGRLGYD
 901 FSIRTPCTPA RWSDFDEEMT SAWEALCTAY CGENYGSTEL DALETVRDAI
 951 LRMTYYWYNF MPLARGTAVT GFVVLLGLLL AANMEFTETI PKGLQIDWEA
1001 ILNVEPGSFV DSVKSWLYPS LKINTSWRDH TEISSAFSTT GAVVAALSTY
1051 ND
```

Figure IV-2

Figure IV-2. *SRFR1* encodes a predicted TPR protein. **(A)** (Top) Gene model for *SRFR1*. Exons are indicated by boxes, introns by lines. The position of the premature stop codons in *srfr1-1* (at 3764 bp) and *srfr1-2* (at 5430 bp) are indicated by asterisks. (Middle) DNA and deduced amino acid sequence of *SRFR1*. Mutated bases are indicated in red. (Bottom) Point mutations in *srfr1-1* and *srfr1-2* visualized by CAPS markers PCR products digested with DdeI (left) and XmnI (right). Approximate band sizes are indicated in bp. For the *srfr1-1* marker, primers used were 5′-AGGCTGGTCTTTAAAGCAGT-3′ and 5′-TCAATGCTCAAGCCAATAGA-3′; for *srfr1-2* 5′-GATTGTCCTGGATTCTTACCA-3′ and 5′-AGTCAGGGTTCTGAAGTAACA-3′. **(B)** Genotyping of *srfr1-1* x *srfr1-2* F2 plants. Analysis of *srfr1-1*x *srfr1-2* F2 plants with allele-specific CAPS markers shows that both mutant alleles are present in the F1 progeny. (Left) *srfr1-1* CAPS marker. (Right) *srfr1-2* CAPS marker. Genomic DNA was isolated from Col-0 (C), RLD (R), two stocks of *srfr1-1* (*1-1*) and *srfr1-2* (*1-2*), and bulked F2 plants derived from three F1 plants. For the cross, *srfr1-1* was the female parent, and *srfr1-2* the male parent. Four out of four F1 plants were scored as susceptible to DC3000*(avrRps4*), and in the F2 generation the phenotype segregated as 66 susceptible to 82 resistant (Kwon *et al*., 2004). Together, these data show that *srfr1-1* and *srfr1-2* display interallelic complementation. **(C)** Predicted SRFR1 protein sequence. Amino acids that constitute TPRs are underlined. Conserved motifs of unknown function in the C-terminus are double underlined. Amino acids at position 522 (Q) and 701 (W) are substituted by stops in *srfr1-1* and *srfr1-2*, respectively, and are shown in bold.

markers that distinguish the mutant alleles from wild-type (Figure IV-2A). These CAPS markers also showed that both *SRFR1* mutant alleles were present in the *srfr1-1* x *srfr1-2* population, confirming interallelic complementation of *srfr1-1* and *srfr1-2* (Figure IV-2B). *SRFR1* is a single-copy gene in *Arabidopsis*.

The predicted SRFR1 protein is 1052 amino acids long (Figure IV-2C). Depending on the stringency, protein motif searches identified 7 to 10 TPR repeats in the N-terminal part of SRFR1, with the terminal capping repeat (D'Andrea and Regan, 2003) positioned at amino acids 534-567, just C-terminal of the *srfr1-1* mutation. TPRs are highly degenerate and bind other proteins of very diverse structure (D'Andrea and Regan, 2003). The C-terminal 485 amino acids form a sequence of unknown function that is conserved among SRFR1-like proteins in plants and other eukaryotes (Figure IV-2C), but does not appear to occur outside this protein family.

Basis for resistance in *srfr1-1* and *srfr1-2* plants

In plants as in other organisms, mRNAs with premature stop codons can be subject to the nonsense-mediated decay pathway (Arciga-Reyes *et al.*, 2006). We therefore measured *srfr1-1* and *srfr1-2* mRNA abundance and found that they were approximately half of wild-type *SRFR1* mRNA levels in RLD and Col-0 (Figure IV-3A). This indicates that *srfr1-1* and *srfr1-2* are not null alleles and is consistent with interallelic complementation observed with *srfr1-1* and *srfr1-2*. Interestingly, mutations in *SRFR1* caused approximately two-fold higher expression of *rps4-RLD* (Figure IV-3B), indicating a possible correlation between

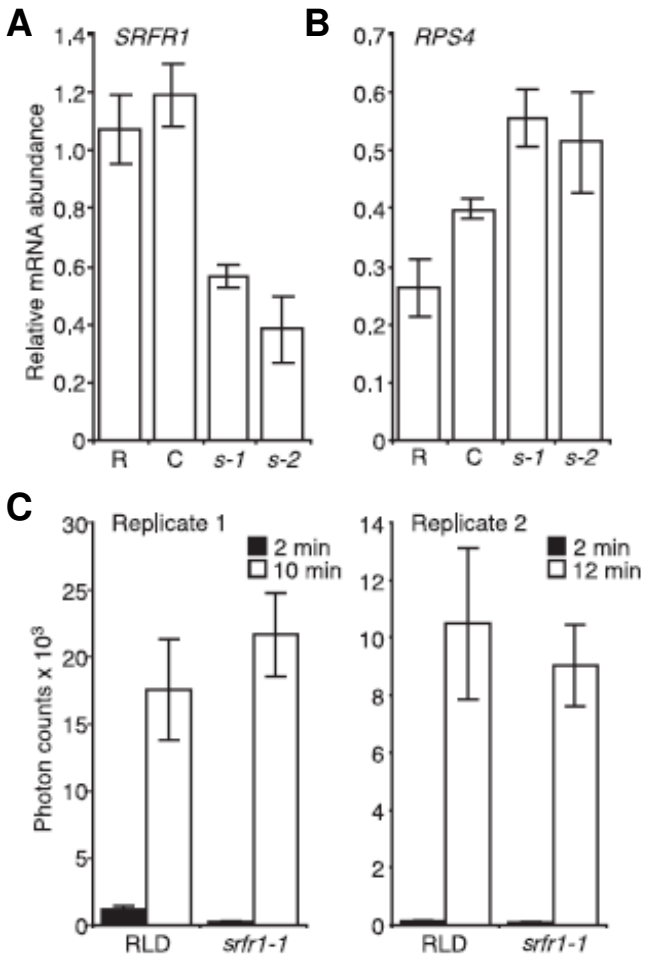

A
SRFR1
Relative mRNA abundance
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
R
C
s-1
s-2
B
RPS4
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
R
C
s-1
s-2
C
Replicate 1
2 min
10 min
Photon counts x 10³
30
25
20
15
10
5
0
RLD
srfr1-1
Replicate 2
2 min
12 min
14
12
10
8
6
4
2
0
RLD
srfr1-1

Figure IV-3

Figure IV-3. *SRFR1* and *RPS4* expression levels and oxidative burst response to the peptide elicitor flg22. **(A,B)** Real-time quantitative RT-PCR analysis of **(A)** *SRFR1* and **(B)** *RPS4* expression in RLD (R), Col-0 (C), *srfr1-1* (*s-1*) and *srfr1-2* (*s-2*) plants. Values represent averages of gene expression levels normalized with *SAND* expression levels from three biological replicates, and error bars denote standard deviation. Equivalent results were obtained by normalization with *UBQ10* expression levels (data not shown). **(C)** Reactive oxygen species production in response to 10 nM flg22 in RLD wild-type and *srfr1-1* plants. Results from two replicate experiments are shown. Reactive oxygen production at 10 min (replicate 1) and 12 min (replicate 2) represent peak production for both RLD and *srfr1-1*. Values represent averages from 6 (replicate 1) and 5 (replicate 2) samples, and error bars denote standard error. Based on two-tailed, two-sample equal variance t-tests, the difference in peak response to flg22 between RLD and *srfr1-1* is not statistically different ($p>0.4$ in replicate 1, $p>0.6$ in replicate 2).

SRFR1 and *R* gene expression levels. However, previous work showed that two-fold differences in functional *RPS4-Ler* or non-functional *rps4-N195D* transgene expression levels in RLD did not influence the resistance phenotype (Zhang and Gassmann, 2003). Premature stop codon-containing exons in *srfr1* mRNA could also be removed by changes in pre-mRNA splicing. To test this, we determined the splicing pattern of *srfr1* mRNA by reverse transcription PCR. We did not find any evidence for changes in splicing in the mutant alleles (data not shown). We conclude that the *srfr1-1* and *srfr1-2* phenotypes are not based on drastic changes in *SRFR1* gene expression. The stability and possible function of mutant srfr1 protein, and the function of the C-terminus in wild-type SRFR1, warrant further study.

To test whether the level of basal resistance is altered in *srfr* mutants, we measured reactive oxygen species production in RLD and *srfr1-1* plants in response to the peptide elicitor flg22. Consistent with the full susceptibility of *srfr* mutants to virulent DC3000 (Figure IV-1A), we found no difference in the response to flg22 (Figure IV-3C). However, resistance in *srfr* mutants fully required EDS1, a central regulator of effector-triggered immunity mediated by R proteins of the Toll/interleukin-1 receptor (TIR)-nucleotide binding site (NBS)-leucine-rich repeat (LRR) class (Aarts *et al*., 1998). As shown in Figure IV-4, F4 plants homozygous for the *eds1-1* and *srfr1-1* mutations and for the *rps4-RLD* allele were susceptible to DC3000(*avrRps4*). In the F3 generation with plants homozygous for *srfr1-1* and *rps4-RLD* but segregating for *eds1-1*, siblings with wild-type copies of *EDS1* were resistant (data not shown). Combined with the

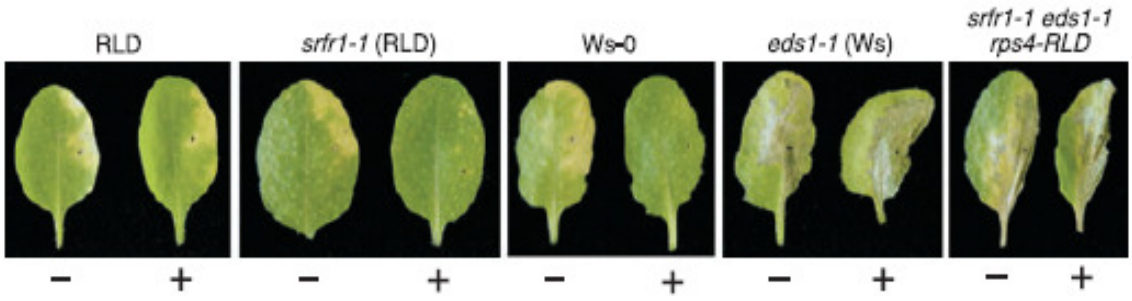


Figure IV-4

Figure IV-4. *srfr1*-mediated resistance requires *EDS1*. The accession of wild-type plants and the genotype of mutant plants (back-ground accession in parentheses) are indicated above each panel. The right half of individual leaves were infiltrated with DC3000(empty vector) (-) or DC3000(*avrRps4*) (+) at a density of 106 cfu/ml. Symptoms were recorded 5 days post inoculation. Symptoms with the *eds1-1 srfr1-1 rps4-RLD* triple mutant are representative for leaves from a total of 40 out of 40 F4 plants derived from four F3 plants homozygous for *eds1-1*, *srfr1-1* and *rps4-RLD*.

lack of elevated basal resistance and of constitutive expression of *PR1* (Kwon *et al.*, 2004), these results support the hypothesis that mutations in *SRFR1* potentiate an *R* gene-mediated response triggered by *avrRps4*.

Expression of *SRFR1*

Based on data in the Genevestigator database (https://www.genevestigator.ethz.ch/at/) (Zimmermann *et al.*, 2004), *SRFR1* is expressed in all plant tissues at all stages and is not strongly regulated by various treatments, including pathogens. To examine *SRFR1* expression at the sub-tissue level, we constructed transcriptional fusions with the *SRFR1* native promoter and the β-glucoronidase (*GUS*) gene. As shown in Figure IV-5, the *SRFR1* promoter directs expression of the reporter gene in all tissues at all stages of development, except in very young flowers (Figure IV-5j,k) and older siliques (Figure IV-5l). The very broad expression pattern is consistent with electronic Northern data on *SRFR1*.

Phylogenetic analysis of SRFR1

Highly similar predicted SRFR1-like proteins were identified in plants, including rice (*Oryza sativa*, 63% amino acid identity) using the BLAST algorithm (Altschul *et al.*, 1997). Outside the plant kingdom, SRFR1-like proteins with significant similarity were found in other eukaryotes such as slime molds and amoeba (*Dictyostelium discoideum*, *Entamoeba histolytica*; 30% identity/50% similarity) and in sea urchin and vertebrates, including the human TTC13 protein (26%

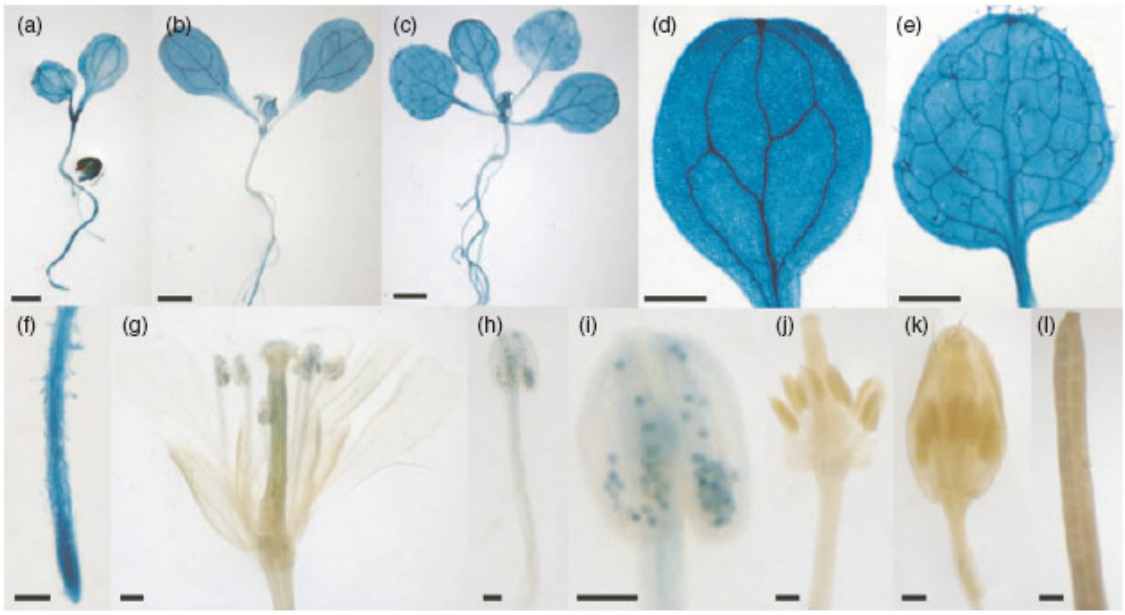


Figure IV-5

Figure IV-5. *SRFR1* is ubiquitously expressed. *SRFR1* expression as indicated by a GUS reporter driven by the *SRFR1* promoter in 3-day (a), 5-day (b) and 7-day (c) old seedlings, a 7-day old cotyledon (d), leaf (e) and root (f), flower (g), anther (h), pollen grains (i, close-up of h), young flowers (j,k), and silique (l). Scale bars: 2 mm (a-c), 500 μm (d,e,g,j-l), 100 μm (f,h,i).

identity, 46% similarity) (Figure IV-6A). These sequence similarities span the whole length of the SRFR1 protein, not just the TPR domain, and identify proteins of similar length but unknown function in these organisms.

Genes encoding SRFR1-like proteins are absent in the fully sequenced genomes of *Saccharomyces cerevisiae*, *Drosophila melanogaster*, or *Caenorhabditis elegans*, and in eubacteria and archea. However, using PSI-BLAST significant sequence similarity was found between the SRFR1 TPR domain and the TPR domains of *C. elegans* and *D. melanogaster O*-linked *N*-acetylglucosamine transferases (OGTs), and *S. cerevisiae* Ssn6 (Figure IV-6B). OGTs and ScSsn6 function in transcriptional repressor complexes in which the TPR domains interact with diverse proteins such as transcription factors or transcriptional corepressors (Jackson and Tjian, 1988; Lubas and Hanover, 2000; Smith and Johnson, 2000; Yang *et al.*, 2002). The closest relatives of SRFR1 in the *Arabidopsis* proteome, SPINDLY (SPY) and SECRET AGENT (SEC) are more similar to OGTs (Figure IV-6A) and share the C-terminal *N*-acetylglucosamine transferase domain, which is absent in SRFR1. In contrast to the alignment of full-length proteins, where distinct clades based on diverged C-termini are evident, alignment of TPR domains alone show a closer sequence relationship between AtSRFR1, ScSsn6 and OGTs (Figure IV-6B). The TPR protein AtSGT1b functions as a co-chaperone of R proteins (Shirasu and Schulze-Lefert, 2003). However, amino acid sequences of TPRs in SRFR1-like and SGT1-like proteins are distinct (Figure IV-6B).

