Alternative Splicing and mRNA Levels of the Disease Resistance Gene RPS4 Are Induced during Defense Responses\textsuperscript{1,W,OA}

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The Arabidopsis (Arabidopsis thaliana) disease resistance protein RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4) activates defenses in response to bacterial pathogens expressing \textit{avrRps4} in a gene-for-gene specific manner. The RPS4 gene produces multiple transcripts via alternative splicing of two regular introns flanking exon 3 and a cryptic intron within exon 3. We showed previously that RPS4-mediated resistance requires the combined presence of transcripts encoding both full-length and truncated open reading frames. Here, we demonstrate that alternative splicing of RPS4 undergoes dynamic changes specifically during the resistance response. Furthermore, RPS4 expression was induced by the presence of AvrRps4 in an \textit{EDS1}-dependent manner. Interestingly, inducible alternative splicing was not limited to the \textit{avrRps4-RPS4} interaction, indicating that regulation of alternative splicing may be a general response to prime the plant stress response system. Intron-deficient transgenes lacking only one intron were previously shown to be nonfunctional. Here, we establish quantitatively that the absence of one intron had no effect on the splicing frequency of remaining introns. Given the lack of functionality of single intron-deficient transgenes, this suggests that the products of individual transcripts have distinct functions during RPS4-triggered resistance. Transient expression of truncated RPS4 proteins in \textit{Nicotiana benthamiana} induced hypersensitive response-like cell death in the absence of AvrRps4. Interestingly, different truncated proteins had markedly differing stability. In summary, RPS4 function is regulated at multiple levels, including gene expression, alternative splicing, and protein stability, presumably to fine-tune activity and limit damage inflicted by activated RPS4 protein.

Alternative splicing is widespread in eukaryotic organisms and significantly contributes to proteomic complexity (Graveley, 2001; Reddy, 2001; Brett et al., 2002; Kazan, 2003; Kriventseva et al., 2003; Iida et al., 2004). A recent genome-wide analysis in Arabidopsis (Arabidopsis thaliana) with an expanded set of ESTs concluded that the incidence of alternative splicing is approximately 22% (Wang and Brendel, 2006), with intron retention the major mechanism of alternative transcript generation (Ner-Gaon et al., 2004; Wang and Brendel, 2006). Interestingly, stress response genes were overrepresented among transcripts that showed intron retention (Ner-Gaon et al., 2004). This raises the possibility that the incidence of intron retention in plants is underestimated because many stress response genes have very low steady-state expression levels. Notably, to date the Arabidopsis RESISTANCE TO PSEUDOMONAS SYRINGAE4 (RPS4) gene is not represented in EST databases.

Several mechanisms of alternative splicing have been reported for plant RESISTANCE (R) genes belonging to the TOLL/INTERLEUKIN-1 RECEPTOR (TIR)-nucleotide-binding site (NBS)-Leu-rich repeat (LRR) class (Whitham et al., 1994; Lawrence et al., 1995; Gassmann et al., 1999; Jordan et al., 2002; Vidal et al., 2002; Borhan et al., 2004; Schornack et al., 2004). The majority of TIR-NBS-LRR (TNL) R gene alternative transcripts possess premature stop codons that lead to truncation of open reading frames (ORFs). These transcripts encode TIR-NBS (TN) proteins or TN proteins with only the first several LRRs (TNxL; Whitham et al., 1994; Lawrence et al., 1995; Gassmann et al., 1999; Jordan et al., 2002; Vidal et al., 2002; Borhan et al., 2004; Schornack et al., 2004). For both the tobacco (Nicotiana tabacum) N and the Arabidopsis RPS4 gene, alternative transcripts were required for rapid and complete R gene-mediated resistance (Dinesh-Kumar and Baker, 2000; Zhang and Gassmann, 2003; Schornack et al., 2004). For both the tobacco (Nicotiana tabacum) N and the Arabidopsis RPS4 gene, alternative transcripts were required for rapid and complete R gene-mediated resistance (Dinesh-Kumar and Baker, 2000; Zhang and Gassmann, 2003). The Arabidopsis genome also contains TIR- and TN-encoding genes that evolved independently and are not evolutionary remnants of TNL R genes (Meyers et al., 2002). These findings suggest that the plant genome harbors a large number of TIR and TN protein-encoding
genes and alternative TNL R gene transcripts, but their role in disease resistance or other plant functions is unknown.

Alternative splicing of R genes, mainly reported for TNL genes, is not limited to this class of R genes. The barley (*Hordeum vulgare*) coiled-coil (CC)-NBS-LRR gene *Mla13* is alternatively spliced in the 5'-untranslated region, and its splicing pattern changes during the resistance response (Halterman et al., 2003), regulating the expression of MLA13 protein during the infection process (Halterman and Wise, 2006). Similarly to Arabidopsis *RPP5* (Parker et al., 1997), a putative truncated MLA6 protein is encoded by a truncated gene family member (Halterman et al., 2001). Tobacco *NRG1*, a CC-NBS-LRR gene that is required for *N* gene-mediated resistance to *Tobacco mosaic virus*, produces two mRNAs: full-length *NRG1* mRNA and a truncated form with its 3’ end within intron 1 (Peart et al., 2005). Interestingly, transcript alteration in the absence of introns was reported for the maize (*Zea mays*) *Rp1-D* rust resistance gene (Ayliffe et al., 2004). In this case, transgenic barley and wheat (*Triticum aestivum*) expressing *Rp1-D* accumulated a truncated transcript arising from premature cleavage and polyadenylation, but no full-length transcript. Intriguingly, this alternative processing also occurred in maize, albeit at very low frequency (Ayliffe et al., 2004).

