Resistance to the *Pseudomonas syringae* Effector HopA1 Is Governed by the TIR-NBS-LRR Protein RPS6 and Is Enhanced by Mutations in *SRFR1*[1][W][OA]

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The *Pseudomonas syringae*-Arabidopsis (*Arabidopsis thaliana*) 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 (TNL) class. Comparative studies with the coiled-coil-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 TNL 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 *ENHANCED DISEASE SUSCEPTIBILITY1*, indicating that the corresponding resistance gene *RPS6* belongs to the TNL 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 TNL resistance 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. 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 TNL resistance pathway in Arabidopsis.

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 (*Arabidopsis thaliana*) 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 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 Leu-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

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that shows sequence similarity with the cytosolic domain of the animal innate immune transmembrane receptors Toll/Interleukin-1 receptor (TIR). A second class of NBS-LRR R proteins possesses an N-terminal coiled-coil 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 coiled-coil-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* (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 *P. syringae* pv maculicola strain 4326 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 *hopA1 susceptible* 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-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 1,100 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 d 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 Wassilewskija-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 four mutants classified as *hopA1 susceptible* (*hps*) that bred true and showed consistent chlorosis to DC3000(*hopA1*). Backcrossing *hps* mutants to RLD and to each other established that the mutations were recessive and fell into three complementation groups (Table 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.

Table I. *Genetic analysis of hps mutants*

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

Map-Based Cloning of *RPS6*

The *rps6-1* and *rps6-2* mutants were susceptible to DC3000 and DC3000(*hopA1*) (Fig. 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 outcross 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 five Arabidopsis chromosomes and analysis of chromosome break points placed the *RPS6* locus on the bottom of chromosome 5 between the markers RPS4 and NGA129 (Supplemental Table S1). For fine-scale mapping, approximately 300 *hopA1*-susceptible F2 plants were selected, and additional mapping markers were identified by testing simple sequence length polymorphism (SSLP) and CAPS markers at bacterial artificial chromosome (BAC) ends (Supplemental Table S2). The *RPS6* locus was confined between markers MUGCAPS05 and MUGSSLP07, a 0.35-Mb interval (Fig. 2A).

Based on the physical location of putative Arabidopsis resistance genes (Meyers et al., 2003), seven TNL genes on the two BAC clones MPL12 and K11I1 were identified within the genomic interval defined by MUGCAPS05 and MUGSSLP07 (Fig. 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 seven 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 seven candidate genes, only the pSHK103 construct containing At5g46470 changed the *rps6* mutant-susceptible phenotype to the parental *hopA1*-resistant phenotype (Fig. 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 approximately 100-fold lower bacterial growth than *rps6-1* mutant plants (Fig. 2C). Disease assays with these lines also showed complementation by At5g46470 (Supplemental Fig. S1). The lines shown in these figures were representative of a total of five out of five 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*.

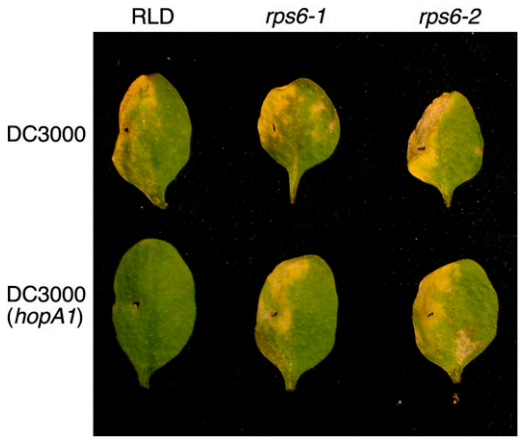

Figure 1. *rps6* mutants are susceptible to DC3000(*hopA1*). Disease symptoms of parental RLD (left), *rps6-1* (middle), and *rps6-2* (right) syringe inoculated with DC3000 (top row) or DC3000(*hopA1*) (bottom row). Leaves infiltrated with DC3000 and DC3000(*hopA1*) came from the same plant and remained attached until disease symptoms (leaf chlorosis) were recorded 5 d after inoculation. Only the left 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′-RACE. This showed that the *RPS6* transcript is approximately 5.8 kb long and contains nine exons (Fig. 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 polyadenylation 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 BioArray 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 1,127 amino acids (Fig. 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 pathogen *A. 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