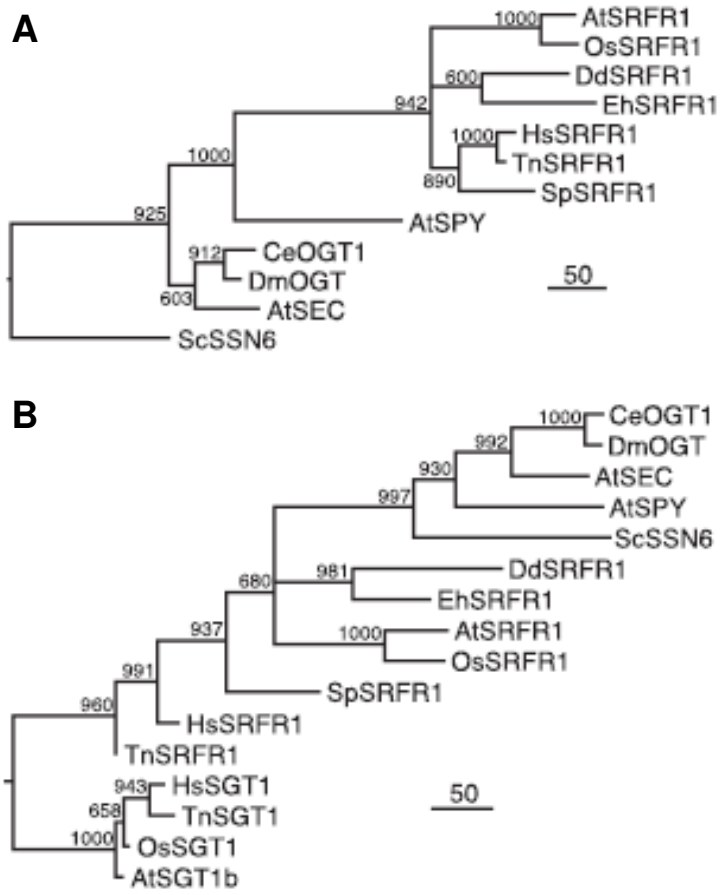

A
1000
AtSRFR1
OsSRFR1
600
DdSRFR1
EhSRFR1
942
1000
HsSRFR1
TnSRFR1
890
SpSRFR1
1000
925
AtSPY
912
CeOGT1
DmOGT
603
AtSEC
50
ScSSN6
B
1000
CeOGT1
992
DmOGT
930
AtSEC
997
AtSPY
ScSSN6
981
DdSRFR1
680
EhSRFR1
937
1000
AtSRFR1
OsSRFR1
991
SpSRFR1
960
HsSRFR1
TnSRFR1
943
HsSGT1
658
TnSGT1
50
1000
OsSGT1
AtSGT1b

Figure IV-6

Figure IV-6. SRFR1 shows sequence similarity to proteins that regulate transcription. **(A)** Cladogram of full-length proteins. **(B)** Cladogram of TPR domains. Scale bar indicates sequence distance expressed as the mean number of sequence differences per 100 amino acid positions as calculated by the Kimura distance formula. Numbers represent bootstrap values in support of the indicated branch points from 1000 iterations. Species abbreviations are: At (*A. thaliana*), Os (*O. sativa*), Dd (*D. discoideum*) Eh (*E. histolytica*), Hs (*Homo sapiens*), Tn (*Tetraodon nigroviridis*, puffer fish), Sp (*Strongylocentrotus purpuratus*, purple sea urchin), Ce (*C. elegans*), Dm (*D. melanogaster*), Sc (*S. cerevisiae*).

Localization of SRFR1 in *Nicotiana benthamiana* leaf cells

Based on phylogenetic analysis, we hypothesized that SRFR1 functions as a scaffold protein in a transcriptional repressor complex. *In silico* analysis of SRFR1 localization was suggestive of nuclear localization despite the absence of a canonical nuclear localization signal. To experimentally test SRFR1 localization, we constructed translational fusions with GFP at the N-terminus of SRFR1 driven by the cauliflower mosaic virus 35S promoter. This construct complemented *srfr1-1* and *srfr1-2* (Figure IV-8), but did not provide a detectable fluorescence signal in transgenic plants. 35S:*GFP* control and 35S:*GFP-SRFR1* were expressed in *N. benthamiana* leaf cells using *Agrobacterium*-mediated transient expression. GFP-SRFR1 was only detected when an *Agrobacterium* strain delivering the viral silencing suppressor HcPro was co-infiltrated, and this strain was consequently also used with GFP controls. As shown in Figure IV-7A, GFP was localized throughout the cytoplasm and nucleus. With GFP-SRFR1, we observed punctate localization in the nucleus, perinuclear region and cytoplasm. The perinuclear localization of GFP-SRFR1 is more evident in cells with lower expression (Figure IV-7B). Western blots confirmed that the GFP-SRFR1 localization as exemplified in Figure IV-7 was based on full-length protein expression in *N. benthamiana* leaves (Figure IV-7C).

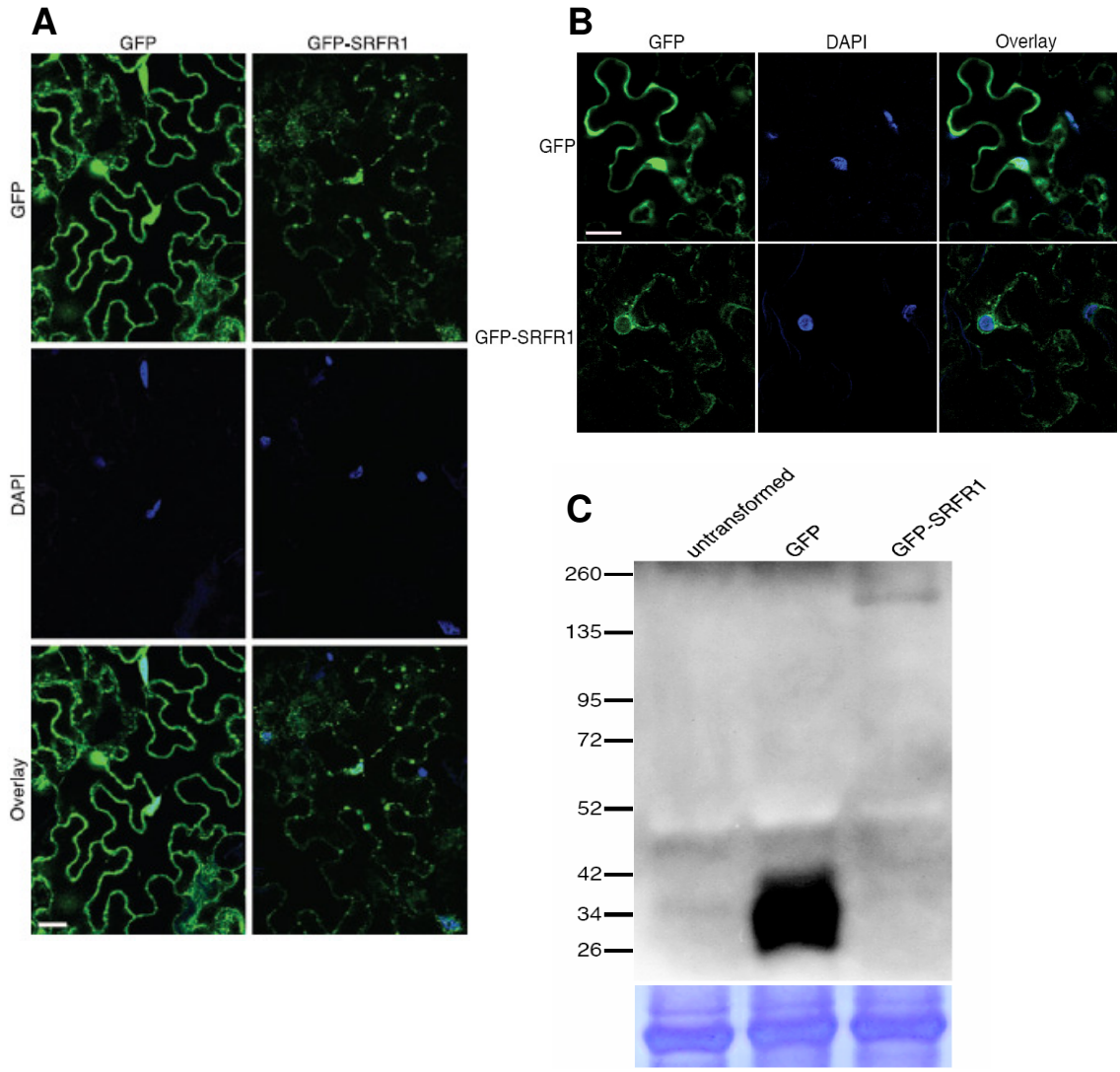

A
GFP
GFP-SRFR1
GFP
DAPI
Overlay
B
GFP
DAPI
Overlay
GFP
GFP-SRFR1
C
untransformed
GFP
GFP-SRFR1
260
135
95
72
52
42
34
26

Figure IV-7

Figure IV-7. SRFR1 localizes to the nucleus and the cytoplasm. **(A)** GFP control (left column) and GFP-SRFR1 (right column) were transiently expressed in *N. benthamiana* leaves. Confocal images were taken three days after *Agrobacterium* infiltration. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Results are representative of four independent experiments. Scale bar: 20 µm for all panels. **(B)** Perinuclear localization of GFP-SRFR1. GFP control (top row) and GFP-SRFR1 (bottom row) detected by confocal fluorescence microscopy. DAPI was used to stain nuclei. Scale bar: 20 µm for all panels. **(C)** Detection of full-length GFP-SRFR1 in *N. benthamiana* by western blot. The GFP and GFP-SRFR1 proteins were transiently expressed in *N. benthamiana* via agro-infiltration. Both samples were co-infiltrated with an *Agrobacterium* strain delivering the viral silencing suppressor Hc-Pro. At 3 days post inoculation, total protein was extracted from three leaf discs obtained from each infiltrated patch and also from a non-transformed leaf. The extract was subjected to immunoblot analysis with rabbit anti-GFP primary and HRP-conjugated goat anti-rabbit secondary antibodies. The same blot was stained with Coomassie staining solution to show equal loading of protein samples. No GFP-SRFR1 was detected in the absence of Hc-Pro (data not shown).

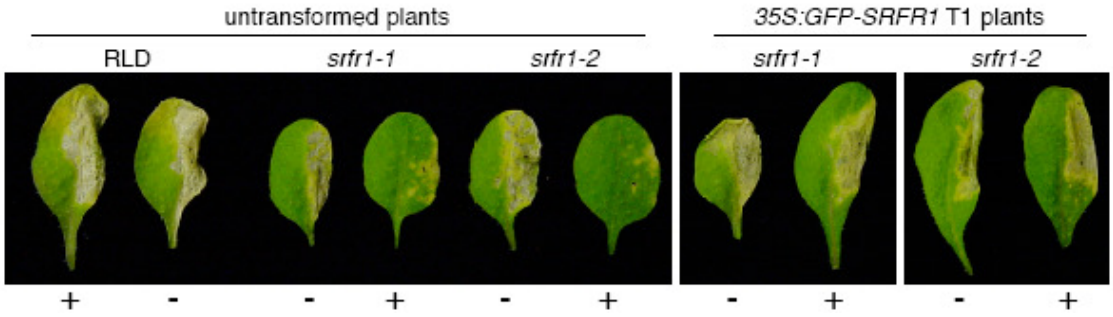

untransformed plants
35S:GFP-SRFR1 T1 plants
RLD
srfr1-1
srfr1-2
srfr1-1
srfr1-2
+ - - + - + - + - +

Figure IV-8

Figure IV-8. Complementation of *srfr1* mutants with a *35S:GFP-SRFR1* construct. Individual leaves of untransformed control plants (left) and transgenic plants (right) of the indicated genotype were infiltrated with a suspension of virulent DC3000 (–) or DC3000(*avrRps4*) (+) at a density of 10^6 cfu/ml. Symptoms were recorded 5 days after inoculation. Disease symptoms in the transgenic plants as depicted in the image are representative of 5 out of 9 *srfr1-1* and 9 out of 21 *srfr1-2* independent transgenic T1 plants.

Discussion

We have used a genetic suppressor approach to identify SRFR1, a negative regulator of ETI to *avrRps4* in Arabidopsis accession RLD. This accession possesses a natural missense mutation in the *avrRps4*-cognate *R* gene *RPS4* (Hinsch and Staskawicz, 1996; Gassmann *et al*., 1999; Zhang and Gassmann, 2003). Based on the presence of TPRs in the predicted SRFR1 protein, we hypothesize that SRFR1 exerts its regulatory function by interacting with other proteins. However, TP repeats show high variability, and TPR proteins are involved in virtually every aspect of cell biology. The presence of TPRs alone therefore does not suggest a particular regulatory function, for example *R* gene expression or R protein stabilization. Notably, there is little sequence similarity between the TPR domains of SRFR1 and the R protein co-chaperone SGT1b. In addition, none of the SRFR1-like proteins in other eukaryotes has an assigned function. Phylogenetic analysis indicates that the SRFR1 TPR domain shows sequence similarity to proteins in *S. cerevisiae* and *C. elegans* that function as transcriptional regulators.

The *C. elegans* OGT-1 (*O*-linked *N*-acetylglucosamine transferase) protein has an N-terminal TPR domain, but is distinct from SRFR1 at the C-terminus. OGT proteins have a C-terminal catalytic domain that functions in glycosylating Ser and Thr sidechains with N-acetylglucosamine. It is thought that this glycosylation is in direct competition with phosphorylation by Ser/Thr protein kinases and thus may downregulate kinase substrates (Wells *et al*., 2001). The

TPR domain is required for OGT multimerization and binding of target proteins such as transcription factors (Jackson and Tjian, 1988; Lubas and Hanover, 2000). Interestingly, mammalian OGT was found to be bi-functional: a truncated OGT lacking the catalytic *N*-acetylglucosamine transferase domain was still able to repress transcription by binding, via the OGT TPR domain, the transcriptional corepressor mSin3A (Yang *et al.*, 2002). Amino acid sequence similarity between SRFR1 and OGT1 therefore is suggestive of a role of SRFR1 in transcriptional repression despite the absence of a recognizable catalytic domain in SRFR1.

The *S. cerevisiae* protein with highest similarity to SRFR1 is Ssn6, also known as Cyc8. Ssn6 functions in a general transcriptional repressor complex with Tup1, a protein with WD40 repeats (Smith and Johnson, 2000). The function of Ssn6 in this complex is the binding, again via its N-terminal TPR domain, of transcriptional regulators that possess DNA binding domains. Although these transcription factors can also make contact with Tup1, in some cases Ssn6 appears to be essential for directing the Tup1 repressor function to the appropriate regions in the genome. Arabidopsis does not appear to have a Ssn6-like protein with sequence similarity across the whole protein. The Arabidopsis OGTs SEC and SPY show highest sequence similarity in the TPR domain to ScSsn6. AtSPY is a negative regulator of gibberellin signaling (Sun and Gubler, 2004), but the mechanism of regulation is unknown.

Based on this phylogenetic analysis, we hypothesize that SRFR1-like proteins function as scaffold proteins in a transcriptional repressor complex that has diverged considerably from the canonical ScSsn6-ScTup1 complex, perhaps

to regulate a very particular subset of responses such as non-self recognition. Consistent with this hypothesis, a sub-pool of SRFR1 was localized to the nucleus when transiently expressed in *N. benthamiana*. The punctate nucleocytoplasmic and perinuclear localization of SRFR1 is reminiscent of rat (Kreppel *et al.*, 1997) and *C. elegans* OGT (Lubas *et al.*, 1997). The nucleus contains several punctate compartments that are associated with diverse functions such as regulation of transcription and pre-mRNA processing (Dellaire *et al.*, 2003). Further experiments are required to determine whether SRFR1, like OGT, has functions in both the nuclear and cytoplasmic compartment, or whether relocalization of SRFR1 serves to regulate activity of this protein.

In our model, activation of ETI by *avrRps4* would overcome the repressor effect of SRFR1 in the presence of a fully functional RPS4 R protein, whereas in the accession RLD a missense mutation in RPS4 (Zhang and Gassmann, 2003) prevents ETI activation. Identification of genes that are regulated by the putative SRFR1 transcriptional repressor complex, and whether base-line expression or inducibility of these genes is elevated, will require further study. Conceivably, these genes could encode proteins that effect resistance against bacterial pathogens (Kalde *et al.*, 2007), or proteins that function in AvrRps4 detection and resistance signaling, or both. Either way, *srfr* plants would be closer to a threshold for ETI activation. Previously, we did not detect qualitative differences in *PR1* expression between mutant and wild-type plants in uninduced tissue or tissue induced with salicylic acid (Kwon *et al.*, 2004). The two-fold increase in

RPS4 mRNA in *srfr1-1* and *srfr1-2* mutants detected here may indicate that other *R* and defense genes are slightly upregulated in the mutant background.

In both the *srfr1-1* and *srfr1-2* mapping population, *srfr1*-mediated resistance segregated with a ratio that is expected if resistance depends on a recessive and a dominant gene. This conclusion was supported by observing segregation of dominant resistance in select F3 families derived from resistant F2 plants in the mapping population. Genotyping of resistant F2 and F3 plants showed that the dominant gene is not the *rps4*-RLD allele. We therefore proposed that RLD possesses another R protein with weak affinity for *avrRps4* that is able to trigger effective ETI when SRFR1 is non-functional (Kwon *et al*., 2004). In a study of the complex *Rp1* locus in maize, it was found that most recombinant haplotypes with novel resistance specificities showed weaker resistance against maize rust biotypes recognized by the parental haplotypes, indicating that paralogs conferring weaker resistance were masked by strong *R* genes in the parents (Smith and Hulbert, 2005). Interestingly, recent work identified the *Arabidopsis TAO1* TIR-NBS-LRR gene as a weak resistance gene for the effector AvrB that additively contributes to the major resistance function governed by the coiled-coil-NBS-LRR gene *RPM1*. The weak resistance function of *TAO1* was not evident in bacterial growth assays with *rpm1* plants (Eitas *et al*., 2008). We have crossed *srfr1* plants to mutants in *RPS2*, *RPM1* and *NDR1*, and have tested double-mutants with *avrRpt2*, *avrRpm1*, or both, respectively. These double mutants were as susceptible as the corresponding single mutants (data

not shown), indicating that mutations in *SRFR1* do not enhance resistance to *avrRpm1* or *avrRpt2* in the corresponding *R* gene mutants.

There is increasing evidence for a direct connection between the activity of some R proteins and transcriptional regulation. RPM1 interacts with TIP49a, a conserved protein that interacts with the TATA binding protein complex. Interestingly, reduction in *TIP49a* expression increases ETI to *H. parasitica* isolates by *RPP5* and *RPP2* (Holt *et al.*, 2002). The *Arabidopsis* MOS3 nucleoporin and the MOS6 importin alpha3 protein are required for resistance activation by the constitutively active TIR-NBS-LRR R protein mutant snc1 (Palma *et al.*, 2005; Zhang and Li, 2005). The *R* gene *RRS1* constitutes a fusion of a TIR-NBS-LRR gene and a WRKY transcription factor gene (Deslandes *et al.*, 2002). Intriguingly, RRS1 localizes to the nucleus in the presence of the corresponding effector protein PopP2 (Deslandes *et al.*, 2003). Several effector proteins have hallmarks of transcriptional regulators and are localized to the nucleus (van den Ackerveken *et al.*, 1996; Zhu *et al.*, 1999; Kay *et al.*, 2007). Recent results also demonstrate nuclear localization of subpools of the barley MLA10, the tobacco N and the *Arabidopsis* RPS4 protein (Burch-Smith *et al.*, 2007; Shen *et al.*, 2007; Wirthmueller *et al.*, 2007). In addition, MLA10 was found to interact with WRKY transcription factors upon elicitation, presumably to regulate defense gene transcription (Shen *et al.*, 2007). The study of SRFR1 function should provide further insight into the regulation of the transcriptional reprogramming process that occurs during the plant ETI response, and provide models for regulatory functions of SRFR1-like proteins in animals.