Alternative splicing can be constitutive, leading to stable ratios of transcript variants (TVs), or dynamic and tightly regulated in response to developmental, physiological, and biochemical cues (Lopez, 1998). The ratio of alternative versus regular *N* transcript increases approximately 60-fold after viral infection, and transgenic tobacco plants expressing both transcripts in a stable 1:1 ratio display incomplete resistance to a similar degree as plants expressing only the regular transcript (Dinesh-Kumar and Baker, 2000). In the case of *RPS4*, we previously showed that coexpression of intron-deficient and artificially truncated *RPS4* transgenes partially reconstituted *RPS4* gene function (Zhang and Gassmann, 2003). However, this result did not clarify whether complete resistance requires a narrowly defined stable ratio of regular and alternative *RPS4* transcripts or dynamic alternative splicing.

To address this, we developed an approach to accurately quantify multiple *RPS4* transcripts simultaneously. We found that the alternative transcript retaining intron 3 was rapidly up-regulated during the resistance response in wild-type Columbia (Col-0) plants, whereas the abundance of other alternative *RPS4* transcripts remained constant. We also found that *RPS4* expression is induced by *AvrRps4* in an *RPS4*- and *EDS1*-dependent manner. However, these responses were not limited to *avrRps4* or *RPS4*. Truncated *RPS4* proteins mimicking the protein products of *RPS4* alternative transcripts induced hypersensitive response (HR)-like cell death in the absence of *AvrRps4* when transiently expressed in *Nicotiana benthamiana*. Interestingly, different truncated proteins had markedly differing stability. We conclude that *RPS4* function is regulated at multiple levels, including gene expression, alternative splicing, and protein stability, presumably to fine-tune activity and limit damage inflicted by activated *RPS4* protein.

**RESULTS**

**Alternative Splicing of RPS4 Pre-mRNA**

At measurable frequencies, *RPS4* introns 2 and 3 are retained and a cryptic intron within exon 3 is spliced out (Gassmann et al., 1999; Zhang and Gassmann, 2003), theoretically resulting in eight *RPS4* TVs (Fig. 1). We refer to TV3 as the regular transcript because it contains a full-length ORF and was the predominant TV based on previous reverse transcription (RT)-PCR experiments (Zhang and Gassmann, 2003), whereas other TVs are collectively referred to as alternative transcripts. TV1, TV2A, TV2B, TV3, and TV4 were identified in several independent RT-PCR experiments and were sequenced (Gassmann et al., 1999; Zhang and Gassmann, 2003), confirming the structures shown in Figure 1. TV6, identified via fluorescence-aided detection in the course of this study, has not been verified by sequencing. TV5 and TV7 have not been identified to date, indicating that either they are very rare or they are not generated in planta.

**Figure 1.** Schematic diagram of the *RPS4* gene and predicted TVs. Exons are shown as boxes. Retained introns are shown as black lines and spliced introns are shown as diagonal lines. The cryptic intron within exon 3 is indicated by diagonal hatching. PCR primers are indicated by arrows. The forward primer is labeled with the fluorescent dye 56-FAM. *RPS4* TV designations are indicated on the left. The regular transcript TV3 encodes full-length *RPS4* and is underlined. Stars represent premature stop codons identified in each alternative transcript. On the right are indicated the size of PCR-amplified TV fragments (bp) and the average abundance of TVs relative to TV3 in uninoculated Col-0 leaf tissue from three biological replicates ±SD (%). n.d., Not detected. See text for details.
Quantifying in Planta RPS4 Transcript Profiles

Our previous study did not resolve whether RPS4 relies on temporal regulation of transcript abundance for complete function (Zhang and Gassmann, 2003). To address this, we attempted to quantify TV abundance by real-time PCR. However, the complexity of RPS4 pre-mRNA splicing and the degeneracy of RPS4 introns 2 and 3 and flanking sequences precluded this approach. Therefore, we developed an alternative method that relied on the predicted size differences of TVs and high-resolution separation of RT-PCR products on a capillary genetic analyzer for quantification (see “Materials and Methods”). Validation experiments using plasmid mixtures of cloned RPS4 transcripts as PCR templates showed that this approach reproducibly quantified expected RPS4 TV ratios (Supplemental Fig. S1; Supplemental Table S1). By integrating the splice status of two introns and a cryptic intron, this method has the advantage of not presupposing independence of individual splicing events and should therefore be generally applicable to quantification of complex alternative transcript products.