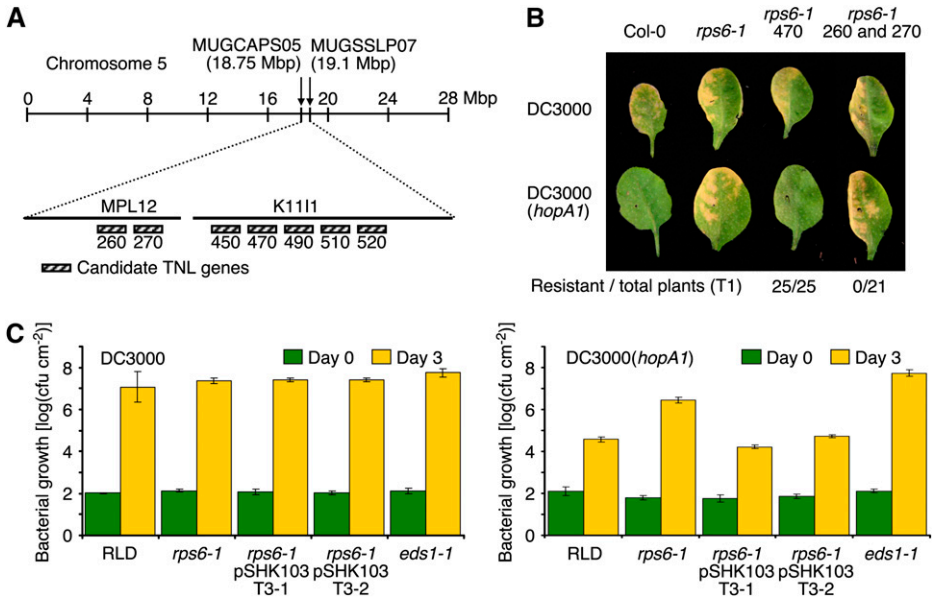

Figure 2. Map-based cloning of *RPS6*. A, Schematic diagram of the *RPS6* locus on chromosome 5. Two genetic markers confining *RPS6*, MUGCAPS05 and MUGSSLP07, are indicated by arrows (top). Seven candidate TNL genes located on BAC clones MPL12 and K11I1 within the *RPS6* locus are indicated by hatched 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 TNL 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 cfu/mL. The number of resistant T1 plants are indicated below the figure. Disease symptoms were recorded 5 d 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 SD. This experiment was repeated twice with similar results.

(Fig. 3B). The *rps6-1* allele has an A to G transition at base number 1,563 (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 bp change in *rps6-1* can be visualized by a CAPS marker (Supplemental Fig. S2). 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 (Fig. 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 (Fig. 4, B and C). Control reactions without reverse transcriptase showed that these bands did not arise from genomic DNA contamination (Fig. 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 (Fig. 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 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 no. 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

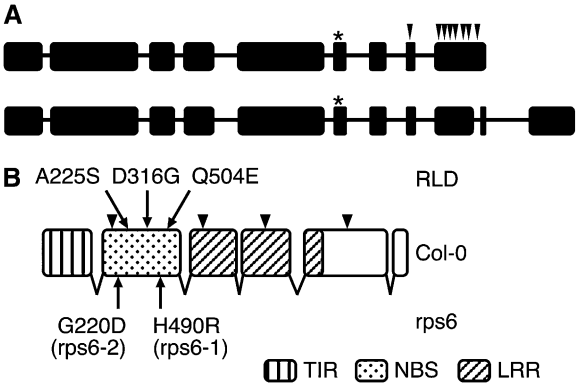

Figure 3. *RPS6* encodes a member of the TNL class of R proteins. A, *RPS6* gene model as experimentally verified by reverse transcription PCR and 3′-RACE (top) compared with the TAIR8 gene model (bottom). Exons are indicated by black boxes, introns by lines, and stop codons by asterisks. Positions of polyadenylation 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.