Materials and Methods

Map-based cloning and complementation of *srfr1* mutants

Screening of F2 plants from crosses of *srfr1-1* or *srfr1-2* and *rps4-1*, and genotyping of resistant F2 plants or F3 families were performed as described previously (Kwon *et al*., 2004). BAC clones F6G17 and F19F18 were provided by the Arabidopsis Biological Resources Center (ABRC). BAC DNA was partially digested with HindIII and subcloned into the cosmid binary vector pCLD04541 (Bancroft *et al*., 1997). The genomic subclone pSHK101 containing At4g37450, At4g37460 and At4g37470 was generated by digesting BAC F6G17 with KpnI and cloning the 13.6 kb band into pCAMBIA2300. The *SRFR1*-specific clone pSHK102 was generated by digesting pSHK101 with SpeI and cloning the 9 kb band into the compatible XbaI site of pCAMBIA2300.

Clones were transferred to *Agrobacterium tumefaciens* strain GV3101 by electroporation. Homozygous *srfr1-1* and *srfr1-2* plants were transformed by floral dip (Clough and Bent, 1998) and selected as described (Zhang and Gassmann, 2003). For disease assays, plants were grown in a Conviron E-15 growth chamber (Controlled Environments, Winnipeg, Manitoba, Canada) with an 8 h light/16 h dark cycle at 22 °C and 70% humidity with a light intensity of 90-140 μmol m^{-2} sec^{-1}. Five-week-old plants were infiltrated with a bacterial suspension of 1×10^6 cfu ml^{-1} in 10 mM $MgCl_2$ using a needle-less syringe. For *in planta* bacterial growth measurements, plants were infiltrated as above with

5×10^4 cfu ml^{-1} bacteria, and growth was quantified as described previously (Kwon *et al.*, 2004).

Elicitor response

Reactive oxygen production was measured as described (Heese *et al.*, 2007). Briefly, leaf disks of 0.25 cm^2 from 4-5 week -old plants were cut into 4 pieces and floated on sterile water in wells of a 96-well plate overnight. The next day, water was replaced with 100 µl oxidative burst assay solution containing 200 µM luminol (Sigma, St. Louis, MO), 20 µg/ml horseradish peroxidase (Sigma), and 10 nM flg22. Reactive oxygen species production was measured in a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA).

Epistasis analysis

srfr1 plants were crossed to *eds1-1* (Falk *et al.*, 1999). Using molecular markers, F2 plants homozygous for *rps4-RLD* and *srfr1*, and heterozygous for *eds1-1* were chosen. Resistance to AvrRps4 and the genotype at the *EDS1* locus were monitored in the F3 generation. F3 plants with a wild-type copy of *EDS1* were found to be resistant. Susceptible F3 plants were propagated to the F4 generation and retested.

Real-time quantitative PCR

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA). For RT-PCR experiments, first-strand cDNA was synthesized from 2µg of total RNA

using an oligo$(dT)_{15}$ primer and M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions. Real-time qRT-PCR was performed using an ABI 7500 system and SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, UK) with the following cycling conditions: 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles of 95 ºC for 15 s and 59 ºC for 1 min. Primers used were: 5'-CTGGATATGCCTCACTAGAAG-3' and 5'-CACTGGGTCACAAGGCTCTG-3' for *SRFR1*; 5'-CCTAACATTATGGGCATCATCA-3' and 5'-CCGCCTTCACAATTTCATTGA-3' for *RPS4*; 5'-AACTCTATGCAGCATTTGATCCACT-3' and 5'-TGATTGCATATCTTTATCGCCATC-3' for *SAND* family gene (At2g28390); 5'-GGCCTTGTATAATCCCTGATGAATAAG-3' and 5'-AAAGAGATAACAGGAACGGAAACATAGT-3' for *UBQ10* (At4g05320). Primer sequences for *SAND* and *UBQ10* were taken from (Czechowski *et al*., 2005). The *SAND* family gene was previously found to be expressed approximately 25-fold lower than *UBQ10* (Czechowski *et al*., 2005) and is therefore closer to the expression levels of *SRFR1* and *RPS4*. In addition, it was found to be a stable reference gene during basal defense responses to chitin (Libault *et al*., 2007). PCR efficiencies were calculated using linear regression in the LinRegPCR software (Ramakers *et al*., 2003) and were found to be >1.7 for all samples, with R^2>0.995. The cycle threshold (Ct) value for the *SAND* family gene was subtracted from the Ct value for *SRFR1* and *RPS4*. Because sample-to-sample variability for PCR efficiencies was small and comparable between reference and

test genes, expression values (Exp) for *SRFR1* and *RPS4* were calculated using the equation Exp = $2^{-\Delta Ct}$.

Phylogenetic analyses

Protein sequences were aligned using the MegAlign software in the Lasergene package (DNASTAR, Madison, WI) with the following parameters: ClustalW, protein weight matrix Gonnet 250, gap penalty 10, no gap length penalty. Phylogenetic trees were constructed by the neighbor-joining method implemented in ClustalW. For alignment of TPR domains, proteins were analyzed using pfam (http://www.sanger.ac.uk/Software/Pfam/) (Finn *et al.*, 2006). Amino acid sequences encompassing the first to the last TPR with a trusted match, including intervening non-TPR sequences, were aligned as above. Accession numbers for the aligned proteins: NP_195462 (AtSRFR1); NP_001058749 (OsSRFR1); XP_645222 (DdSRFR1); XP_652971 (EhSRFR1); Q8NBP0 (HsSRFR1, TTC13); CAG12970 (TnSRFR1); XP_787775 (SpSRFR1); O18158 (CeOGT1); NP_523620 (DmOGT); Q9M8Y0 (AtSEC); AAC49446 (AtSPY); NP_009670 (ScSsn6); NP_006695 (HsSGT1); CAF97651 (TnSGT1); AAF18438 (OsSGT1); NP_192865 (AtSGT1b).

***SRFR1* expression analysis**

For the *SRFR1*:GUS construct, the *SRFR1* promoter (2.5 kb upstream of the start codon) was amplified with the primer pair 5'-GATCAAGCTTACCGTCCTGATCTCTTCT-3' and 5'–

CGCGGATCCTTTGAGCGGTGGTGTCTC–3’ using Ex Taq Polymerase (Takara, Shiga, Japan). The PCR product was digested with HindIII and BamHI and ligated into the binary vector pBI101. GUS staining was performed on transformed Col-0 T2 plants. At least 15 independent transgenic lines were tested and showed similar expression patterns. Untransformed Col-0 control plants were stained for GUS activity in parallel and did not show any staining.

SRFR1 protein localization

The coding region of *SRFR1* was amplified by PCR using first-strand cDNA (see above) as template and the primers 5’-AAAAAGCAGGCTCAATGGCGACGGCGACGGCG-3’ and 5’-AGAAAGCTGGGTAATCGTTGTAAGTGCTAAGCG-3’. The PCR product was cloned into the entry vector pDONR201 using the Gateway BP reaction (Invitrogen). To generate the *GFP-SRFR1* construct, the entry clone was recombined into pMDC43 containing an N-terminal GFP fusion tag using the Gateway LR reaction (Invitrogen). All clones were verified by sequencing.

GFP constructs were electroporated into the *A. tumefaciens* strain C58C1 harboring the virulence plasmid pCH32. For transient expression in *N. benthamiana* leaf cells (Bendahmane *et al*., 2000), *Agrobacterium* cultures grown overnight were pelleted and resuspended in 10 mM $MgCl_2$. The *Agrobacterium* strain expressing the silencing suppressor HcPro was a kind gift from Peter Moffett (Boyce Thompson Institute for Plant Research) and was co-infiltrated with both GFP and GFP-SRFR1 constructs to boost GFP-SRFR1 expression. Leaves

from four week-old *N. benthamiana* plants were co-infiltrated with a mixed suspension of *Agrobacterium* in which each strain was adjusted to an optical density of 0.3 at 600 nm. Three days after *Agrobacterium* infiltration, the same area was re-infiltrated with DAPI solution (0.1 M Na_2HPO4, 1 mM EDTA, 0.1% Triton X-100, DAPI 1 μg/ml). A small section of the infiltrated area was excised and used for confocal microscopy.

Confocal fluorescence images were acquired on a Zeiss LSM 510 META NLO two-photon point-scanning confocal system mounted on an Axiovert 200M inverted microscope with a 40x/1.2 C-Apochromat water immersion objective (Zeiss, Thornwood, NY). DAPI staining was visualized by two-photon excitation with a Chameleon pulsed laser (Coherent Inc., Santa Clara, CA) tuned to 760 nm with emission detection at 435-485 nm using appropriate band-pass filters. GFP was visualized with a 488 nm laser and a 500-550 nm band-pass emission filter.

For western blot analysis, 3 leaf discs from the corresponding infiltrated patch were excised with a microcentrifuge tube cap. The leaf discs were macerated in 200 μl of 8 M urea. Fifty μl of 5X loading dye was added to the suspension, the sample boiled for 5 minutes and centrifuged for 5 minutes at maximum speed. The supernatant was collected in a fresh tube and used for western blot analysis. Western blots were performed with rabbit anti-GFP primary antibodies (Invitrogen) and goat anti-rabbit-HRP conjugated secondary antibodies (Rockland Immunochemicals) and visualized with ECL Plus chemiluminescent kit (GE Healthcare Biosciences).

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Chapter V

SRFR1 interacts with three TCP transcription factor family members and may function in a transcriptional repressor complex

Abstract

Plant immunity is mediated in large part by specific interactions between a host resistance (*R*) gene and a pathogen avirulence (*avr*) gene. *Avr* genes encode effectors for pathogen fitness, so this immune response is now named effector trigger immunity (ETI). ETI needs to be tightly controlled both positively and negatively to enable normal plant growth, because constitutively activated defense response caused by excessive ETI is detrimental to the host. In previous work, we reported that mutations in *SRFR1* (*Suppressor of rps4-RLD 1*), identified in a suppressor screen, reactivated *avrRps4*-triggered immunity. Resistance in *srfr1* mutants was dependent on the known positive ETI regulator *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY 1*), suggesting that *SRFR1* is a negative regulator of *avrRps4*-dependent ETI in Arabidopsis. SRFR1 encodes a pioneer TPR (Tetratricopeptide Repeat) protein conserved between plants and animals. The SRFR1 TPR domain has significant sequence similarity to those of the *Saccharomyces cerevisiae* Ssn6 and *Caenorhabditis elegans* OGT (*O*-linked *N*-acetylglucosamine transferase) proteins, which function as transcriptional repressors. SRFR1 was targeted to the nucleus in *Nicotiana benthamiana*. Here we show that mRNA levels of defense related genes are upregulated in *srfr1* mutants. SRFR1 physically interacts with three members of the TCP (TEOSINTE BRANCHED1, CYCLOIDEA and PCF) transcription factor family, TCP8, TCP20 and TCP22, in a yeast-two hybrid system. In addition, SRFR1, TCP8, TCP20 and TCP22 dimerize and interact with each other in the nucleus of *Nicotiana*

benthamiana. With phylogenetic analysis, the nuclear localization and TCP interactors, we propose that SRFR1 functions in a transcriptional repressor complex that balances plant resistance activation and suppression.

Introduction

Plants are hosts to pathogens such as viruses, bacteria, fungi and nematodes (Staskawicz et al., 1995). Plants possess mechanically and chemically pre-formed defense layers, but this is not enough to halt the growth of pathogens. Through evolutionary pressure, plants developed a surveillance system to generate efficient defense mechanisms. To recognize the pathogen- derived molecules, so-called PAMPs (pathogen associated molecular patterns), plants evolved a primary immune system, PAMP-triggered immunity (PTI) or basal defense. Pathogens overcame this primary immune layer and suppress PTI by delivering virulence factors (effector proteins) into plant cells. In the co-evolution of the plant-microbe interaction, plants in turn developed resistance (R) proteins to monitor the pathogen effectors, a process now referred to as effector-triggered immunity (ETI) and originally described in the gene-for-gene hypothesis (Flor, 1971; Chisholm et al., 2006; Jones and Dangl, 2006). Thus, the combination of ETI and PTI determines the degree of host plant defenses against microbial pathogen attack (Tao et al., 2003).

ETI can lead to localized programmed cell death called the hypersensitive response (HR) (Goodman and Novacky, 1994; Greenberg and Yao, 2004; Chisholm et al., 2006). Even when ETI is not accompanied by HR, the detrimental effects of the resistance response can be evident as chlorosis in Arabidopsis (Gassmann, 2005). As a result, stunted growth and poor seed-set are common phenotypes associated with constitutive ETI responses.

Consequently, the plant must fine-tune its response to pathogens by exerting tight positive and negative control (McDowell and Simon, 2006).

There are several reports that transcription factors control the defense response by positively or negatively regulating the defense transriptome, as exemplified with the WRKY family (Eulgem, 2005; Eulgem and Somssich, 2007). TCP proteins have recently been recognized to function as plant specific transcription factors and to date have mainly been implicated in plant developmental pathways as regulators of morphological traits (Cubas et al., 1999; Cubas, 2002; Kosugi and Ohashi, 2002; Navaud et al., 2007). The TCP protein family was named after TEOSINTE BRANCHED 1 (TB1) in maize, CYCLOIDEA (CYC) in *Anthirrinum majus* and PCF in rice. It can be divided into two subfamilies, TB1/CYC (TCP-C) and PCF (TCP-P), based on the structure of the basic-Helix-Loop-Helix (b-HLH) DNA-binding domain (Cubas et al., 1999; Navaud et al., 2007). TB1s in maize and rice repressed lateral branching (Doebley et al., 1995; Doebley et al., 1997; Takeda et al., 2003) and CYC in *Anthirrinum majus* functioned in control of floral asymmetry (Luo et al., 1996; Luo et al., 1999) suggesting that the TCP-C subfamily plays role in plant architecture or in shoot branching (Nath et al., 2003; Cubas, 2004). Rice PCF1 and PCF2, the first identified TCP-Ps, were reported to affect cell growth and proliferation and were known to bind site II motifs (TGGGCY, Y=C or T), which is the *cis*-acting element of genes related to the cell cycle, ribosomal protein synthesis and axillary bud outgrowth (Kosugi et al., 1995; Kosugi and Ohashi, 1997, 2002; Tremousaygue et al., 2003; Tatematsu et al., 2005). Whereas most of the TCP-

C proteins contain a R domain which forms a hydrophilic α–helix, the TCP-P family does not have R-domain (Cubas, 2002).

There are twenty four members of the TCP protein family in Arabidopsis (Cubas, 2002) (Figure V-3C). Increasingly, biological functions of Arabidopsis TCP proteins are being reported even though they are not well studied. AtTCP20 (At3g72010) binds site II motifs and is involved in control of the cell cycle, cell expansion and plant shape (Tremousaygue et al., 2003; Li et al., 2005; Herve et al., 2009). AtTCP16 (AT3G45150) plays a pivotal role in the early stage of pollen development (Takeda et al., 2006). AtTCP14 (AT3G47620) is important for seed germination via the control of embryonic growth potential (Tatematsu et al., 2008). In addition, Koyama and collaborators suggested that repression of AtTCP3 (AT1G53230) causes abnormal shoot lateral organ formation because it negatively regulates the expression of boundary specific genes (Koyama et al., 2007).

By performing a genetic suppressor screen, we previously identified mutants with specifically enhanced responses to the *P. syringae* effector protein AvrRps4 (Kwon et al., 2009). The Arabidopsis accession RLD is a natural mutant in the cognate R protein RPS4 and is fully susceptible to DC3000 expressing *avrRps4* (Hinsch and Staskawicz, 1996; Gassmann et al., 1999). Mutations in RLD in a gene we called *SUPPRESSOR OF rps4-RLD 1* (*SRFR1*) enhanced resistance to DC3000(*avrRps4*), but not to virulent DC3000. The mutant *srfr1* alleles were recessive, suggesting that genetically *SRFR1* functions as a negative regulator of AvrRps4-triggered immunity (Kwon et al., 2004). *SRFR1*

encodes a novel tetratricopeptide repeat-containing protein that is conserved between plants and animals. To date none of the proteins in other organisms have an assigned function. SRFR1 orthologs appear to be absent in *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and *Drosophila melanogaster*. While proteins that share amino acid sequence similarity over the whole length of SRFR1 are absent in these organisms, the SRFR1 TPR domain shares some sequence similarity with the TPR-containing transcriptional repressors ScSSN6 and CeOGT1 (Kwon et al., 2009).

In an effort to understand the molecular and biological mechanism of SRFR1, we measured the expression levels of defense related genes in *srfr1* mutants and identified SRFR1-interactors using yeast two-hybrid screening. The TCP proteins TCP8, TCP20 and TCP22 were identified as SRFR1 interactors *in vivo* as well as *in vitro* and their interactions occurred in the nucleus. Additionally, the loss and gain of function of three TCP proteins and SRFR1 were described to identify their biological relevance. Considering the previous study (Kwon et al., 2009) and this study, we propose that SRFR1 functions in a transcriptional repressor complex of plant defense genes by interacting with TCP proteins in the nucleus that fine-tune the Arabidopsis defense response.

Results

Altered expression of defense genes in *srfr1* mutants

Initial characterization of *srfr1* mutants by RNA gel blots showed that the defense gene *PR1* is not constitutively upregulated, consistent with the absence of elevated resistance to virulent DC3000 (Kwon et al., 2004). After cloning of *SRFR1*, we quantified the expression of *RPS4* in the mutants and determined that *RPS4* mRNA levels, which are induced approximately 10-fold by *avrRps4* in resistant wild-type plants (Zhang and Gassmann, 2007), are approximately two-fold higher in uninduced *srfr1* mutants compared to uninduced wild-type plants (Kwon et al., 2009) This raised the possibility that other defense-related genes are also slightly upregulated in *srfr1* mutants, albeit not highly enough to trigger constitutive activation of plant defenses. This would be consistent with our model that *srfr1* mutants are closer to a threshold for defense activation.