In untreated Col-0 leaf tissue, TV3, TV2A, and TV4 were robustly quantifiable. Relative to TV3, abundance of TV2A was 4.0% ± 1.0% and of TV4 1.7% ± 0.5% (Figs. 1 and 2). TV1, TV2B, and TV6 were detectable, but of such low abundance that accurate quantification was not always feasible. We estimated the upper limit for each of these transcripts at 2%. TV5 and TV7 were not detected. TV2A was therefore the most abundant alternative transcript in planta, suggesting that intron 3 is more recalcitrant to splicing than intron 2.

**TV2A Levels Are Increased during the Resistance Response**

We next quantified TV abundance relative to TV3 in inoculated wild-type Col-0. As in uninoculated tissue, TV1, TV2B, and TV6 were detectable, but of such low abundance at all time points after inoculation that accurate quantification was not feasible. When inoculated with buffer (data not shown) or virulent Pseudomonas syringae pv tomato strain DC3000, weak TV2A induction was observed at 1 h postinoculation (hpi), but not at later time points (Fig. 2). This suggests a minor nonspecific response caused by tissue flooding or infiltration damage at this early time point. The amount of TV4 was stable throughout the experiment. In marked contrast to TV4 and TV2A control inoculations, TV2A abundance increased to almost 80% relative to TV3 at 1 hpi when tissue was inoculated with DC3000(avrRps4) (Fig. 2). A second peak in TV2A abundance was observed at 12 hpi. Based on TV2A values at 8 and 12 hpi in individual experiments, we assume that the timing of this peak is more variable, leading to a larger error associated with TV2A levels at these time points. Statistical analysis using two-way ANOVA on pooled data from the three biological replicates showed that the values at 1 and 12 hpi with DC3000(avrRps4) were significantly different from those with all other treatments or time points (P < 0.01).

**RPS4 Is Induced by avrRps4 in an EDS1-Dependent Manner**

Besides the dynamic regulation during pre-mRNA splicing, the absolute abundance of alternative RPS4 transcripts could also be up-regulated via induction of RPS4 gene expression. Although generally considered to be constitutively expressed, several R genes have recently been reported to be induced by pathogens (see “Discussion”). We therefore quantified overall RPS4 mRNA abundance by real-time PCR using primers flanking the constitutively spliced intron 1 (see “Materials and Methods”). In wild-type Col-0 plants, RPS4 expression was weakly induced by inoculation with buffer or virulent DC3000 (Fig. 3A). In fact, RPS4 was found to be induced about 2- to approximately 4-fold under various biotic stresses by querying the digital northern database (Zimmermann et al., 2004; Toufighi et al., 2005), suggesting that the RPS4 induction we measured occurs under multiple

![Figure 2. Generation of the alternative RPS4 transcript TV2A is dynamic. The amount of TV2A and TV4 transcripts as a percentage of the amount of TV3 is shown at indicated time points after inoculation of Col-0 with virulent DC3000 or DC3000 expressing avrRps4. Tissue for the zero time point was harvested immediately prior to inoculation. Data represent averages of three biological replicates, with error bars denoting 30%](image-url)
conditions. In contrast to this nonspecific induction, RPS4 was induced significantly higher (ANOVA test, \( P < 0.01 \)) after leaf inoculation with DC3000(\textit{avrRps4}) (Fig. 3A). The RPS4 mRNA levels shown in Figure 3A correspond to an induction of 17.4 ± 5.9-fold at 1 hpi and 23.5 ± 8.1-fold at 12 hpi compared to mRNA levels prior to inoculation (averages from three biological replicates ±SD). Together with data presented in Figure 2, we concluded that RPS4 induction coupled with dynamic regulation of RPS4 splicing induces TV2A over 100-fold at 1 and 12 h after inoculation.

We next tested the impact of DC3000(\textit{avrRps4}) on RPS4 expression in a set of accessions or mutants. Like other TNL R genes, RPS4 resistance requires a functional \textit{EDS1} gene, whereas CC-NBS-LRR gene resistance in general requires \textit{NDR1} (Aarts et al., 1998). Based on the results in Figure 3A, expression levels were measured at 12 hpi. RPS4 was induced in the resistant Wassilewskija-0 and Landsberg \textit{erecta} (Ler) accessions and the resistant \textit{ndr1}-1 mutant to similar levels as those in Col-0 plants (Fig. 3B). RPS4 was induced to an intermediate level in susceptible RLD plants (ANOVA test, \( P < 0.01 \)), comparable to levels observed in Col-0 mock inoculated or inoculated with virulent DC3000 (see Fig. 3A), and was <2-fold induced in \textit{edsl1}-1 mutant plants (Fig. 3B). This showed that maximal RPS4 induction by AvrRps4 is dependent on a functional RPS4 and \textit{EDS1} gene, but not on \textit{NDR1}. Furthermore, RPS4 is weakly induced by basal resistance responses governed by \textit{EDS1}.