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. To exclude that the differential Arabidopsis response to DC3000 and DC3000($hopA1_{Pss61}$) 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 (Supplemental Fig. S3). 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 (Fig. 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 that shows resistance to all of the commonly used laboratory isolates of *H. parasitica* (J.M. McDowell, personal communication). The molecular characterization of RLD *rps6* mutants therefore enabled us to test the spectrum of *srfr1*-mediated resistance in a uniform genetic back-

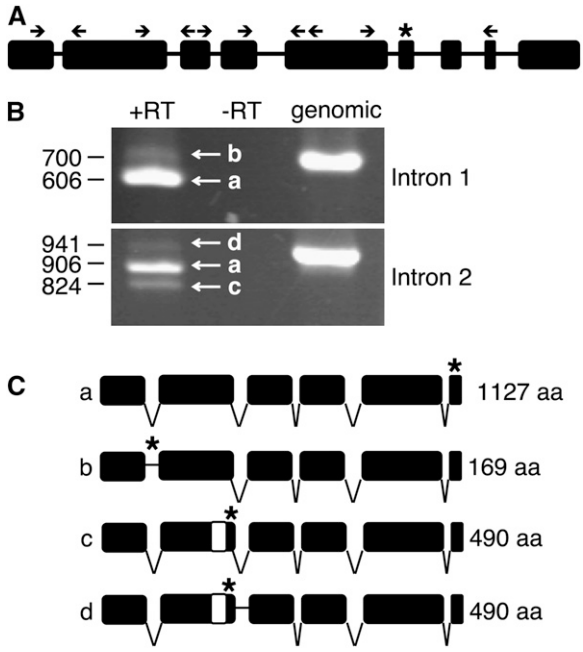

Figure 4. Alternative splicing of *RPS6*. A, Schematic diagram of the *RPS6* gene structure. PCR primer pairs flanking introns are indicated by black arrows above the diagram. B, PCR with primers flanking introns 1 (top) and 2 (bottom) yields multiple products, indicated by white arrows. Reverse transcription 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 bp 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 (aa) lengths of the corresponding predicted RPS6 proteins are shown to the right of the diagrams.

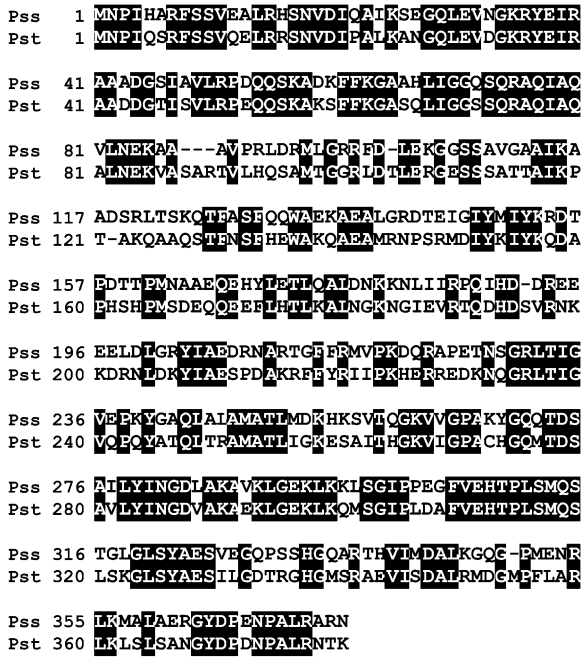

Figure 5. Protein sequence alignment of HopA1 from *P. syringae* pv syringae strain 61 (Pss, top) and DC3000 (Pst, bottom). Sequences were aligned with ClustalW using the Gonnet 250 amino acid weight matrix. Identical amino acid residues are shaded black.

ground 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* (Fig. 6). While virulent DC3000 grew to comparable high levels in all plant lines, DC3000(*hopA1*) growth was restricted to levels approximately 1,000-fold lower in resistant RLD and *srfr1-1*, both of which have a functional *RPS6* gene. Similar to Figure 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 is larger [50- to 100-fold lower DC3000 (*avrRps4*) levels on *srfr1-1* than on RLD; Kwon et al., 2004; Supplemental Fig. S4], 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 (Fig. 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* double mutants did not increase compared to growth in the *srfr1-1* single mutant (Supplemental Fig. S4). 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