Several defense-related genes were indeed upregulated in uninduced *srfr1-1* and *srfr1-2* leaf tissue, including *PR1, PR2, PDF1.2, EDS1, PAD4* and *RPS4* (Figure V-1A). Also, the expression level of *PR1* in the mutants was significantly more induced compared to that of wild type plants after treatment with salicylic acid (SA). The level of *PR1* induction in uninduced *srfr1* mutants was still considerably lower than in SA-induced tissue from a separate batch of wild-type or mutant plants (Figure V-1B).

Isolation of proteins that physically interact with SRFR1

SRFR1 encodes a pioneer TPR (Tetratricopeptide Repeat) protein conserved between plants and animals. The SRFR1 TPR domain has sequence similarity to that of the *Saccharomyces cerevisiae* Ssn6 protein, which interacts with DNA-

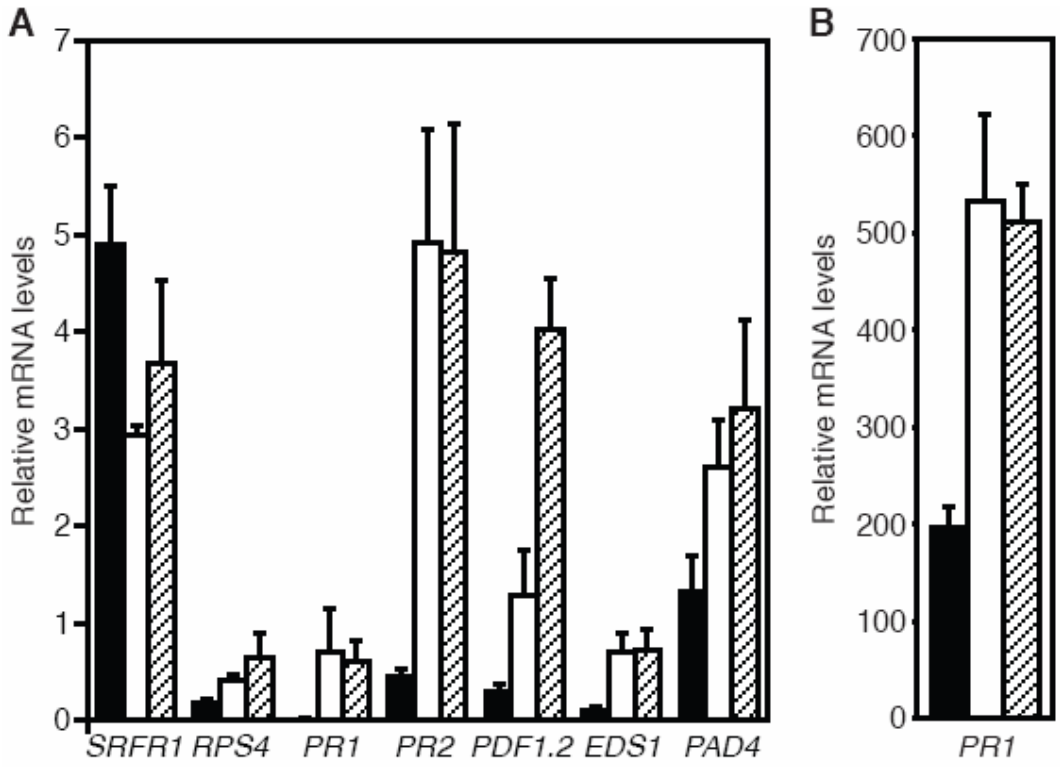

A
Relative mRNA levels
0
1
2
3
4
5
6
7
SRFR1
RPS4
PR1
PR2
PDF1.2
EDS1
PAD4
B
Relative mRNA levels
0
100
200
300
400
500
600
700
PR1

Figure V-1

Figure V-1. Defense gene mRNA levels are higher in *srfr1* mutants than in the wild-type. Total RNA was isolated from three biological replicates. mRNA levels were quantified by real-time quantitative reverse transcription PCR and were normalized using *SAND* gene (At2g28390) mRNA levels as an internal standard. Error bars denote standard error. **(A)** Defense gene mRNA levels in uninduced tissue of RLD wild-type (closed bars), *srfr1-1* (open bars) and *srfr1-2* (hatched bars). **(B)** *PR1* mRNA levels 24h after induction by spraying leaf tissue of RLD (closed bars), *srfr1-1* (open bars) and *srfr1-2* (hatched bars) with 1.5 mM SA. Note difference in scale.

binding proteins and negatively regulates downstream genes. In addition, SRFR1 was targeted to the nucleus in *Nicotiana benthamiana* leaf cells. Therefore, we hypothesized SRFR1 may function in a transcriptional repressor complex that balances plant resistance activation and suppression (Figure V-2).

In order to identify possible SRFR1 interactors, we performed a yeast two-hybrid screen using a pool of Arabidopsis transcription factor (TF) cDNA library as prey and Arabidopsis SRFR1 as bait. We cloned *SRFR1* constructs into the Invitrogen low-copy bait vector pDEST32. Apart from full-length SRFR1 ($SRFR1_{1-1052}$) bait, we constructed TPR domain-specific $SRFR1_{1-567}$ and $SRFR1_{298-653}$, and C-terminal domain-specific $SRFR1_{568-1052}$ baits (Figure V-3A). Screening the prey library of over 1,400 transcriptional regulators with all 4 bait constructs identified 18 positive clones representing 9 distinct putative interactors. Retesting by co-transformation with bait and prey vectors eliminated all but two interactors, At1g53230 (TCP3) and At3g27010 (TCP20). Interactions were strongest with $SRFR1_{298-653}$. No interactors were found using the C-terminal $SRFR1_{568-1052}$ bait. TCP3 and TCP20 belong to separate classes among the TCP transcription factor family and show sequence similarity only in the conserved TCP domain (Cubas et al., 1999).

We therefore re-screened 17 Arabidopsis TCP TFs with the full-length SRFR1 bait using individual co-transformations. Interestingly, $SRFR1_{1-1052}$ interacted not only moderately with TCP20 but also strongly with TCP8 and TCP22 (Figure V-3B). We also specifically screened the WRKY prey sub-library with $SRFR1_{1-1052}$ because of the known function of WRKY transcription factors in

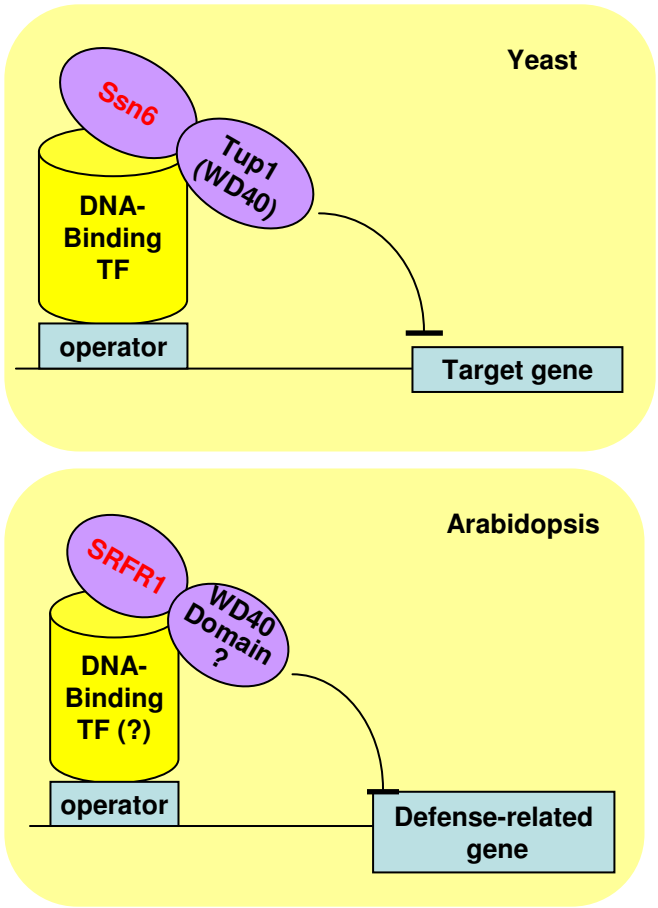
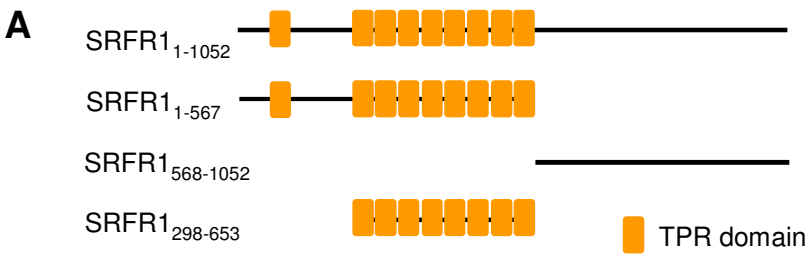
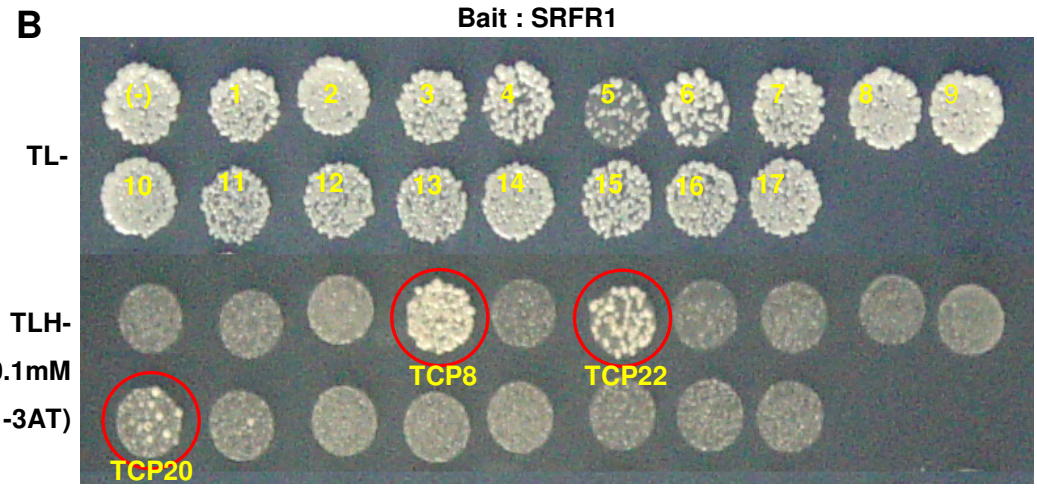

Yeast
Ssn6
Tup1
(WD40)
DNA-
Binding
TF
operator
Target gene
Arabidopsis
SRFR1
WD40
Domain
?
DNA-
Binding
TF (?)
operator
Defense-related
gene

Figure V-2

Figure V-2. Proposed model for SRFR1 in a transcriptional repressor complex. (Top) The Ssn6 transcriptional repressor complex in yeast. Ssn6–Tup1 is known to interact with DNA-binding proteins and to repress downstream genes that function in many different pathways. (Bottom) Model of a SRFR1 transcriptional repressor complex in Arabidopsis. SRFR1 negatively regulates defense-related genes by interacting with DNA-binding transcription factors.


A
SRFR1 1-1052
SRFR1 1-567
SRFR1 568-1052
SRFR1 298-653
TPR domain


B
Bait : SRFR1
TL-
TLH-
(0.1mM
-3AT)
(-)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
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16
17
TCP8
TCP22
TCP20

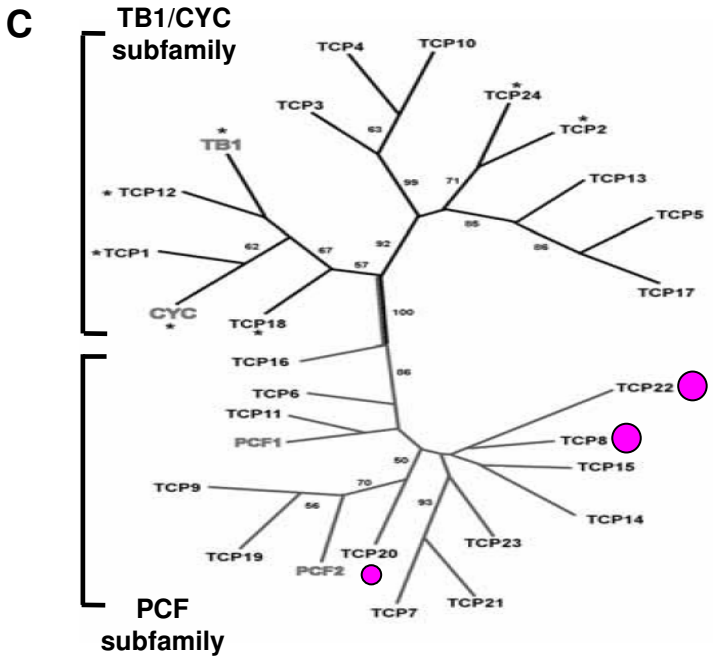

C
TB1/CYC
subfamily
PCF
subfamily
TCP4
TCP10
TCP24
TCP3
TCP2
TB1
TCP12
TCP13
TCP5
TCP1
TCP17
CYC
TCP18
TCP16
TCP6
TCP22
TCP11
PCF1
TCP8
TCP15
TCP9
TCP14
TCP19
TCP20
TCP23
PCF2
TCP7
TCP21

Figure V-3

Figure V-3. SRFR1 interacts with three members of the TCP transcription factor family. **(A)** Schematic diagram of the SRFR1 full-length protein and its derivatives that were used in yeast two-hybrid screens as baits. Orange boxes indicate tetratricopeptide repeats (TPR), and numbers denote the amino acids of SRFR1 used as baits. **(B)** SRFR1 interacts with TCP8, TCP20 and TCP22 in the yeast two-hybrid system. $SRFR1_{1-1052}$ was used as bait with the following prey constructs: pEXP AD-502 (negative control) (-), 1. TCP24, 2. TCP3. 3. TCP8, 4. TCP15, 5. TCP22, 6. TCP10, 7. TCP09, 8. TCP13, 9. TCP4, 10. TCP20, 11. TCP14, 12. TCP2, 13. TCP17, 14. TCP7, 15. TCP6, 16. TCP19, 17. TCP1. Upper two rows: absence of Trp and Leu (selection for prey and bait vectors). Lower two rows: absence of Trp, Leu and His (selection for bait and prey interaction). 0.1 mM 3-AT (3-amino-1,2,4-triazole) was added to inhibit low-level constitutive expression of the *HIS3* reporter. **(C)** Phylogenic analysis of Arabidopsis TCP genes (Cubas, 2002). The reference genes TB1, CYC, PCF1 and PCF2 were included. TCP domains were aligned with CLUSTALW. The three SRFR1 interactors are indicated by a pink circle.

defense response regulation (Eulgem et al., 2000; Eulgem and Somssich, 2007). However, no SRFR1 interactors were identified among the WRKY transcription factors.

In silico analysis of TCP8, TCP20, and TCP22

All three TCP protein members, TCP8, TCP20, and TCP22 belong to the PCF (TCP-P) subfamily of the plant-specific TCP protein family and TCP8 and TCP22 are closely related (Figure V-3C). Annotation of *TCP8* in the Arabidopsis Information Resource database (TAIR; http://www.arabidopsis.org/) indicates that the open reading frame predicts a protein of 401 amino acids. The theoretical isoelectric point is 6.45 and the calculated molecular weight is 42471 Da. The TCP domain, which is predicted to form a non-canonical basic-Helix-Loop-Helix (bHLH) domain, is found at amino acid residues 60-114 in TCP8 (Figure V-4). According to the TAIR8 database, *TCP8* is alternatively spliced, producing an in-frame deletion of protein positioned at amino acids 93-116, and therefore affecting the TCP domain. The predicted truncated TCP8 protein is 377 amino acids in length and 39924.3 Da in weight. Based on data in the AtGenExpress Visualization Tool (http://jsp.weigelworld.org/expviz/expviz.jsp) (Schmid et al., 2005), expression levels of *TCP8* are similar in all organs and at all stages, except for a significantly decreased transcript level in 6 week old pollen. *TCP8* transcript is not strongly regulated by various treatments, including pathogens.

TCP20 encodes a protein 185 amino acids long. The mature protein has a molecular weight of 33,390.8 Da and an isoelectric point of 7.25. The TCP


TCP8 ---MDLSDIRNNNNDTAAVATGGGARQLVDASLSIVPRSTPPEDSTLATTSSTATAT--- 54
TCP22 MNQNSSVAEATLQLNSGEKPSPGSIPFISSGQHGNISTSATSSTSTSSGSALAVVKS--- 57
TCP20 MDPKNLNRHQVPNFLNPPPPPRNQGLVDDDAASAVVSDENRKPTTEIKDFQIVVSASDKE 60
TCP8 ------------TTKRSTKDRHTKVDGRGRRIRMPALCAARVFQLTRELGHKSDGETIEW 102
TCP22 ------------AVKKPTKDRHTKVDGRGRRIRMPAMCAARVFQLTRELGHKSDGETIEW 105
TCP20 PNKKSQNQNQLGPKRSSNKDRHTKVEGRGRRIRMPALCAARIFQLTRELGHKSDGETIQW 120
TCP8 LLQQAEPAIVAATGTGTIPANFSTLSVSLRSSG-STLSAPPSKSVP---LYGALGLTHHQ 158
TCP22 LLQQAEPAIIASTGTGTIPANFSTLNASLRSGGGSTLFSQASKSSSSPLSFHSTGMSLYE 165
TCP20 LLQQAEPSIIAATGSGTIPA--------------SALASSAATSNHHQGGSLTAGLMISH 166
TCP8 YDEQGGGG--------VFAAHTSPLLGFHHQLQHHQNQNQNQDPVETIPEGENFSRKRYR 210
TCP22 DNNGTNGSSVDPSRKLLNSAANAAVFGFHHQMYPPIMSTERNPNTLVKPYREDYFKEPSS 225
TCP20 DLDG---------------GSSSSGRPLNWGIGGGEGVSRSSLPTGLWPNVAGFGSGVPT 211
TCP8 SVDLSKENDDRKQNENKSLKESE-TSGPTAAPMWAVAPPSRSGAGNTFWMLPVPTTAGNQ 269
TCP22 AAEPSESSQKASQFQEQELAQGRGTANVVPQPMWAVAPGTTNGG-SAFWMLPMSGSGGRE 284
TCP20 TGLMSEGAGYRIGFPGFDFPGVG-------------------------HMSFASILGGN- 245
TCP8 MESSSNNNTAAGHRAPPMWPFVNSAGGGAGGGGGAATHFMAGTGFSFPMDQYRGSPLQLG 329
TCP22 QMQQ--------QPGHQMWAFNPGN---------------------YPVGTGRVVTAPMG 315
TCP20 --HNQMPGLELG 255
TCP8 SFLAQPQPTQNLGLSMPDSNLGMLAALNSAYSRGGNANANAEQANNAVEHQEKQQQSDHD 389
TCP22 SMMLG---GQQLGLGVAEGNMAAAMRG----SRGDGLAMTLDQHQHQLQHQEPNQSQASE 368
TCP20 LSQEG-----NVGVLNPQSFTQIYQQMGQAQAQAQGRVLHHMHHNHEEHQQESGEKDDSQ 310
TCP8 DDSREENSNSSE 401
TCP22 NGGDDKK----- 375
TCP20 GSGR-------- 314

Figure V-4

Figure V-4. Comparison of deduced amino acid sequences of the Arabidopsis SRFR1 interacting-TCP transcription factors performed by EBI-ClustalW . Aligned from top to bottom are TCP8 (AGI no At1g58100), TCP22 (At1g72010) and TCP20 (At3g27010). Identical amino acids are indicated by asterisks under the sequences. Colons and semi-colons show conserved substitutions and semi-conserved substitutions, respectively. A basic-Helix-Loop-Helix (bHLH) domain is boxed-in. Bold characters in blue show the amino acids deleted by *TCP8* alternative splicing.