### Specificity of RPS4 Alternative Splicing

We next tested whether RPS4 is the only gene that undergoes changes in alternative splicing in response to \textit{avrRps4} and whether \textit{avrRps4} is the only effector gene that induces these changes. First, we chose two genes other than RPS4 that were shown to be alternatively spliced via intron retention by Ner-Gaon et al. (2004). \textit{At2g22750} encodes a member of the \( \alpha/\beta \)-hydrolase family of enzymes that shows similarity to the tobacco methyl salicylate esterase SABP2 (Kumar and Klessig, 2003; Forouhar et al., 2005). \textit{At2g22750} may therefore function in Arabidopsis defense responses. \textit{At4g07410} encodes a WD-40 repeat protein gene that induces these changes. First, we chose two genes other than RPS4 that were shown to be alternatively spliced via intron retention by Ner-Gaon et al. (2004). \textit{At2g22750} encodes a member of the \( \alpha/\beta \)-hydrolase family of enzymes that shows similarity to the tobacco methyl salicylate esterase SABP2 (Kumar and Klessig, 2003; Forouhar et al., 2005). \textit{At2g22750} may therefore function in Arabidopsis defense responses. \textit{At4g07410} encodes a WD-40 repeat protein

### Removal of Individual Introns Has No Measurable Effect on Splicing of Remaining Introns

RPS4 function was fully compromised by removal of introns 2 or 3 alone. This was striking because qualitatively all the remaining expected alternative transcripts were detected from intron-deficient RPS4-
Experiments were performed and analyzed as described in A.

DC3000(\textit{avrRps4}) analysis of alternative transcript induction in Col-0 in response to \textit{avrRps4} in Col-0 and RLD in response to DC3000(\textit{avrRpt2}). Leaf tissue was harvested immediately prior to (0) or 12 hpi. Numbers at left denote DNA size standards (bp). Numbers below each lane indicate band frequency of alternative transcripts relative to the regular transcript. Values were determined by densitometry and represent the averages (av) and so of three biological replicates. B. RT-PCR analysis of alternative transcript induction in Col-0 in response to DC3000(\textit{avrRps4}) (a4), DC3000(\textit{hopA1}) (hA), and DC3000(\textit{avrRpt2}) (a2). Experiments were performed and analyzed as described in A.

Figure 4. Inducible alternative splicing is not limited to the \textit{avrRps4}-\textit{RPS4} interaction. A, RT-PCR analysis of alternative transcript induction in Col-0 and RLD in response to DC3000(\textit{avrRps4}). Leaf tissue was harvested immediately prior to (0) or 12 hpi. Numbers at left denote DNA size standards (bp). Numbers below each lane indicate band brightness of alternative transcripts relative to the regular transcript. Values were determined by densitometry and represent the averages (av) and so of three biological replicates. B, RT-PCR analysis of alternative transcript induction in Col-0 in response to DC3000(\textit{avrRps4}) (a4), DC3000(\textit{hopA1}) (hA), and DC3000(\textit{avrRpt2}) (a2). Experiments were performed and analyzed as described in A.

Ler transgenes and no aberrant alternative splicing occurred (Zhang and Gassmann, 2003). This suggested that removing one intron might quantitatively affect the splicing frequency of the remaining introns and therefore have an influence on the relative accumulation of alternative transcripts. Additionally, the failure to detect TV5 and TV7 in this study indicated that the splicing of the cryptic intron and intron 2 are coupled or that the cryptic intron is spliced at measurable frequency only after the splicing of intron 2.

To address these open questions, we quantified \textit{RPS4} transcript abundance in transgenic plants expressing intron-deficient \textit{RPS4} transgenes during the infection. For this purpose, we introduced intron-deficient \textit{RPS4-Ler} transgenes into the \textit{rps4-1} knockout line (Kwon et al., 2004) because the endogenous \textit{rps4}-\textit{RLD} in the original transgenic lines prevented us from quantifying transgene-derived transcripts efficiently (Zhang and Gassmann, 2003). Consistent with our previous results, disease assays in stable transgenic plants showed that \textit{rps4-1-R4L} (containing a wild-type genomic clone of \textit{RPS4-Ler}) was resistant, whereas \textit{rps4-1-R4L-i2r}, \textit{rps4-1-R4L-i3r}, and \textit{rps4-1-R4L-i23r} plants (genomic \textit{RPS4-Ler} clone with intron 2, intron 3, or introns 2 and 3 removed, respectively) were fully susceptible (data not shown).