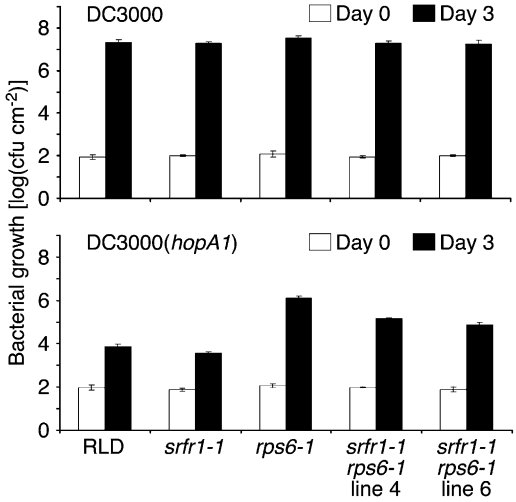

Figure 6. Mutations in *SRFR1* enhance *hopA1*-triggered immunity in *rps6* mutants. In planta bacterial growth was measured in RLD, *srfr1-1*, *rps6-1*, and two independent F3 *srfr1-1 rps6-1* double mutants on day 0 (white columns) and day 3 (black columns) after inoculation with DC3000 (top) and DC3000(*hopA1*) (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 SD. This experiment was repeated three times with similar results.

largely nonfunctional. 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 turnover 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 *Nicotiana glutinosa N* gene were nonfunctional (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′-untranslated region 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′-CCTGGTAATACCCG-CAAATC-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 (*Arabidopsis thaliana*) 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.) under an 8 h light/16 h dark cycle at 24°C, 70% relative humidity, and a light intensity of 90 to 140 μmol photons $m^{-2}\,s^{-1}$. For dip inoculations, rosettes of 3- to 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 d postinoculation 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 1,100 M1 plants from 67 different pools were dip inoculated with DC3000(*hopA1*) and scored for disease symptoms 5 d 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 χ^2 test with one degree of freedom.

The BAC clones K11I1 and MPL12 were obtained from the Arabidopsis Biological Resources Center. To generate subclones containing At5g46260, At5g46270, At5g46450, At5g46470, At5g46490, At5g46510, and At5g46520, the BAC clones were partially digested with *Hin*dIII, and DNA was subcloned into the cosmid binary vector pCLD04541. In the case of the *RPS6*-specific genomic subclone pSHK103, a 10 kb band from a partial digest of BAC K11I1 with *Hin*dIII encompassed At5g46470 including 3.1 kb upstream of the start codon and 3 kb downstream of the stop codon. Subclones were transferred to *Agrobacterium 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 reverse transcriptase (Promega). To test splicing variation, PCR was performed using a primer pair flanking introns (5′-TGAGGAAACAGACCGGAGAC-3′ and 5′-CAAGATGTGCTAGATG-CTTTAGCGG-3′ for intron 1; 5′-GTGGGAGTAGAATCATTGTG-3′ and 5′-AGCAGAGCAAACTTGAGAAG-3′ for intron 2; 5′-GCTTCGACTATT-TGCCCTCTAGACT-3′ and 5′-AACAACCCAAGTTTTGTGGAAGTCC-3′ for introns 3 and 4; 5′-ACAGCGGACATTCCCTCTAA-3′ and 5′-GGGACTC-TATCTCCCTACCT-3′ for intron 4; and 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.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_124017.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Disease assays on complemented *rps6-1* transgenic lines.

Supplemental Figure S2. Point mutations in *rps6-1* and *rps6-2*, and visualization of the mutation in *rps6-1* by CAPS marker.

Supplemental Figure S3. DC3000 expressing $hopA1_{DC3000}$ from vector pML123 does not trigger resistance in Arabidopsis.

Supplemental Figure S4. Mutations in *RPS6* do not affect *srfr1*-mediated resistance to DC3000(*avrRps4*).

Supplemental Table S1. Recombination frequency between *rps6-1* and genetic markers on chromosome 5.

Supplemental Table S2. *RPS6* fine-mapping markers on the bottom of chromosome 5.

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