domain of TCP20 encompasses amino acid position 78 to 132. Data from digital Northern (AtGenExpress Visualization Tool) indicate *TCP20* is highly expressed in response to 10μM cycloheximide. However, the profiles of expression were not altered at developmental stages and after treatment with other biotic and abiotic stresses.

The *TCP22* cDNA comprises 1,709 bp nucleotides, including a 5'-flanking region of 277 bp and a 3' flanking region of 304 bp, encoding a protein with 377 amino acids. The theoretical isoelectric point and calculated molecular mass of TCP22 are 8.98 and 39598.7 Da, respectively. In TCP22, the TCP domain found at amino acid residues 63-117. TCP22 is not represented on the ATH1 chip. Comparisons of the amino acid sequences among three members of TCP proteins were performed by EBI-ClustalW (http://www.ebi.ac.uk/Tools/clustalw/) (Larkin et al., 2007) and are presented in Figure V-4. Sequence identities between TCP8 and TCP22, TCP8 and TCP20, and TCP22 and TCP20 were 30%, 29% and 27%, respectively.

Subcellular localization of TCP8, TCP20, and TCP22

To identify the subcellular localization of TCP8, TCP20, and TCP22, we used an *Agrobacterium*-mediated transient expression system in *Nicotiana benthamiana* leaf cells. Computational analyses of the predicted protein sequences revealed that TCP8, TCP20, and TCP22 may be nuclear proteins (http://wolfpsort.seq.cbrc.jp/) (Horton et al., 2007) despite the absence of a canonical nuclear localization signal. To corroborate the nuclear localization,

TCP8, TCP20, and TCP22 were fused in frame with green fluorescent protein (GFP) at the N-terminus of the proteins of interest. Fusion proteins were expressed under the control of the CaMV 35S promoter in *Nicotiana benthamiana* leaf cells. As shown in Figure V-5, the control GFP was uniformly distributed throughout the nucleus and cytoplasm. However, GFP-TCP8, GFP-TCP20, and GFP-TCP22 fusion proteins were predominantly targeted to the nucleus. Intriguingly, GFP-TCP8 and GFP-TCP22 were localized not only in the nucleoplasm but also in nuclear speckles (Figure V-5).

SRFR1 interacts with three TCP TF family members *in planta*

SRFR1 interacted with TCP8, TCP20 and TCP22 in yeast (Figure V-3B). Also, SRFR1 and the TCP proteins were localized to the nucleus (Figure V-5). To test the *in planta* interactions of SRFR1 with the 3 TCP proteins, we performed bimolecular fluorescence complementation (BiFC). BiFC enables the determination of protein-protein interactions in living cells as well as the subcellular localization of the interacting protein complexes (Citovsky et al., 2006; Ohad et al., 2007). SRFR1 was fused to the N-terminal domain of the yellow fluorescent protein (YFP) (nY-SRFR1) and TCP8, TCP20 and TCP22 were fused to the C-terminal domain of the YFP (cY-TCP8, cY-TCP20 and cY-TCP22). As shown in Figure V-6, SRFR1 interacted with TCP8, TCP20 and TCP22, emitting YFP signals in the nucleus. Moreover, the YFP signals were detected in nuclear speckles in the interactions of SRFR1-TCP8 and SRFR1-TCP22. We obtained the same results by using the reverse combinations of cY-SRFR1 and nY-TCP8,

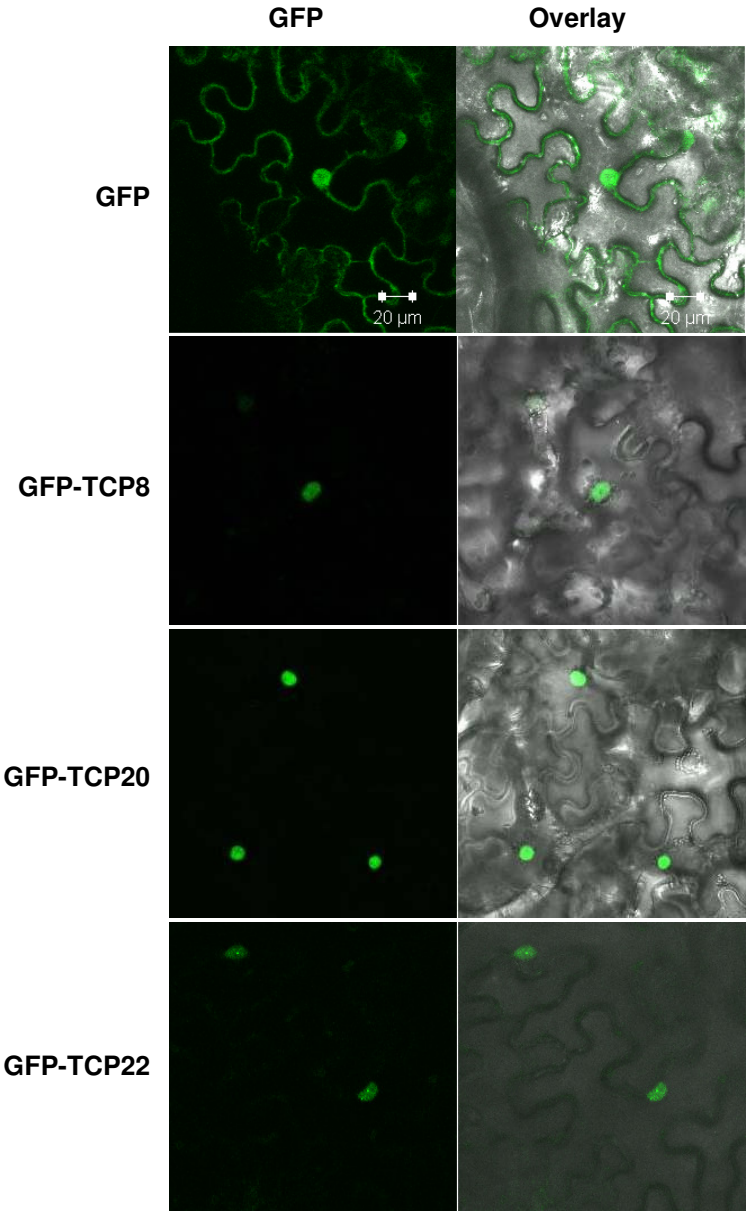


Figure V-5

Figure V-5. Nuclear localization of three TCP proteins. *GFP and GFP-TCP8, GFP-TCP20* and *GFP-TCP22* fusion constructs (from top to bottom) were transiently expressed in *N. benthamiana* leaves using Agrobacterium. Cells expressing fusion proteins were analyzed 3 days after infiltration by a Zeiss LSM 510 META NLO two-photon point-scanning confocal microscope under GFP fluorescence (left) and GFP/bright field overlay (right).

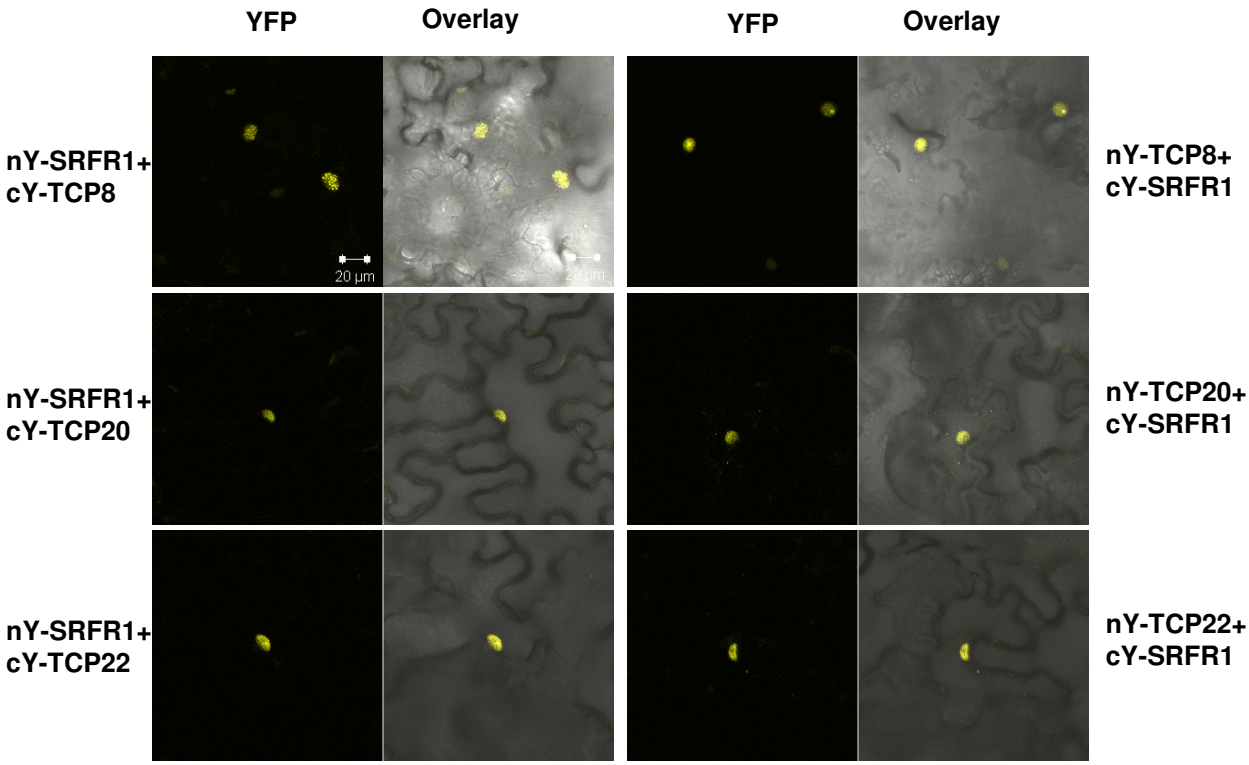

YFP
Overlay
YFP
Overlay
nY-SRFR1+
cY-TCP8
20 µm
nY-TCP8+
cY-SRFR1
nY-SRFR1+
cY-TCP20
nY-TCP20+
cY-SRFR1
nY-SRFR1+
cY-TCP22
nY-TCP22+
cY-SRFR1

Figure V-6

Figure V-6. Interaction of SRFR1 with three TCP proteins *in planta*. BIFC indicates SRFR1 and SRFR1-interacting proteins, TCP8, TCP20 and TCP22, are co-localized to the nucleus in *N. benthamiana* leaf epidermal cell. (Left) : Co-infiltration of *nYFP-SRFR1* with *cYFP-TCP8, cYFP-TCP20* and *cYFP-TCP22* (from top to bottom). (Right) : Co-infiltration of *cYFP-SRFR1 with nYFP-TCP8, nYFP-TCP20* and *nYFP-TCP22* (from top to bottom). Cells expressing fusion proteins were analyzed 3 days after infiltration with a Zeiss LSM 510 META NLO two-photon point-scanning confocal microscope.

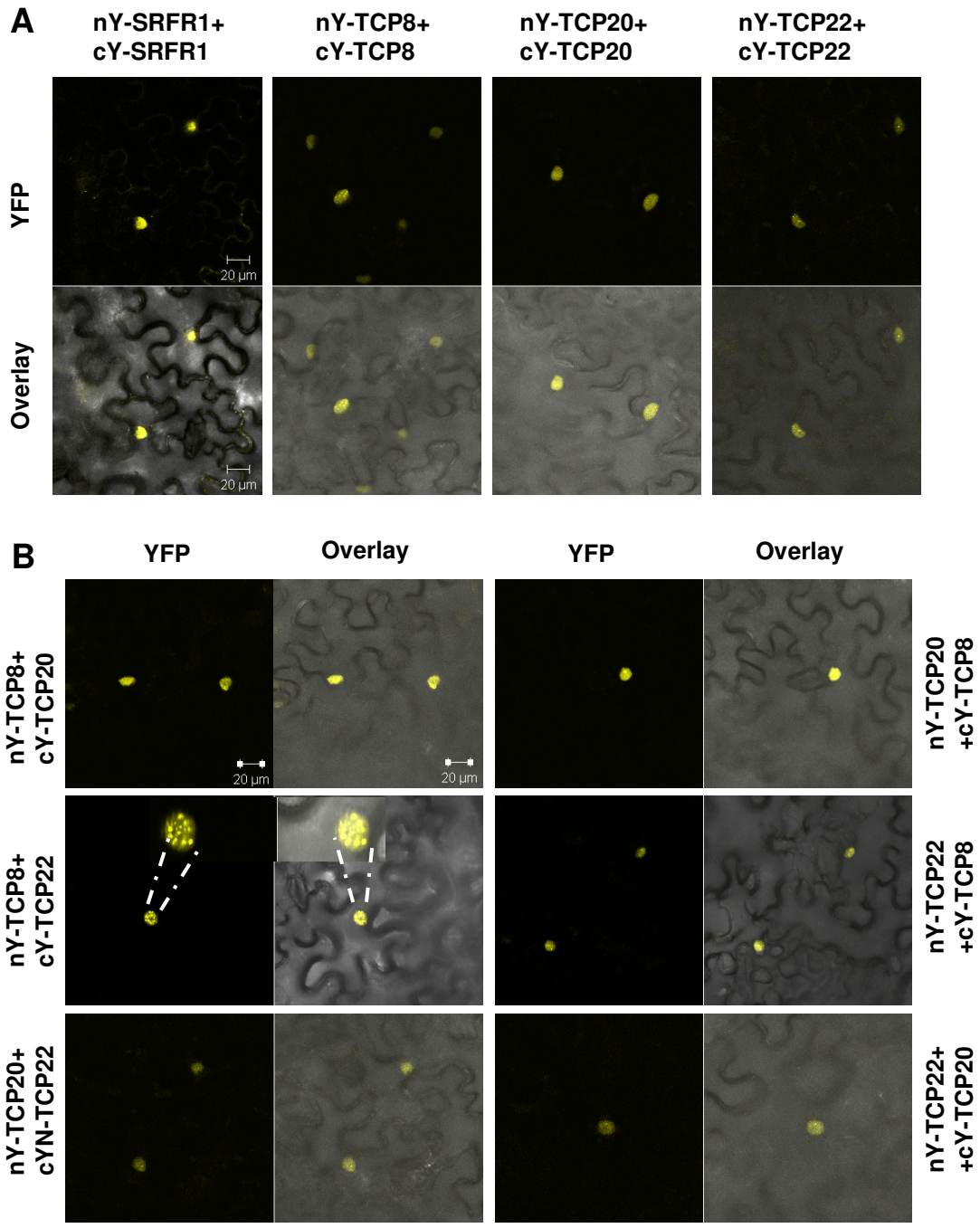

A
nY-SRFR1+
cY-SRFR1
nY-TCP8+
cY-TCP8
nY-TCP20+
cY-TCP20
nY-TCP22+
cY-TCP22
YFP
Overlay
20 µm
20 µm
B
YFP
Overlay
YFP
Overlay
nY-TCP8+
cY-TCP20
nY-TCP20
+cY-TCP8
nY-TCP8+
cY-TCP22
nY-TCP22
+cY-TCP8
nY-TCP20+
cYN-TCP22
nY-TCP22+
+cY-TCP20
20 µm
20 µm

Figure V-7

Figure V-7. Homodimerization and heterodimerization of SRFR, TCP8, TCP20 and TCP22 *in planta*. **(A)** BIFC indicates dimerized SRFR1, TCP8, TCP20 and TCP22, are targeted to the nucleus in *N. benthamiana* leaf epidermial cells. Co-infiltration of *nYFP-SRFR1* with *cYFP-SRFR1, nYFP-TCP8* with *cYFP-TCP8, nYFP-TCP20* with *cYFP-TCP20* and *nYFP-TCP22* with *cYFP-TCP22* (from left to right). **(B)** BIFC indicates that TCP8, TCP20 and TCP22, interact with each other and are targeted to the nucleus in *N. benthamiana* leaf epidermal cells. (Left) Co-infiltration of *nYFP-TCP8* with *cYFP-TCP20, nYFP-TCP8* with *cYFP-TCP22, nYFP-TCP20* with *cYFP-TCP22* (from top to bottom). (Right) Co-infiltration of *nYFP-TCP20* with *cYFP-TCP8, nYFP-TCP22* with *cYFP-TCP8, nYFP-TCP22* with *cYFP-TCP20* (from top to bottom). Cells expressing fusion proteins were analyzed 3 days after infiltration with a Zeiss LSM 510 META NLO two-photon point-scanning confocal microscope.