After inoculating with DC3000(\textit{avrRps4}), the relative amount of TV2A in the TV2A-generating transgenic plants, \textit{rps4-1-R4L} and \textit{rps4-1-R4L-i2r}, displayed a dynamic pattern similar to that in Col-0 plants except that the initial levels prior to inoculation were higher and the peak levels at 1 and 12 hpi were lower (Table I). Consistent with previous results, the relative accumulation of TV2A at 1 and 12 hpi was observed only when inoculated with DC3000(\textit{avrRps4}) (Table I). The relative amount of TV2B in transgenic plants \textit{rps4-1-R4L} and \textit{rps4-1-R4L-i3r} was low and similar to that in Col-0 plants (data not shown). TV4 was present at low levels in all transgenic plants, including \textit{rps4-1-R4L-i3r} and \textit{rps4-1-R4L-i23r} plants, and no significant relative accumulation was observed (Table I; data not shown). Interestingly, TV4 was produced in \textit{rps4-1-R4L-i23r}, whereas TV5 and TV7 were not detected in any of these transgenic plants. This indicates that splicing of the cryptic intron is not coupled to splicing of intron 2; rather, it appears to be a stepwise process that depends on the absence of intron 2. In conclusion, these data rule out the possibility that intron removal affects the splicing frequency of the remaining introns. In addition, \textit{RPS4} gene expression was similarly induced in intron-deficient transgenic \textit{rps4-1} plants (Table II), indicating that these transgenes express normally compared to the corresponding genomic clone and that the removal of introns has no influence on the induction of \textit{RPS4} expression.

### Transient Expression of Truncated \textit{RPS4} Proteins

Based on the above results, the nonfunctionality of transgenes with single introns removed suggests that, in total, a minimal initial amount of alternative transcripts is required to activate resistance or that individual transcripts have different functions during the resistance response. Previously, alternative transcripts were proposed to function on the protein level rather than as RNAs (Zhang and Gassmann, 2003). The premature stop codons identified in detected alternate \textit{RPS4} transcripts (Fig. 1) lead to three truncated ORFs putatively encoding a TN protein (\textit{RPS4T0N}) by TV1 and TV2B, a TN protein containing one-half of the first LRR (\textit{RPS4T1N/2}) by TV4 and TV6, and a TN protein containing the first four LRRs (\textit{RPS4T4N}) by TV2A. These truncated proteins are here collectively referred to as \textit{RPS4T0N} proteins. We focused on \textit{RPS4T0N} and \textit{RPS4T4N} as the two most sequence-diverse truncated proteins with different transcript dynamics.

To facilitate the detection of truncated \textit{RPS4T0N} proteins, epitope-tagged \textit{RPS4} constructs were generated. The first construct had an N-terminal fusion of the FLAG epitope in a genomic \textit{RPS4} (\textit{gRPS4}) sequence (Fig. 5A). The N-terminal epitope in these constructs is necessary to preserve natural intron retention and detection of C-terminally truncated \textit{RPS4T0N} proteins. The other two constructs had a C-terminal FLAG tag fused to \textit{RPS4} cDNA (\textit{cRPS4}) sequences corresponding to naturally occurring alternative \textit{RPS4} transcripts (Fig. 5A).
Stable transgenic Arabidopsis plants in the knock-out line (rps4-1) were generated using these tagged RPS4 constructs driven by the 35S promoter. The N-terminally FLAG-tagged full-length RPS4 protein was detected in several independent T1 transgenic lines (Fig. 5B). Furthermore, a weak specific band was observed that possibly corresponded to RPS4

TN0L in these T1 plants (data not shown). Given the low abundance of alternative RPS4 transcripts in uninduced tissue, the lack of strong signal from truncated proteins is not surprising. However, T1 transformants were highly stunted and showed severely reduced viability, preventing propagation of these lines and a more thorough analysis. No tagged proteins were detected when constructs were driven by the native RPS4 promoter.

In parallel, tagged constructs under the control of the 35S promoter were expressed in N. benthamiana leaves using Agrobacterium-mediated transient expression assays. RPS4

TN0L proteins were not detected from A. tumefaciens leaves in the absence of AvrRps4 (Fig. 5C), suggesting that these constructs were functional. We were able to detect the RPS4

TN0L protein when transiently expressed from a 35S: cRPS4

TN0L-FLAG construct (Fig. 5D), indicating that at least one RPS4

TN0L protein is stable in planta. Interestingly, even though RPS4

TN4L induced HR-like cell death, the RPS4

TN4L protein was not detected (Fig. 5D). We verified by real-time PCR quantification that mRNAs for the full-length and the two truncated RPS4 proteins accumulated to similar levels in N. benthamiana leaves (Supplemental Fig. S2). In the previous report by Zhang et al. (2004), a hemagglutinin-tagged version corresponding to RPS4

TN4L was detected in tobacco, whereas a protein corresponding to RPS4

TN0L was not. We have sequenced our FLAG-tagged constructs twice and used two independent A. tumefaciens transformants with identical results (data not shown). At present, we cannot explain this discrepancy.

**DISCUSSION**

**RPS4 Pre-mRNA Splicing Is under Dynamic Regulation**

In this study, we showed that the alternative splicing of RPS4 pre-mRNA is tightly regulated during avrRps4-triggered resistance. The relative abundance of the dominant alternative transcript TV2A was up-regulated within 1 h during the resistance response. Therefore, RPS4-mediated resistance might require not only the combined presence of multiple transcripts

| Table II. Increase in RPS4 mRNA in intron-deficient transgenic rps4-1 plants

| hpi | Inoculum | Plant Line |
|-----|----------|------------|
|     |          | rps4-1-R4L | rps4-1-R4L-i2r | rps4-1-R4L-i3r | rps4-1-R4L-i23r |
| 1   | −        | 2.5 ± 0.4  | 1.9 ± 0.8  | 2.3 ± 0.8  | 1.6 ± 1.1  |
|     | +        | 5.2 ± 2.4  | 4.1 ± 1.1  | 6.3 ± 1.9  | 7.8 ± 4.1  |
| 12  | −        | 2.9 ± 0.8  | 4.0 ± 1.6  | 2.2 ± 1.0  | 3.9 ± 0.8  |
|     | +        | 12.7 ± 3.1 | 9.5 ± 2.3  | 7.7 ± 2.3  | 9.7 ± 4.1  |