nY-TCP20 and nY-TCP22. YFP signals were not detected in infiltrations of individual constructs (nY-SRFR1, cY-SRFR1 nY-TCP8, cY-TCP8, nY-TCP20, cY-TCP20, nY-TCP22 and cY-TCP22) (data not shown).

In addition, not only SRFR1 but also TCP8, TCP20, and TCP22, were shown to homodimerize in the nucleus (Figure V-7A). Interestingly, TCP8 and TCP20, TCP8 and TCP22, and TCP20 and TCP22 interacted with each other to form heterodimers in the nucleus (Figure V-7B). Furthermore, TCP8-TCP22 complexes were localized in nuclear speckles.

Phenotypes of *TCP8* over-expression and knockout plants

We hypothesized SRFR1 may function as a negative regulator of AvrRps4-triggered immunity by forming a host protein complex with TCP8, TCP20 and TCP22. As a first step to identify the biological function of TCP8, we analyzed a homozygous T-DNA knockout line of *TCP8* (*tcp8-1*, SAIL_656_F11). *In planta* bacterial growth assays indicated that the growth of DC3000 (*avrRps4*) in *tcp8-1* was similar to growth in wild type Col-0, and all plant lines were fully susceptible to virulent DC3000 (Figure V-8A).

To examine a possible gain-of-function phenotype, we constructed *Myc-TCP8* driven by the cauliflower mosaic virus 35S promoter. Interestingly, we detected a full length and truncated Myc-TCP8 when *Myc-TCP8* was transiently expressed in *N. benthamiana* leaf cells, supporting the predicted alternative splicing of *TCP8* by TAIR8. *HA-TCP8* also produced full length and truncated TCP8 proteins (Figure V-8B). We isolated homozygous *TCP8* over-expression

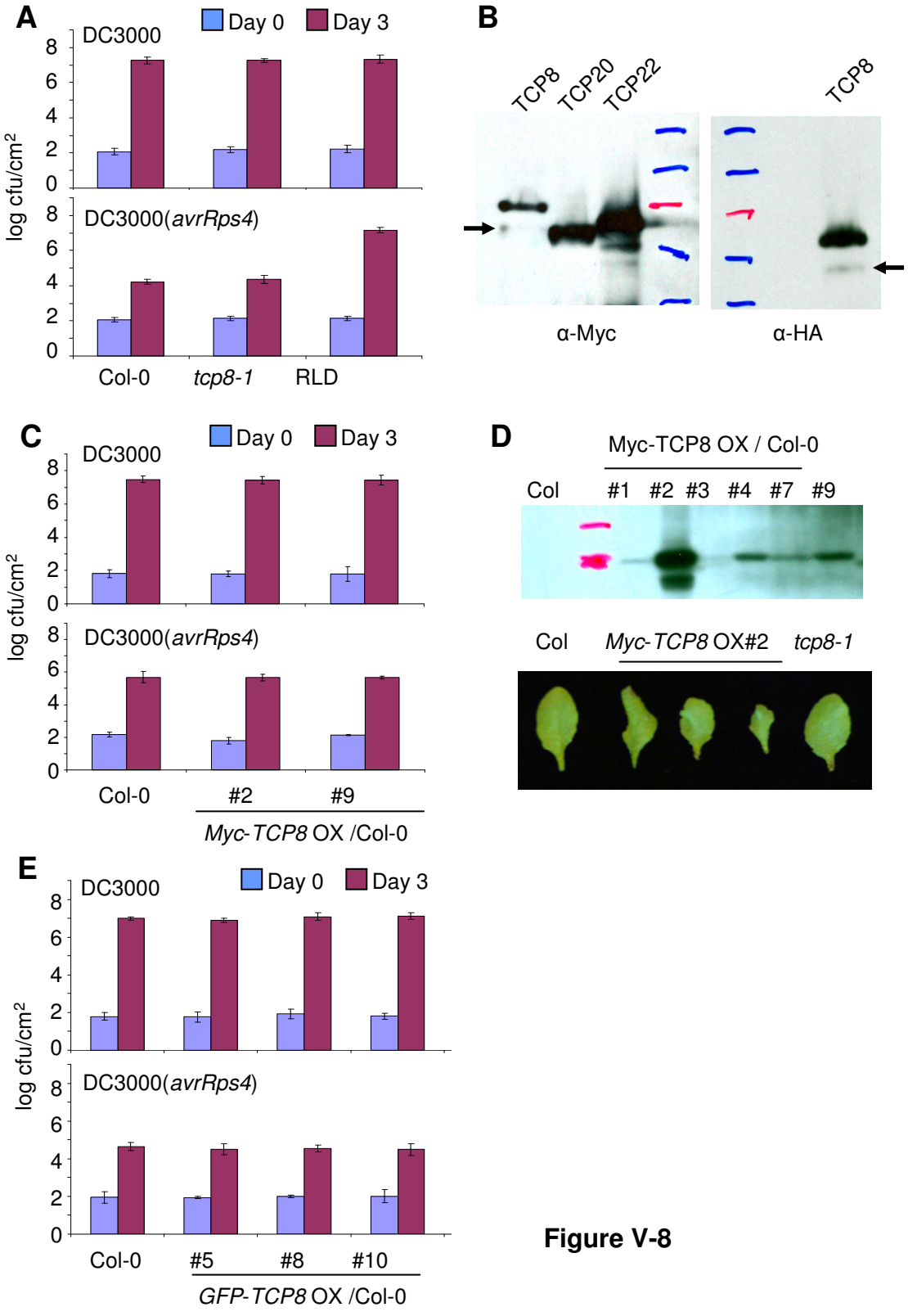

A
Day 0
Day 3
DC3000
log cfu/cm2
DC3000(avrRps4)
Col-0
tcp8-1
RLD
B
TCP8
TCP20
TCP22
TCP8
α-Myc
α-HA
C
Day 0
Day 3
DC3000
log cfu/cm2
DC3000(avrRps4)
Col-0
#2
#9
Myc-TCP8 OX /Col-0
D
Myc-TCP8 OX / Col-0
Col
#1
#2
#3
#4
#7
#9
Col
Myc-TCP8 OX#2
tcp8-1
E
Day 0
Day 3
DC3000
log cfu/cm2
DC3000(avrRps4)
Col-0
#5
#8
#10
GFP-TCP8 OX /Col-0

Figure V-8

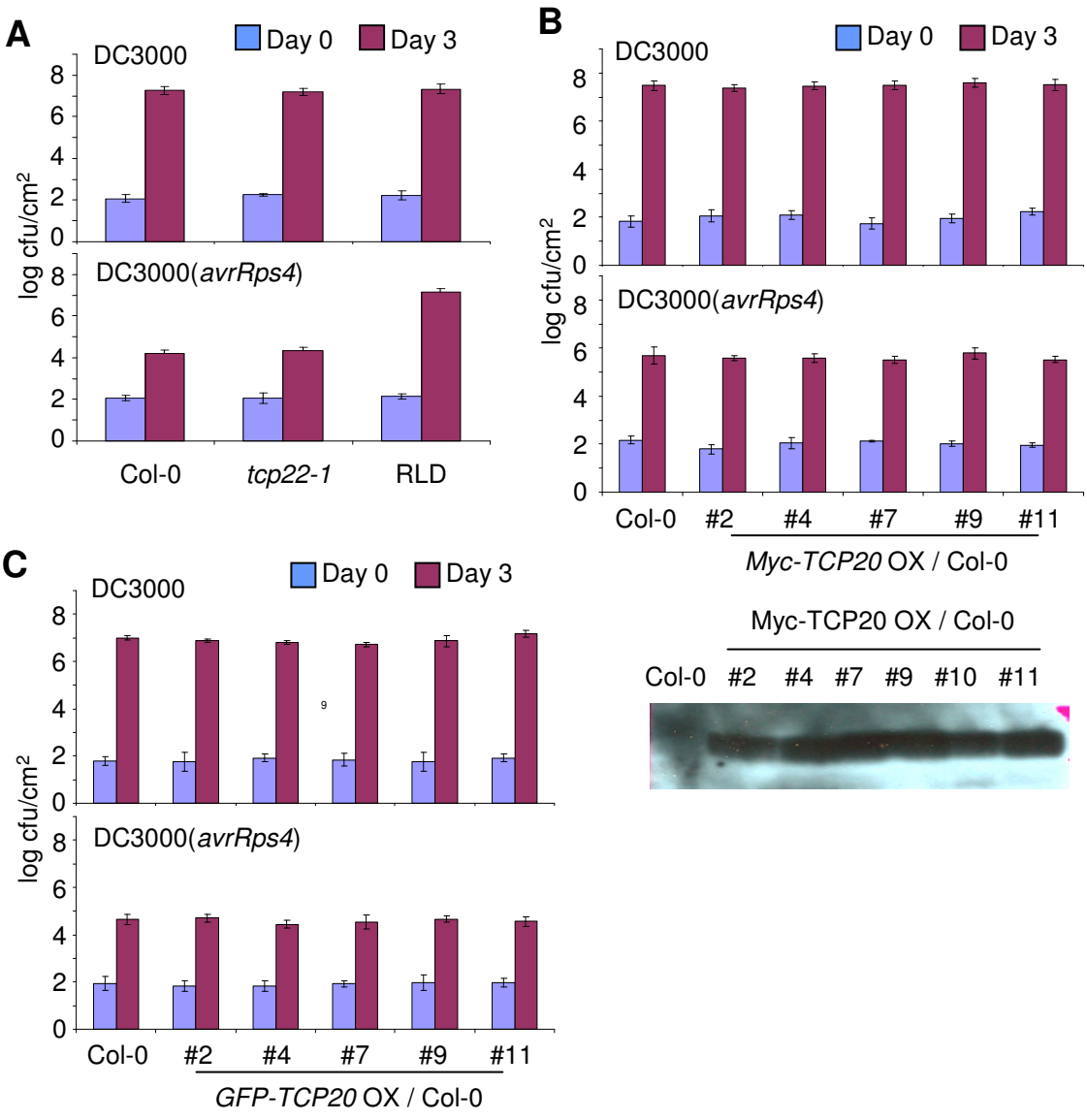
Figure V-8. Bacterial growth assays in *TCP8* knockout (K/O) and overexpression (OX) plants. **(A)** *In planta* bacterial growth was measured in Col-0, *tcp8-1* and RLD plants on day 0 (blue columns) and day 3 (purple columns) after inoculation with DC3000 (top) and DC3000(*avrRps4*) (bottom). **(B)** Detection of TCP8, TCP20 and TCP22 protein in *N. benthamiana* by western blot. Myc-TCP8, Myc-TCP20, Myc-TCP22 (left) and HA-TCP8 fusion constructs (right) were transiently expressed in *N. benthamiana.* Total protein was extracted from three leaf discs at 3 days post inoculation and was subjected to immunoblot analysis with horseradish peroxidase (HRP)-conjugated A-14 (left) and HA (right). Arrows indicate the presence of truncated TCP8 protein. **(C)** *In planta* bacterial growth was measured in Col-0 and the T3 generation of two independent *Myc-TCP8* OX lines on day 0 (blue columns) and day 3 (purple columns) after inoculation with DC3000 (top) and DC3000(*avrRps4*) (bottom). **(D)** (top) Western blots of total extracts of Col-0 and T2 generation of six independent *Myc-TCP8* OX lines probed with HRP-conjugated A-14. (bottom) Phenotypes of four-week old Arabidopsis leaves of wild-type Col-0, *Myc-TCP8* OX line #2 and *tcp8-1* K/O plants. The *Myc-TCP8* OX #2 line showed dwarfed and curled phenotypes compared to Col-0 and *tcp8-1*. **(E)** *In planta* bacterial growth was measured in Col-0 and the T3 generation of three independent *GFP-TCP8* OX lines on day 0 (blue columns) and day 3 (purple columns) after inoculation with DC3000 (top) and DC3000(*avrRps4*) (bottom). In all bacterial growth tests above, plants were inoculated with a bacterial suspension at a density of $5x10^4$ cfu/mL. Values represent averages of cfu/cm^2 leaf tissue from triplicate samples, and error bars denote standard deviation.

lines (*Myc-TCP8*-OX, *GFP-TCP8*-OX) in Col-0. Western blot confirmed that Myc-TCP8 proteins were stably expressed (Figure V-8D). Additionally, *Myc-TCP8*-OX #2 representing the highest expression of TCP8 showed a stunted and dwarfed phenotype compared to wild-type Col-0 and *tcp8-1* (Figure V-8D). However, *Myc-TCP8*-OX did not alter the bacterial growth after inoculation with virulent DC3000 and avirulent DC3000(*avrRps4*) (Figure V-8C). We obtained the same results in *GFP-TCP8*-OX lines (Figure V-8E). These results suggest that AvrRps4-triggered immunity was not altered in TCP8 loss- and gain-of function plants.

Phenotypes of *TCP20* over-expression and *TCP22* knockout plants

We characterized the *TCP22* T-DNA knockout line (*tcp22-1,* SALK_027490) in Col-0. Growth of DC3000(*avrRps4*) in *tcp22-1* was similar to that in Col-0 and all lines were equally susceptible to virulent DC3000 (Figure V-9A). We also measured the effects of overexpression of *TCP20* in wild type Col-0. TCP20 was highly expressed in transgenic *Myc-TCP20* over-expressing plants (*Myc-TCP20*-OX) (Figure V-9B) and transiently expressed in *N. benthamiana* leaf cells (Figure 8B). Growth of virulent and *avrRps4*-expressing DC3000 in both *Myc-TCP20* OX and *GFP-TCP20* OX plants was similar to that in wild-type Col-0 (Figure V-9B and Figure V-9C). We did not observe any apparent growth differences between Col-0 and *Myc-TCP20* OX lines (data not shown).

Overexpression of *SRFR1* does not alter AvrRps4-triggered immunity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Day 0
Day 3
DC3000
log cfu/cm2
DC3000(avrRps4)
Col-0
tcp22-1
RLD
B
Day 0
Day 3
DC3000
log cfu/cm2
DC3000(avrRps4)
Col-0
#2
#4
#7
#9
#11
Myc-TCP20 OX / Col-0
Myc-TCP20 OX / Col-0
Col-0
#2
#4
#7
#9
#10
#11
C
Day 0
Day 3
DC3000
log cfu/cm2
DC3000(avrRps4)
Col-0
#2
#4
#7
#9
#11
GFP-TCP20 OX / Col-0

Figure V-9

Figure V-9. Bacterial growth assays in *TCP22* knockout (K/O) and *TCP20* overexpression (OX) plants. **(A)** *In planta* bacterial growth was measured in Col-0, *tcp22-1* and RLD plants on day 0 (blue columns) and day 3 (purple columns) after inoculation with DC3000 (top) and DC3000(*avrRps4*) (bottom). **(B)** (top) *In planta* bacterial growth was measured in Col-0 and the T3 generation of two independent *Myc-TCP20* OX lines on day 0 (blue columns) and day 3 (purple columns) after inoculation with DC3000 (top) and DC3000(*avrRps4*) (bottom). (bottom) Western blots of total extracts of Col-0 and the T2 generation of six independent *Myc-TCP20* OX lines probed with HRP-conjugated with A-14. (bottom) **(C)** *In planta* bacterial growth was measured in Col-0 and the T3 generation of three independent *GFP-TCP20* OX lines on day 0 (blue columns) and day 3 (purple columns) after inoculation with DC3000 (top) and DC3000(*avrRps4*) (bottom). In all bacterial growth tests above, plants were inoculated with a bacterial suspension at a density of $5x10^4$ cfu/mL. Values represent averages of cfu/cm^2 leaf tissue from triplicate samples, and error bars denote standard deviation.

SRFR1 negatively regulates AvrRps4-triggered immunity which leads to the speculation that overexpression of *SRFR1* could increase the severity of a bacterial disease. We overexpressed *SRFR1* in RLD, *srfr1-1* and *srfr1-2* by using the *35S-GFP-SRFR1* construct which was used in the localization experiment (Kwon et al., 2009). Homozygous *SRFR1*-overexpressing (*GFP-SRFR1*-OX) plants in RLD had no altered growth phenotypes. As shown in Figure V-10A, *in planta* bacterial growth curve assays suggested that the *GFP-SRFR1*-OX construct complemented *srfr1-1 and srfr1-2*, indicating that this construct is functional. However, RLD plants overexpressing *SRFR1* (*GFP-SRFR1*-OX/RLD) had no increase of bacterial growth compared to RLD in response to DC3000 and DC3000(*avrRps4*) (Figure V-10B). These results demonstrate that overexpressing *SRFR1* did not boost plant susceptibility to virulent and avirulent bacteria.