4Numbers represent fold increases in RPS4 mRNA levels normalized with RPS4 mRNA levels prior to inoculation and quantified by real-time PCR using the expression level of EF-1a as an internal standard. Data represent averages ±sd from two (−) and three (+) biological replicates.

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TV2A is the most abundant alternative transcript and is the only alternative transcript that displayed significant dynamic regulation during the resistance response, suggesting that TV2A has an important function in addition to TV3 in RPS4-mediated resistance. This is consistent with the fact that RLD-R4L-i3r and rps4-1-R4L-i23r plants, which cannot produce TV2A, are fully susceptible to DC3000(avrRps4) (Zhang and Gassmann, 2003). However, the function of TV2A is not sufficient to replace other alternative transcripts because transgenic plants containing R4L-i2r, which do not produce TV2B, but do produce TV2A dynamically, were equally susceptible. We hypothesize therefore that individual alternative transcripts fulfill separate functions before and during the disease resistance response. Specifically, in this model, the sum of alternative transcripts is proposed to function in priming the system for activation prior to the response, whereas up-regulation of TV2A functions in amplifying the response after activation. Alternatively, the different truncated RPS4 proteins, which differ in the number of LRRs, have distinct nonredundant functions in RPS4-mediated resistance.

In support of a required threshold of alternative transcripts prior to pathogen recognition, we did not detect a compensatory increase in abundance of remaining alternative transcripts in susceptible plants containing intron-deficient transgenes. In other words, because of the inability to produce certain TVs, the total amount of alternative transcripts in intron-deficient transgenic plants is lower than that in wild-type Col-0 or rps4-1 transgenic plants containing a genomic clone of RPS4. These data also suggest that the artificial removal of one intron has no effect on the splicing frequency of the other introns. With the exception of the cryptic intron, splicing of a given RPS4 intron is therefore an independent event determined by intrinsic local sequence features.

**RPS4 Gene Induction by avrRps4**

Several disease resistance genes have been reported to be induced during the resistance response (Yoshimura et al., 1998; Shirano et al., 2002; Halterman et al., 2003; Xiao et al., 2003; Chandra-Shekara et al., 2004; Levy et al., 2004; Gu et al., 2005). We report here that RPS4 expression was significantly induced by DC3000(avrRps4) in an EDS1-dependent manner. The second wave of RPS4 gene induction may be based on an EDS1-dependent positive feedback loop linking salicylic acid accumulation and the induction of several TNL R genes (Rustérucci et al., 2001; Shirano et al., 2002; Xiao et al., 2003; Chandra-Shekara et al., 2004). RPS4 induction, together with the regulation of RPS4 splicing, results in a rapid increase of TV2A by more than 100-fold upon pathogen recognition. The combined regulation of RPS4 gene expression and pre-mRNA splicing might rapidly boost RPS4-mediated defense signaling. Interestingly, the two peaks of RPS4 induction coincided with the two peaks of TV2A

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**Figure 5.** Detection of epitope-tagged RPS4 proteins. A, FLAG-tagged RPS4 constructs. RPS4 sequences are shown schematically: Exons are shown as colored boxes and domains are specified by different colors. Introns are shown as black lines. B, Detection of full-length N-terminally FLAG-tagged RPS4 protein (arrow) in stable Arabidopsis transformants in the T1 generation. Five independent transformants are shown. C, A. tumefaciens strain GV3101 containing the indicated FLAG-tagged RPS4 constructs was infiltrated into fully expanded leaves of N. benthamiana, with empty vector (pBIB-Kan-35S) as a negative control. HR-like responses (necrotic regions) were photographed 4 dpi. D, Detection of C-terminally FLAG-tagged RPS4TN6L (arrow) after Agrobacterium-mediated transient expression in N. benthamiana. Arrowhead indicates the expected size of FLAG-tagged RPS4TN6L. This experiment was repeated three times with two independent A. tumefaciens transformants. Protein was isolated 36 hpi before necrotic symptoms appeared. Numbers in B and D indicate positions of molecular mass markers (kD).
accumulation, raising the possibility that splicing of intron 3 is a rate-limiting step in RPS4 pre-mRNA processing or that transcription and splicing are regulated by a coordinated mechanism (Kornbluht, 2005). Maximal induction of RPS4 gene expression and of TV2A generation was dependent on avrRps4. Interestingly, induction also occurred in transgenic plants expressing RPS4-i2r, which are susceptible to DC3000(avnRps4). This observation indicates that full-length RPS4 protein is sufficient for induction of RPS4 and regulation of alternative transcripts, but not for resistance. Future experiments are necessary to determine whether gene regulation and triggering of resistance require different signaling thresholds or whether they are separate processes. In addition, alternative splicing of RPS4, At2g23550, and At4g07410 were also induced in the presence of HopA1 and AvrRpt2, indicating that these responses are not limited to the avrRps4-RPS4 gene-for-gene interaction. Rather, they are likely to be a consequence of priming the plant defense system. Whether this is a direct effect of R protein activity or indirect is an open question. Interestingly, recent studies showed that the R proteins RRS1 in Arabidopsis, MLA in barley, and N in tobacco translocate to the nucleus (Deslandes et al., 2003; Burch-Smith et al., 2007; Shen et al., 2007). Furthermore, MLA interacts with WRKY transcription factors in the nucleus, presumably to derepress gene induction associated with defenses (Shen et al., 2007). The induction of RPS4 expression and alternative splicing upon AvrRpt2 or HopA1 detection could therefore be a general, but direct, consequence of R protein activity.

Possible Function of Truncated R Proteins

Although not exclusively found with TNL genes, alternative splicing and its functional significance has been most prominently reported for this class of R genes. Intriguingly, the mechanism of alternative splicing of plant TNL genes varies, but common to all mechanisms is that they produce transcripts that mainly encode TN proteins (Jordan et al., 2002). Therefore, the study of putative RPS4TNld proteins in planta is highly relevant for understanding TNL R gene-mediated resistance. To date, we have not unequivocally detected RPS4TNld protein generated from a N-terminally tagged genomic RPS4 construct. We have shown here that the initial amount of alternative transcripts is very low (4% or less compared to the regular transcript), most likely precluding direct detection of these truncated proteins in planta.

To address the more general question of whether RPS4TNld proteins are stable in planta, we performed Agrobacterium-mediated transient expression assays in N. benthamiana. Indeed, RPS4TNld was detected in total protein extracts when expressed from the 35S-cRPS4TNld-FLAG construct. In contrast, RPS4TNld encoded by the dominant alternative transcript TV2A, was not detected. However, RPS4TNld and RPS4TNld were equally potent in inducing avrRps4-independent HR-like cell death in N. benthamiana. RPS4TNld is encoded by the up-regulated TV2A transcript and instability of RPS4TNld could function as negative feedback to prevent excessive tissue damage. The HR-like cell death induced by RPS4 overexpression in N. benthamiana was previously shown to require ED51, SG71, and HSP90, strongly suggesting that this HR-like response is not based on general protein toxicity (Zhang et al., 2004).

Given the full susceptibility of plants with single intron-deficient RPS4 transgenes, we propose dual roles for truncated RPS4 proteins. In our model, the sum of truncated RPS4 proteins function in priming the RPS4-dependent resistance response, whereas up-regulated RPS4TNld specifically functions in amplifying the plant defense response. The instability of RPS4TNld results in down-regulation of this disease resistance signal amplification after activation of RPS4-mediated resistance. The lack of resistance with intron-deficient RPS4 transgenes and the induction of HR-like cell death by RPS4TNld proteins are evidence for a positive role of these proteins in RPS4-mediated resistance, possibly by alleviating self-inhibition of full-length RPS4 protein (Zhang and Gassmann, 2003) or by functioning as adaptor proteins (Meyers et al., 2002). Apart from RPS4, transient expression of truncated RPP1 (TN) and RPS5 (CC-NBS) also leads to constitutive cell death, indicating that the N-terminal part of R proteins effects resistance activation and that the LRR inhibits constitutive activity (Weaver et al., 2006; Ade et al., 2007). Rapid up-regulation of truncated RPS4 after pathogen detection is therefore predicted to accelerate the defense response.

This positive role of truncated RPS4 is in contrast to NOD2, a protein that functions in the animal innate immune response. NOD2 contains a nucleotide-binding domain flanked by two N-terminal caspase recruitment domains and a C-terminal LRR domain. NOD2 detects the presence of bacterial cell wall components and activates the nuclear factor-κB transcription factor (Strober et al., 2006). Interestingly, a recent report showed that NOD2 is alternatively spliced and that the alternative transcript encodes a short form of NOD2, NOD2-S, that is truncated prior to the nucleotide-binding domain in the second caspase recruitment domain. NOD2-S interacts with full-length NOD2 and downstream signaling proteins and downregulates nuclear factor-κB activation (Rosenstiel et al., 2006). Interestingly, artificial truncations in NOD2 identified the LRR as an inhibitory domain for constitutive activation of NOD2, consistent with results with R proteins (Tanabe et al., 2004).

To determine the mechanism by which truncated RPS4 proteins positively regulate RPS4-mediated disease resistance, biochemical assays for protein-protein interactions will be required. Given the complexity of possible protein-protein interactions between different RPS4TNld proteins and full-length RPS4, we are further developing the N. benthamiana transient expression system for efficient testing of different
combinations of proteins. \textit{RPS4} is one of the few \textit{Arabidopsis} genes for which intron retention has been shown to be functionally relevant (Haas et al., 2005) and can serve as a model to elucidate the regulatory mechanisms of this important type of alternative splicing in plants.