Sequence analysis of *SRFR1* and *RPS4* promoters

To study potential regulatory cis-elements, *SRFR1* promoter sequences were analyzed with the PLACE (http://www.dna.affrc.go.jp) database at the Advanced Biosciences Computing Center (Higo et al., 1999). A typical TATA box was located at -47 (ttaaata) from the translational initiation codon. Pathogen-induced WRKY DNA-binding proteins have been known to recognize various W-box elements with a TTGAC core sequence in Arabidopsis *NPR1* or with a TGACT element in barley *ISO1* (Eulgem et al., 2000; Yu et al., 2001; Sun et al., 2003). The *SRFR1* promoter contained nine W-boxes within 1050 bp upstream of start

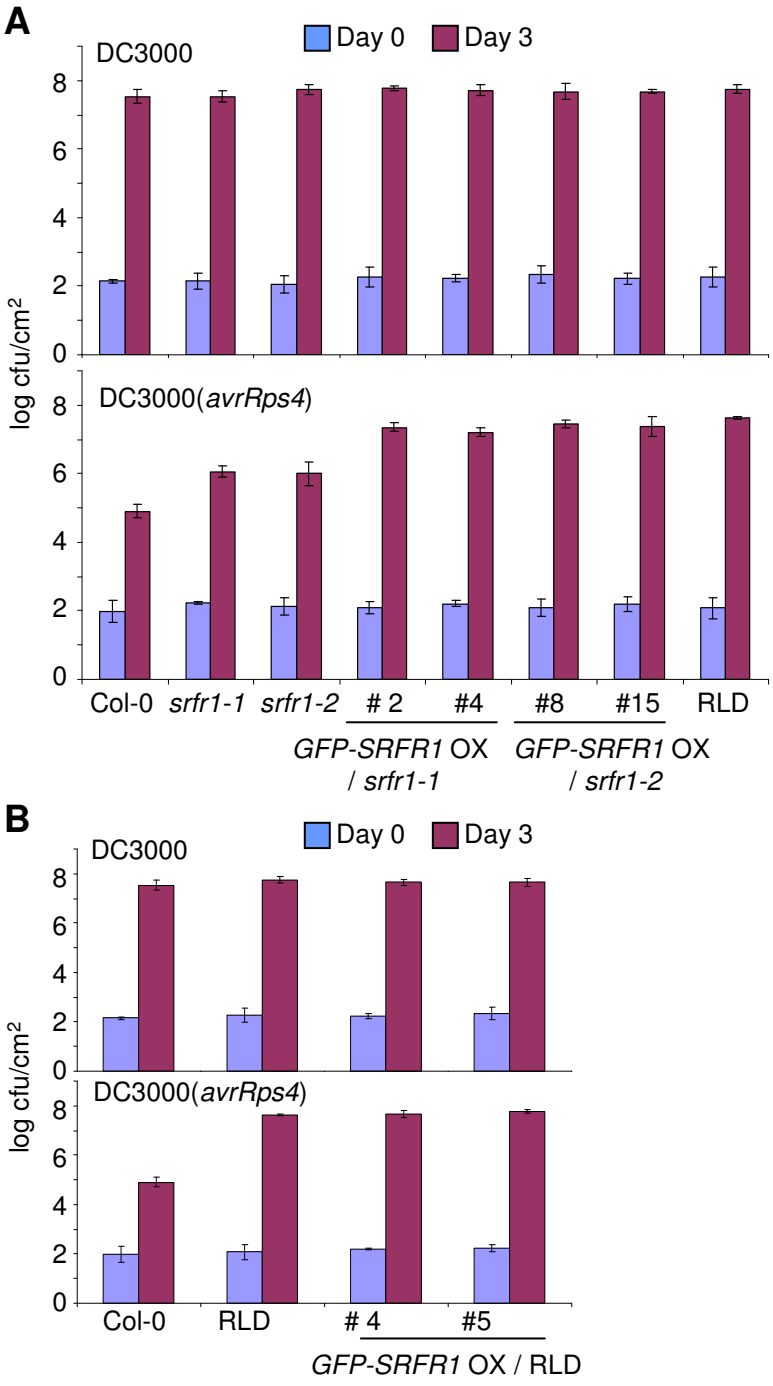
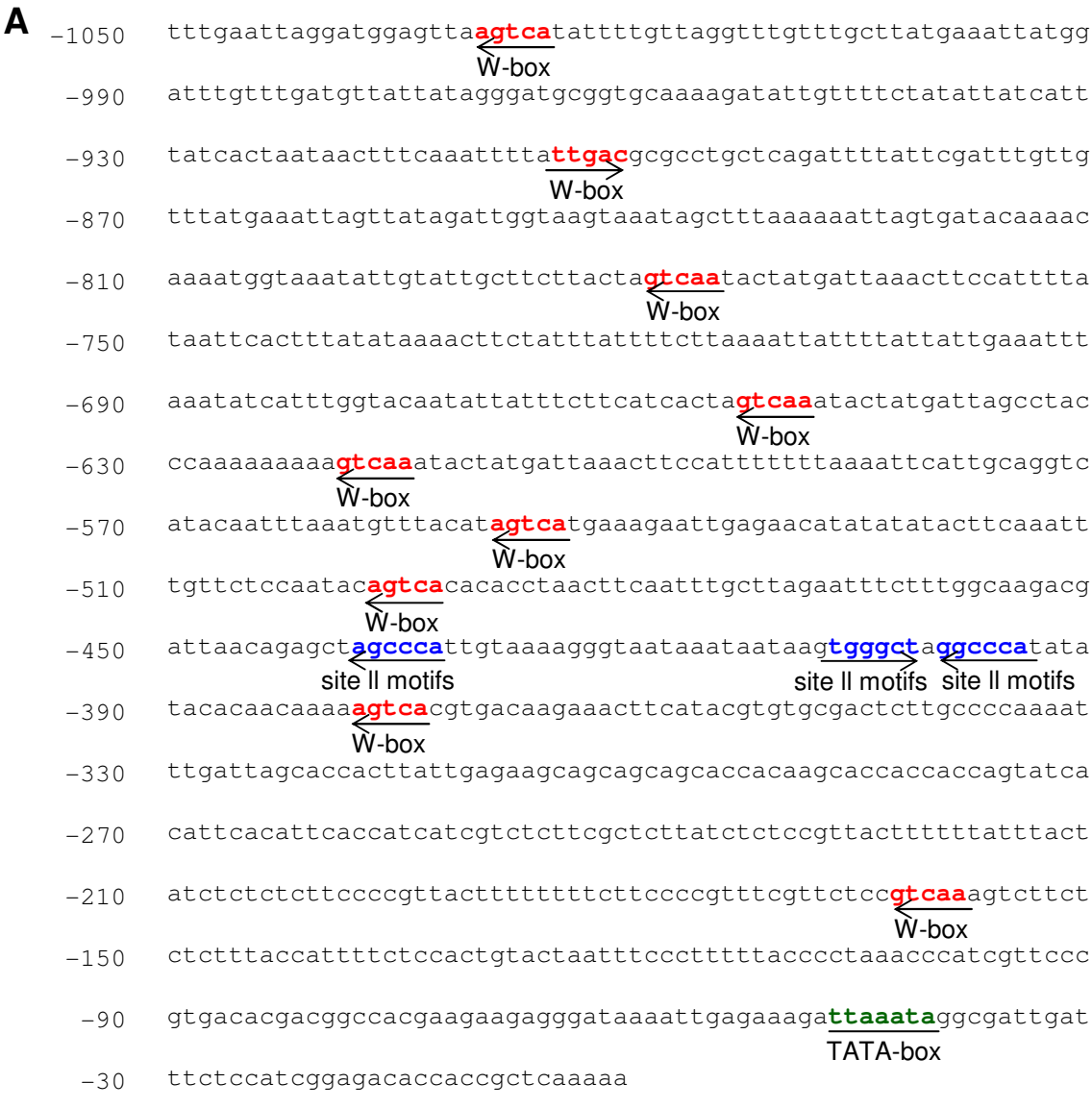

A
Day 0
Day 3
DC3000
DC3000(avrRps4)
log cfu/cm²
0
2
4
6
8
Col-0
srfr1-1
srfr1-2
2
#4
#8
#15
RLD
GFP-SRFR1 OX / srfr1-1
GFP-SRFR1 OX / srfr1-2
B
Day 0
Day 3
DC3000
DC3000(avrRps4)
log cfu/cm²
Col-0
RLD
4
#5
GFP-SRFR1 OX / RLD

Figure V-10

Figure V-10. Bacterial growth assays in *SRFR1* overexpression (OX) plants. **(A)** *In planta* bacterial growth was measured in Col-0, *srfr1-1, srfr1-2,* two independent *GFP-SRFR1* OX T3 generation lines in *srfr1-1*, two independent *GFP-SRFR1* OX lines in *srfr1-2* and RLD plants on day 0 (blue columns) and day 3 (purple columns) after inoculation with DC3000 (top) and DC3000(*avrRps4*) (bottom). **(B)** *In planta* bacterial growth was measured in Col-0, two independent *GFP-SRFR1* OX T3 generation lines in RLD and RLD plants on day 0 (blue columns) and day 3 (purple columns) after inoculation with DC3000 (top) and DC3000(*avrRps4*) (bottom). In all bacterial growth tests above, plants were inoculated with a bacterial suspension at a density of $5x10^4$ cfu/mL. Values represent averages of cfu/cm^2 leaf tissue from triplicate samples, and error bars denote standard deviation.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-570 atacaatttaaatgtttacatagtcatgaaagaattgagaacatatatatacttcaaatt
W-box
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W-box
-450 attaacagagctagcccattgtaaaagggtaataaataataagtgggctaggcccatata
site II motifs
site II motifs
site II motifs
-390 tacacaacaaaaagtcacgtgacaagaaacttcatacgtgtgcgactcttgccccaaaat
W-box
-330 ttgattagcaccacttattgagaagcagcagcagcaccacaagcaccaccaccagtatca
-270 cattcacattcaccatcatcgtctcttcgctcttatctctccgttacttttttatttact
-210 atctctctcttccccgttacttttttttcttccccgtttcgttctccgtcaaagtcttct
W-box
-150 ctctttaccattttctccactgtactaatttccctttttaccccctaaacccatcgttccc
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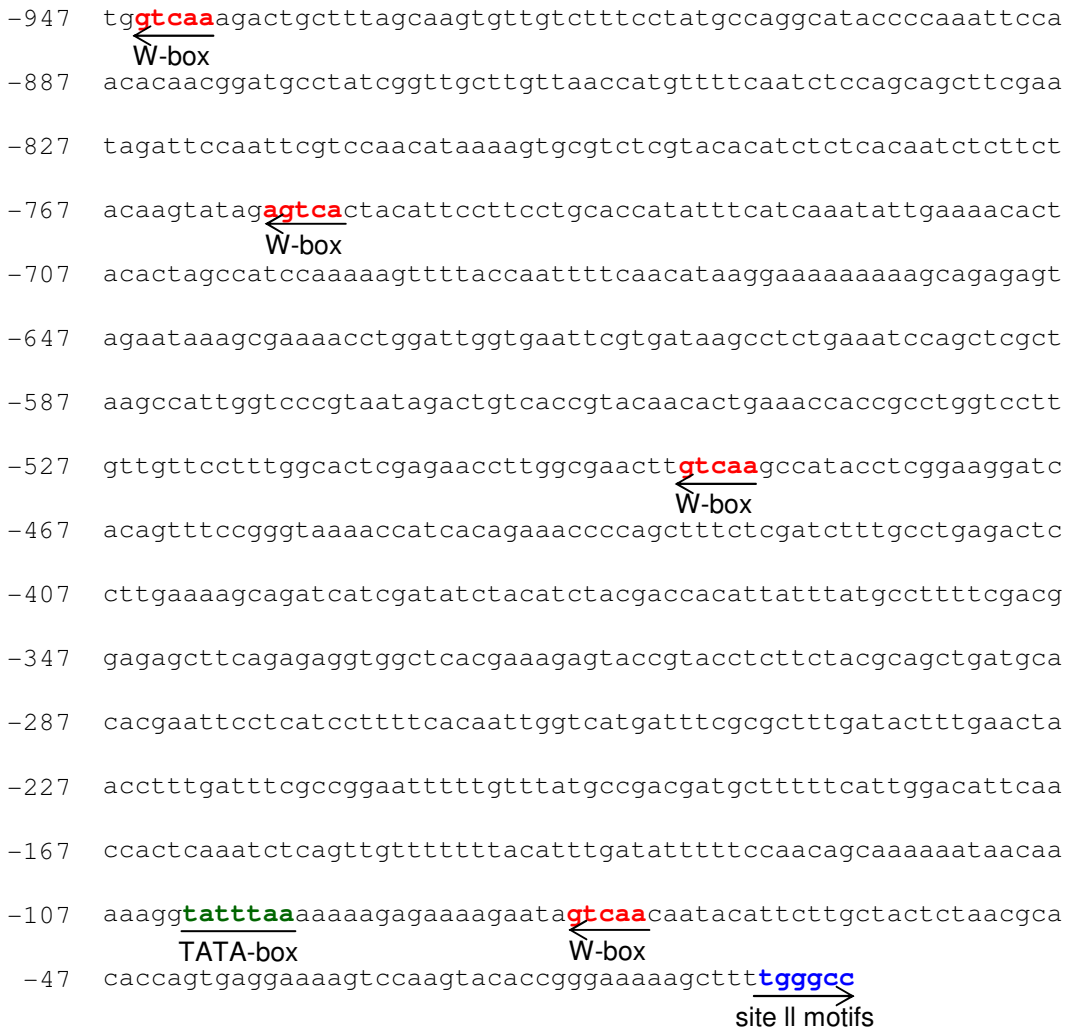
TATA-box
-30 ttctccatcggagacaccaccgctcaaaaa

Figure V-11

B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W-box
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-407 cttgaaaagcagatcatcgatatctacatctacgaccacattatttatgccttttcgacg
-347 gagagcttcagagaggtggctcacgaaagagtaccgtacctcttctacgcagctgatgca
-287 cacgaattcctcatccttttcacaattggtcatgatttcgcgctttgatactttgaacta
-227 acctttgatttcgccggaatttttgtttatgccgacgatgctttttcattggacattcaa
-167 ccactcaaatctcagttgtttttttacatttgatattttccaacagcaaaaaataacaa
-107 aaaggtatttaaaaaaagagaaaagaatagtcaacaatacattcttgctactctaacgca
TATA-box
W-box
-47 caccagtgaggaaaagtccaagtacaccgggaaaaagctttttgggcc
site II motifs

Figure V-11

Figure V-11. Sequence analysis of *SRFR1* and *RPS4* promoters. Nucleotide sequence of the 5'-flanking region of **(A)** *SRFR1* encompassing 1050 base pairs, and **(B)** *RPS4* encompassing a 947 base pairs. W-box (WRKY transcription factor binding site, blue color) and site II motifs (TCP transcription factor binding site, red color) are indicated by arrows.

codon. Site II motifs (TGGGCY, Y=C or T) were identified as a *cis*-acting element recognized by TCP-P proteins including TCP8, TCP20, and TCP22 in Arabidopsis (Tremousaygue et al., 2003; Herve et al., 2009). Site II motifs were not represented in the PLACE database. We examined the *SRFR1* promoter manually and found three Site II motifs at position -468, -407 and -395 (Figure V-11A). Four W-boxes and one Site II motif were located in the promoter of *RPS4* (Figure V-11B).

Discussion

Using a genetic suppressor screen and map-based cloning approach, we previously identified two Arabidopsis *srfr1* (*suppressor of rps4-RLD 1*) mutants, *srfr1-1* and *srfr1-2*, which reactivate resistance to DC3000 expressing *avrRps4* (Kwon et al., 2004). *SRFR1* encodes a pioneer tetratricopeptide repeat (TPR) protein (Kwon et al., 2009). We hypothesize that SRFR1 functions as a negative regulator of AvrRps4-triggered immunity because mutations in *srfr1* are recessive, and these mutants are fully susceptible to virulent DC3000, with specifically enhanced resistance to DC3000(a*vrRps4*). In the presence of a functional RPS4 immune receptor, as in wild-type Col-0 plants, AvrRps4-triggered immunity would overcome the repressor effect of SRFR1 and pass the threshold for effective resistance. In the absence of RPS4, as in the RLD wild type, SRFR1 inhibits weak AvrRps4-triggered immunity. In this model, *srfr1*

mutants are closer to a threshold for defense activation. In addition to the genetic evidence, phylogenic analysis and nuclear localization of SRFR1 supports our hypothesis that SRFR1 functions in a transcriptional repressor complex to negatively regulate AvrRps4-triggered immunity (Kwon et al., 2009).

In this study, we measured the expression levels of defense related genes in *srfr1* mutants by a quantitative real-time PCR. Interestingly, several defense-related genes were significantly increased in *srfr1* mutants in the absence of biotic and abiotic stresses (Figure V-1A). RPS4 belongs to the TNL class of R-proteins, and EDS1, PAD4 and PR proteins are reported to be involved in a TNL-mediated disease resistance response, suggesting that expression of defense genes in downstream of *RPS4*-mediated resistance could be upregulated in *srfr1* mutants. *PR1* expression has been reported to be induced over 1000-fold in resistant plants upon pathogen inoculation (Xiao et al., 2005) and the level of *PR1* induction in uninduced *srfr1* mutants was still considerably lower than in SA-induced tissue from a separate batch of wild-type or mutant plants (Figure V-1B). This perhaps explains why lower levels of *PR1* induction in *srfr1* mutants were previously not detected by RNA gel blot analysis (Kwon et al., 2009). In addition, we have generated *srfr1-3* mutants, homozygous T-DNA knock-out lines for *SRFR1* in the Col-0 wild-type. Interestingly, homozygous *srfr1-3* produced a heavily stunted phenotype, and several defense related genes are upregulated in *srfr1-3* mutants (unpublished data), reminiscent of the Arabidopsis *bon1* mutant phenotype. Mutation in *BON1* activates the *SNC1* TNL *R* gene, leading to constitutive defense responses and reduced plant growth (Yang and Hua, 2004).

Together, these results are supportive of our hypothesis that SRFR1 functions in a transcriptional repressor complex. Whether SRFR1 directly or indirectly downregulates defense gene expression, or whether this regulation involves TNL-independent defense genes, awaits further study.

The TPR domain of Ssn6 in *S. cerevisiae* shows significant similarity to that of SRFR1 (Kwon et al., 2009). Ssn6 physically binds to the WD40 domain containing Tup1 protein. The Ssn-Tup1 complex recognizes DNA-binding TFs to form a general transcriptional repressor complex (Smith and Johnson, 2000). Based on the yeast model, we speculate that SRFR1 may interact with TFs to repress expression of defense-related genes (Figure V-2). Using a yeast two-hybrid system, we screened a specific TF library of Arabidopsis, because TFs are often underrepresented in whole transcription cDNA libraries. Through the primary screen and individual co-transformation, we found that SRFR1 interacts with 3 members of TCP TFs, TCP8, TCP20, and TCP22. Compared to the strong SRFR1 interaction with TCP8 and TCP22, the interaction with TCP20 was moderate or weak (Figure V-3B). The Ssn6-Tup1 protein weakly interacts with DNA-binding TFs in yeast, which are sufficient to repress target genes(Smith and Johnson, 2000), suggesting relatively weak interaction between SRFR1 and TCP20 could be sufficient to function as a repressor complex. Two subfamilies of TCP proteins, TCP-C and TCP-P, were defined based on the structure of a b-HLH DNA-binding domain. Interestingly, all three SRFR1 interactors belong to the TCP-P subfamily of TCP proteins (Figure V-3C). TCP proteins have fairly recently been reported to function as transcription factors, and to date have

mainly been implicated in plant developmental pathways, such as lateral shoot branching, control of floral asymmetry, control of the cell cycle as well as cell expansion, pollen development, and seed germination (Nath et al., 2003; Tremousaygue et al., 2003; Cubas, 2004; Li et al., 2005; Takeda et al., 2006; Koyama et al., 2007; Tatematsu et al., 2008; Herve et al., 2009). However, so far, a functional dichotomy between TCP-C and TCP-P has not been uncovered. Additionally, as far as we know, our study is the first report suggesting a role of TCP proteins, not only in plant defense responses, but anywhere outside of plant development, and identifying a physical interactor of TCP proteins. SRFR1 did not interact with WRKY TFs (data not shown), which are plant-specific TFs mainly involved in plant defense responses (Eulgem et al., 2000; Dellaire et al., 2003; Eulgem and Somssich, 2007). Therefore SRFR1 may specifically interact with TCP-P proteins to function in a transcriptional repressor complex that balances plant resistance activation and suppression, or plant immunity and development.