**MATERIALS AND METHODS**

**Bacterial Strains, \textit{Arabidopsis} Growth, and Inoculations**

\textit{Pseudomonas syringae} pv tomato strain DC3000 containing the empty vector pVSP61 or expressing avrRps4, hopA1, or avrRpt2 were grown as described previously (Zhang and Gassmann, 2003). \textit{Arabidopsis} (\textit{Arabidopsis thaliana}) plants were grown in a GR48 environmental growth chamber (Convion) at 22°C and 70% humidity with an 8-h light/16-h dark cycle. Disease assays on intron-deficient transgenic \textit{rps4-1} plants were performed as described previously (Zhang and Gassmann, 2003). For measuring \textit{RPS4} mRNA levels and quantifying \textit{RPS4} transcript ratios, \textit{Arabidopsis} plants were inoculated at a concentration of 10^7 colony forming units/mL.

**Intron-Deficient Transgenic \textit{rps4-1} Plants**

Construction of binary vectors harboring intron-deficient transgenes, plant transformations, and selection of stable transformants were performed as described previously (Zhang and Gassmann, 2003). Because the T-DNA insertion in the \textit{rps4-1} line occurred in exon 2 upstream of the variably spliced intron, no interference by endogenous transcripts was expected in determining TV abundance arising from intron-deficient transgenes. This was confirmed by real-time PCR measurement of endogenous transcript levels. Real-time PCR was also carried out to quantify transgene expression levels of all lines to choose one representative line for each transgene with similar expression levels for quantifying \textit{RPS4} transcript profiles.

**RNA Manipulation and RT-PCR Analysis**

Total RNA isolated using Tri Reagent (Sigma) was treated with Turbo DNase (Ambion) to remove genomic DNA. mRNA was isolated using poly(A) Purist Mag beads (Ambion) according to the manufacturer’s instructions. RT-PCR experiments were performed using Platinum SYBR Green qPCR SuperMix (Invitrogen) and an Opticon2 real-time PCR machine (MJ Research).

**Generation of Epitope-Tagged \textit{RPS4} Constructs**

The genomic \textit{RPS4}-Lr clone R4L, intron-deficient constructs, and artificially truncated constructs under the native \textit{RPS4} promoter were described previously (Zhang and Gassmann, 2003). To create truncated cDNAs, intron 1 was removed from the artificially truncated \textit{RPS4}-Lr constructs R4L-TN10 and R4L-TN3L (Zhang and Gassmann, 2003) by swapping with cDNA sequence using \textit{Xho}I (upstream of start codon) and EcoRI (within exon 2). For C-terminal fusion with the flag tag, a BamHI restriction site was engineered at the 3’ end using PCR. To generate the FLAG-g\textit{RPS4} construct shown in Figure 5A, the FLAG tag was included in a forward primer and the resulting PCR fragment was cut with \textit{Neo}I and \textit{Bgl}II to insert the FLAG tag after the start codon of R4L. The resulting construct was cut with \textit{Xho}I and SacI and subcloned into the binary vectors pBB-Kan-35S and pBB-Kan.

**\textit{N. benthamiana} Growth and Agrobacterium-Mediated Transient Expression**

\textit{N. benthamiana} plants were grown in an E7/2 environmental growth chamber (Convion) at 22°C with 14-h light/10-h dark cycle. Plants at the age of 6 to 7 weeks were used for experiments. Agrobacterium tumefaciens strain GV3101 containing epitope-tagged \textit{RPS4} constructs was grown overnight in Luria-Bertani broth supplemented with 10 mM MES and 20 µM acetylproline. Bacteria were pelleted and resuspended in a solution containing 10 mM MgCl2, 10 mM MES, and 150 µM acetylproline and the OD was adjusted to 0.8. The Agrobacterium suspension was infiltrated into fully expanded \textit{N. benthamiana} leaves using a needleless syringe. HR-like responses were observed 3 to 5 d after infiltration.

**Protein Extraction and Detection**

Protein extractions followed the procedure described previously (Zhang and Klessig, 1998) with modifications. Briefly, four discs of leaf tissue (approximately 1 cm in diameter) were harvested and ground in protein extraction buffer (100 mM HEPES, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM dithiothreitol, 1% protease inhibitor cocktail [Sigma]), followed by centrifugation at 13,200 rpm for 30 min at 4°C. Total protein was collected from the supernatant and the concentration of total protein was quantified using the Bradford assay (Bradford, 1976). Total protein was separated on a 7.5% SDS-PAGE gel and transferred onto nitrocellulose membrane (Osmonics). Western blots were performed with monoclonal anti-FLAG antibody (Sigma) at a dilution of 1:1,000 and the ECL detection kit (Amersham).
Supplemental Data

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

Supplemental Figure S1. Quantification procedure for RPS4 transcripts.

Supplemental Figure S2. Quantification of RPS4 mRNA transiently expressed in N. benthamiana.

Supplemental Table S1. Quantification of RPS4 transcript clone mixtures.

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