Interactions of SRFR1 with the three TCPs in a yeast two-hybrid system were confirmed by bimolecular fluorescence complementation (BiFC) *in planta* (Figure V-6). BIFC is a noninvasive fluorescence based technique used to identify protein-protein interactions and the subcellular localization of the interacting protein complexes in intact living tissues (Citovsky et al., 2006; Ohad et al., 2007). This technique has several advantages, including minimal equipment, simple methods, a prevention of protein degradation during biochemical cell extraction, and a sensitive detection method, suggesting that

BIFC is the best method to test *in planta* interactions of proteins of interest that are expressed at fairly low levels, such as SRFR1 and TFs. Intriguingly, the three SRFR1-interacting TCP proteins showed homologus (Figure V-7A) and heterologous (Figure V-7B) interactions in the nucleus. Moreover, SRFR1 also forms a nuclear homodimer (Figure V-7A). The bHLH TFs are known to form homodimers and heterodimers to recognize core *cis*-acting elements. The first TCP-P proteins, PCF1 and PCF2, in rice interact with themselves and each other for DNA binding in a yeast two-hybrid system (Kosugi and Ohashi, 1997). These findings support our model that SRFR1 functions in a transcriptional repressor complex with DNA binding TCP proteins. Dimerization of SRFR1 perhaps explains the interallelic complementation found in crosses between *srfr1-1* and *srfr1-2* (Kwon et al., 2004).

As shown in Figure V-3C and Figure V-4, TCP8 and TCP22 are the closest paralogs within the TCP family, but TCP20 is relatively far from them. Nuclear speckles are one of the subnuclear compartments making up the nucleus, and are thought to be storage sites for pre-mRNA splicing factors (Dellaire et al., 2003). TCP8 and TCP22 were shown to localize to nuclear speckles (Figure V-5), and the interaction of the two proteins leads to their strong targeting to nuclear speckles (Figure V-7B). We previously reported that a sub-pool of SRFR1 localized to nuclear speckles, raising the possibility that a SRFR1-TCP8-TCP22 protein complex or TCP8 and TCP22 alone might regulate alternative splicing of defense related genes, including *RPS4* and other TNL *R* genes to fine tune the defense response.

We hypothesized SRFR1 may function as a negative regulator of AvrRps4-triggered immunity by forming a host protein complex with TCP8, TCP20, and TCP22. However, AvrRps4-triggered immunity was not altered in the *TCP8* and *TCP22* knockout plants or the *TCP8* and *TCP20* overexpression plants (Figure V-8 and Figure V-9). We speculate that no phenotype of AvrRps4 in loss- and gain-of function plants can result from probable functional gene redundancy. Again, knockout or overexpression of single *TCP* gene may not be enough to induce resistance or susceptibility. Recently, Herve and colleagues showed that expression of modified TCP20 proteins containing an EAR domain (dominant negative repressor) or a VP16 domain (strong activator) led to severe developmental phenotypes in Arabidopsis (Herve et al., 2009). Further study of double or triple knockout plants of the three TCPs and plants expressing those TCPs with a dominant repressor or activator domain will help determine the biological relevance of TCP proteins.

Because SRFR1 functions as a negative regulator in AvrRps4-triggered immunity, we hypothesize that overexpression of *SRFR1* may downregulate disease resistance. Although overexpression can lead to artifacts, it has been a useful tool to examine downregulation of disease resistance. Overexpression of RIN4 and PPRL, negative regulators of AvrRpt2-triggered immunity, boost the growth of DC3000(*avrRpt2*) and suppress defense responses (Mackey et al., 2003; Katiyar-Agarwal et al., 2006). However, overexpressing *SRFR1* in RLD wild-type did not increase the bacterial susceptibility to virulent and avirulent bacteria (Figure V-10B), suggesting SRFR1 may negatively regulate a defense

response through a different mechanism than RIN4 and PPRL. Alternatively, we can not exclude the possibility that saturation of DC3000(*avrRps4*) growth in RLD, which is naturally highly susceptible to DC3000(*avrRps4*), may prevent detection of an altered phenotype in *SRFR1*-overexpressing RLD plants. Overexpressing *SRFR1* in Col-0 plants displayed a stunting phenotype reminiscent of *srfr1-3* knockout plants (data not shown), and this defective growth inhibits accurate measurement of bacterial growth.

TCP-P proteins recognize a *cis*-acting element called site II motifs (TGGGCY, Y=C or T) to regulate gene expression (Tremousaygue et al., 2003; Herve et al., 2009). Interestingly, *SRFR1* and *RPS4* promoters contain three and one site II motifs, respectively (Figure V-11A and V-11B). The finding of site II motifs in the *SRFR1* and *RPS4* promoters raises the possibility that the SRFR1 interacting TCPs may regulate expression of *SRFR1* by negative or positive feedback, to fine-tune plant defense and growth. Alternatively, the SRFR1-TCPs protein complex may control the expression of the *RPS4 R* gene, which is supported by an increase of *RPS4* transcript levels in *srfr1* mutants (Figure V-1). To carefully study the role of TCP TFs in AvrRps4-triggerd immunity mediated by RPS4 and SRFR1, a detailed analysis will be required.

Materials and Methods

Plant growth and *in planta* bacterial growth curve assay

Pseudomonas syringae pv. tomato strain DC3000 (DC3000) containing the empty vector or expressing *avrRps4*, were grown as described previously (Gassmann et al., 1999). Arabidopsis plants were grown in an E-7/2 reach-in growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) under an 8 h light/16 h dark cycle at 24°C, 70% relative humidity and a light intensity of 90-140 μmol photons m^{-2} s^{-1}. *In planta* bacterial growth assays were performed by syringe infiltration as described (Gassmann et al., 1999; Zhang and Gassmann, 2003). Briefly, leaves of 4-week old plants were infiltrated with bacterial suspensions of 5×10^4 cfu/mL. Leaf discs with a total area of 0.5 cm^2 per sample were ground in 10 mM $MgCl_2$, and solutions were plated in serial dilutions on selective medium in triplicate at the indicated time points.

Real time PCR

Quantitative reverse transcription PCR was perforemd as described previously (Kwon et al., 2009). Briefly, total RNA was extracted from RLD, *srfr1-1* and *srfr1-2* using TRIZOL (Invitrogen, Carlsbad, CA, USA). For RT-PCR experiments, cDNA was synthesized from 2 μg of total RNA using an oligo(dT)15 primer and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's protocols. Real-time qRT-PCR was performed with SYBR GREEN PCR Master Mix and an ABI 7500 system (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. The levels of transcripts were normalized using *SAND* gene (At2g28390) mRNA levels as an internal standard (Kwon et al., 2009). These experiments were performed at least twice with similar results. Table V-1 lists the

Table V-1. Primers used in this study

Primer	Sequences (5'-3')	Purpose
SAND F	AACTCTATGCAGCATTTGATCCACT	qRT-PCR
SAND R	TGATTGCATATCTTTATCGCCATC	qRT-PCR
SRFR1F	CTGGATATGCCTCACTAGAAG	qRT-PCR
SRFR1R	CACTGGGTCACAAGGCTCTG	qRT-PCR
RPS4F	CCTAACATTATGGGCATCATCA	qRT-PCR
RPS4R	CCGCCTTCACAATTTCATTGA	qRT-PCR
PR1 F	GCAATGGAGTTTGTGGTCAC	qRT-PCR
PR1 R	GTTCACATAATTCCCACGAGG	qRT-PCR
PR2 F	ATCTCCCTTGCTCGTGAATC	qRT-PCR
PR2 R	GGATCGTTATCAACAGTGGAC	qRT-PCR
PDF1.2 F	AAGTTGTGCGAGAAGCCAAG	qRT-PCR
PDF1.2 R	CCATGTTTGGCTCCTTCAAG	qRT-PCR
EDS1 F	GACGGGGAAGTAGATGAGAAG	qRT-PCR
EDS1 R	TCATCCATCATACGCTCACG	qRT-PCR
PAD4 F	GAGGAGATCTTTGTTACGGG	qRT-PCR
PAD4 R	TCGCCTCCCACACACTATAA	qRT-PCR
SRFR1 GATE F1	AAAAAGCAGGCTCAATGGCGACGGCGACG GCG	Cloning of SRFR1 entry
SRFR1 GATE F2	AAAAAGCAGGCTCAGTGGAGAAATTTGTTC TTCAAT	Cloning of SRFR1 entry
SRFR1 GATE F3	AAAAAGCAGGCTCAGATTTTCGACTTTCTA GAGGC	Cloning of SRFR1 entry
SRFR1 GATE R1	CGCTTAGCACTTACAACGATTACCCAGCTT TCT	Cloning of SRFR1 entry
SRFR1 GATE R2	TTGACGTGGAACTTGATGCATACCCAGCTT TCT	Cloning of SRFR1 entry
SRFR1 GATE R3	TAACCAAACAGAAGGCGAATTACCCAGCTT TCT	Cloning of SRFR1 entry
TCP8 GATE F1	AA AAA GCA GGC TCA ATG GAT CTC T CC GAC ATC CG	Cloning of TCP8 entry
TCP8 GATE R1	GGAGAACTCAAATAGCTCTGAGTACCCAGC TTTCT	Cloning of TCP8 entry
TCP20 GATE F1	AA AAA GCA GGC TCA ATG GAT CCC AAG AAC CTA AA	Cloning of TCP20 entry
TCP20 GATE R1	TCTCAAGGCTCAGGTCGTTACCCAGCTTTC T	Cloning of TCP20 entry
TCP22 GATE F1	AA AAA GCA GGC TCA ATG AAT CAG A AT TCC TCT GTTG	Cloning of TCP22 entry
TCP22 GATE R1	TGGTGGTGATGACAAAAAGTACCCAGCTTT CT	Cloning of TCP22 entry
EYFP F	CCGGTACCTCTCAACACAACATATA	Cloning of pSBnEY and pSBcEY
nEYFP R	CCGGCGCGCCGTCCTCGATGTTG	Cloning of pSBnEY
cEYFP R	CCGGCGCGCCTTCGTACAGCTC	Cloning of cSBnEY

oligonucleotide sequences used. To measure the induced expression level of *PR1*, Arabidopsis plants were sprayed with 1.5 mM Salicylic acid (SA).

Molecular cloning of plasmids

The coding region of *SRFR1, TCP8, TCP20* and *TCP22* were amplified by PCR using first strand cDNA from Arabidopsis (Col-0), and sub-cloned into the pDONR201 entry vector using BP reaction following the manufacturer's protocols (Invitrogen, Carlsbad, CA, USA). The entry clones were recombined into GATEWAY compatible destination vectors using LR reaction (Invitrogen, Carlsbad, CA, USA) for yeast two-hybrid screening, plant transformation, Agrobacterium-mediated transient expression and western blot. Primer sequences used in cloning are described in Table V-1.

Yeast two-hybrid screening

To construct baits, full length and partial *SRFR1* cDNAs were cloned into pDONR201 and then recombined into Gateway-compatible pDEST32 (Invitrogen, Carlsbad, CA, USA). Primers used for cloning were: SRFR1GATE F1 and SRFR1GATE R1 for $SRFR1_{1-1052}$; SRFR1GATE F1 and SRFR1GATE R2 for $SRFR1_{1-567}$; SRFR1GATE F2 and SRFR1GATE R1 for $SRFR1_{568-1052}$; SRFR1GATE F3 and SRFR1GATE R3 for $SRFR1_{298-653}$ (See details in Table V-1). The prey library used in yeast two-hybrid screening contained over 1,400 Arabidopsis transcriptional regulator full-length cDNAs and is in the CEN-based Gateway-compatible pDEST22 low-copy prey vector (Invitroten, Carlsbad, CA,

USA). The *SRFR1* baits and prey library were transformed into the yeast strain PJ69-4a (MATa *trp1-901 leu2-3,112 ura3-52 his3-200 Δgal4 Δgal80 LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) by a standard yeast transformation procedure (Clonetech, CA, USA) and the transformation mixture was plated on SD media [-Trp-Leu, -Trip-Leu-His (0.1 mM and 0.5 mM 3-AT), -Trp-Leu-Ade-His]. Plates were grown at 30℃ and examined 4 days later. We screened up to approximately $1.7x10^4$ colonies and isolated putative positive clones. Plasmids were isolated from yeast and then sequenced. Each pair of *SRFR1* bait and prey encoding a potential interacting protein was directly co-transformed into PJ69-4a and then screened on selective media to confirm interactions.

Generation and analyses of transgenic Arabidopsis plants

To induce constitutive expression of a gene of interest under the CaMV 35S promoter, entry clones of *TCP8, TCP20, TCP22,* and *SRFR1* were recombined into GATEWAY compatible Myc-pBA (35S-Myc-GW), HA-pBA (35S-HA-GW) and PMDC43 (35S-GFP-GW) vectors. All subclones were transferred to Agrobacterium strain C58C1 and transformed into wild type Col-0 by floral dip (Clough and Bent, 1998). The *GFP-SRFR1* fusion gene was transformed into RLD, *srfr1-1* and *srfr1-2*. Plants were screened on half-strength Murashige and Skoog medium containing 20 μg/mL hygromycin for GFP constructs and Basta for Myc-pBA constructs. T3 homozygous plants were used in bacterial growth curve and western blot experiments.

Agrobacterium-mediated transient expression

Most binary constructs, except the split YFP vector, containing the gene of interest tagged with GFP, Myc and HA were mobilized into the Agrobacterium strain C58C1 containing the virulence plasmid pCH32. The split YFP constructs were electroporated into the *A. tumefaciens* strain GV3101. Bacteria cultured overnight were harvested by centrifugation and resuspended in 10 mM $MgCl_2$ with 100 µM acetosyringone (Sigma-Aldrich, St. Louis, Mo, USA) adjusted to an OD_{600} of 0.3. Agrobacterium was incubated for 2 hr at room temperature and infiltrated into *N. benthamiana* leaves with a 1-ml needleless syringe. *N. benthamiana* plants were placed in a E-7/2 reach-in growth chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) under a 16 h light/8 h dark cycle at 25°C, 70% relative humidity. Tissues were collected three days after infiltration for western blot and confocal microscopy.

Bimolecular Fluorescence Complementation *in planta*

To produce the pSB split YFP vectors for BiFC, the GFP of PMDC43 was replaced by nEYFP and cEYFP to generate pSBnEY (35S-nEYFP-GW) and pSBcEY (35S-cEYFP-GW), respectively. nEYFP and cEYFP were amplified using the primer pairs of EYFP F-nEYFP R and EYFP F-cEYFP R, respectively. EYFP F contained a *KpnI* restriction site with enhancer sequence and nEYFP R and cEYFP R contained a *AscI* site at the 5'-end (See Table V-1). The nEYFP and cEYFP PCR products digested with *KpnI* and *AscI* were sub-cloned into the

PMDC43 *KpnI-AscI* site to produce pSBnEY and pSBcEY, respectively. Finally, pSBnEY-SRFR1 (nY-SRFR1), pSBcEY-SRFR1 (cY-SRFR1), pSBnEY-TCP8 (nY-TCP8), pSBcEY-TCP8 (cY-TCP8), pSBnEY-TCP20 (nY-TCP20), pSBcEY-TCP20 (cY-TCP20), pSBnEY-TCP22 (nY-TCP22) and pSBcEY-TCP22 (cY-TCP22) were obtained by using GATEWAY LR reactions. Agroinfiltration was performed as described above. For co-infiltrations, each strain was adjusted to an optical density of 0.3 at 600 nm and they were mixed. Agrobacterium containing the HcPro silencing suppressor was also co-infiltrated into *N. benthamiana* when *SRFR1* constructs were used. Observations were performed 3 days after infiltration.

Confocal fluorescence microscopy

Plant tissues were viewed directly under a Zeiss LSM 510 META NLO two-photon point-scanning confocal system mounted on an Axiovert 200M inverted microscope with a 40·/1.2 C-Apochromat water immersion. GFP and YFP fluorescence was excited by a 488-nm laser and a 514-nm argon laser, respectively. Samples were detected using a 500- to 550-nm band-pass emission filter.

Western blot analysis

Total protein was extracted from transgenic Arabidopsis and transiently transformed *N. benthamiana* leaves as previously described (Kwon et al., 2009). Briefly, three discs of leaf tissue were ground in 100 µl of 8M urea buffer. 25 µl of

5X loading dye was added to the extracted protein and the samples were boiled for 5 min. The samples were centrifuged for 5 min at 13,200 rpm and the collected supernatant was used for immunoblotting. 40 µl of protein samples were separated on a 8–10% SDS-polyacrylamide gel and were transferred onto immune-blot PVDF membranes (Bio-Rad, Hercules, CA, USA). Immunodetection was performed as described previously (Moffet et al., 2002). HA tag was detected with 1:2000 diluted horseradish peroxidase (HRP)-conjugated HA antibody (Roche, Indianapolis, Indiana, United States) and Myc tag was detected with 1:2000 diluted HRP-conjugated Myc antibody (A-14) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). GFP tag was detected with 1:5000 diluted rabbit anti-GFP primary antibodies (Invitrogen, Carlsbad, CA, USA) and 1:5000 diluted goat anti-rabbit HRP-conjugated secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA). Detected proteins were visualized with an ECL Plus chemiluminescent kit (GE Healthcare, Buckinghamshire, UK).

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VITA

Sang Hee Kim was born October 23, 1975, in Seoul, Korea. He received the following degrees: B.S. in the Department of Agricultural Biology from Korea University at Seoul (2001); M.S. in the Department of Plant Pathology from Korea University at Seoul (2003); Ph. D. in the Division of Plant Sciences from the University of Missouri-Columbia (2009). He is currently working as a postDoc researcher in the Division of Plant Sciences, University of Missouri-Columbia.