

Expression of *RPS4* in tobacco induces an AvrRps4-independent HR that requires EDS1, SGT1 and HSP90

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Summary

The *Arabidopsis RPS4* gene belongs to the Toll/interleukin-1 receptor/nucleotide-binding site/leucine-rich repeat (TIR-NB-LRR) class of plant resistance (*R*) genes. It confers resistance to *Pseudomonas syringae* carrying the avirulence gene *avrRps4*. Transient expression of genomic *RPS4* driven by the 35S promoter in tobacco leaves induces an AvrRps4-independent hypersensitive response (HR). The same phenotype is seen after expression of a full-length *RPS4* cDNA. This indicates that alternative splicing of *RPS4* is not involved in this HR. The extent of HR is correlated with *RPS4* protein levels. Deletion analyses of *RPS4* domains show the TIR domain is required for the HR phenotype. Mutations in the P-loop motif of the NB domain abolish the HR. Using virus-induced gene silencing, we found that the cell death resulting from *RPS4* expression is dependent on the three plant signalling components EDS1, SGT1 and HSP90. All these data suggest that heterologous expression of an *R* gene can result in activation of cell death even in the absence of its cognate avirulence product, and provides a system for studying the *RPS4* domains required for HR.

Keywords: resistance, transient expression, HR, *RPS4*, tobacco, signalling.

Introduction

Plants carry a surveillance system to recognize attacking micro-organisms and to induce effective defence mechanisms. Resistance is often controlled by a gene-for-gene interaction between plant resistance (*R*) genes and pathogen avirulence (*avr*) genes (Dangl and Jones, 2001; Hammond-Kosack and Jones, 1997). Avr recognition by the *R*-gene surveillance system leads to activation of the hypersensitive response (HR), a type of programmed cell death which occurs at or near the site of pathogen entry (Heath, 2000; Morel and Dangl, 1997). The HR is thought to confine the pathogen by stopping it spreading from the site of attempted infection, and is likely to involve active plant metabolism (Levine *et al.*, 1996). Genetic analysis of lesion mimic mutants has permitted the identification of participants and/or regulators of this programme (Dietrich *et al.*, 1997; Greenberg, 1997). In addition to cell death, some specific defence responses are also induced which participate in the resistance process.

These defence responses include the production of reactive oxygen species, activation of genes encoding antimicrobial proteins and of enzymes that promote synthesis of antimicrobial compounds (Hammond-Kosack and Jones, 1996).

Despite the wide range of pathogens that attack plants, only a small number of domains have been identified in *R* proteins. The largest class of *R* proteins is the nucleotide-binding/leucine-rich repeat (NB-LRR) class. This class can be subdivided into two groups according to whether they carry a coiled-coil (CC) domain (*RPS2*, *RPM1*, *RPS5*, *Rx*, *Mi*) or a Toll/interleukin-1 like receptor (TIR) domain (*RPS4*, *RPP1*, *RPP5*, *N*) (Dangl and Jones, 2001; Staskawicz *et al.*, 1995) at their N termini. In addition, the TIR-NB-LRR group can be further divided into two subgroups depending on the presence of a C-terminal non-LRR (CNL) domain (Dodds *et al.*, 2001). Recent studies emphasize the importance of these specific domains for resistance. For example, deletion

and mutation analyses have suggested that the TIR, NB and LRR domains of the N protein play an indispensable role in the induction of the resistance response against tobacco mosaic virus (TMV) (Dinesh-Kumar *et al.*, 2000), the NB domain of RPM1 and the CC and NB domains of RPS2 are critical for the resistance function (Tao *et al.*, 2000; Tornero *et al.*, 2002a). Similarly, the N terminus and LRR regions of the *Mi* gene product are involved in the regulation of localized cell death induced in tomato (Hwang *et al.*, 2000). Truncations of the CNL domain in flax P lead to a loss of function, suggesting a role of this domain in the resistance process (Dodds *et al.*, 2001).

Molecular analyses of genes encoding TIR domain-containing proteins in plants and animals reveal alternatively spliced products in many cases (Jordan *et al.*, 2002). However, the biological relevance of alternative transcripts remains unclear. The tobacco *N* gene was the first reported *R* gene that encodes two transcripts, *N_S* and *N_L*, via alternative splicing of the alternative exon present in intron III. Both these two transcripts and presumably their encoded protein products are necessary to confer complete resistance to TMV (Dinesh-Kumar and Baker, 2000). Recently, *RPS4* has been also shown to have alternative transcripts. These alternative transcripts seem to be required for full *RPS4*-dependent resistance to *Pseudomonas syringae* carrying *avrRps4* (Zhang and Gassmann, 2003). By contrast, transgenic flax plants expressing an intronless *L6* transgene showed *L6* resistance to flax rust indistinguishable from that of the wild-type gene (Ayliffe *et al.*, 1999). All these data were shown at mRNA level, and no protein data have been published so far.

Mutational analyses in *Arabidopsis* led to the identification of signalling components downstream from R protein function (Glazebrook, 2001). *EDS1* encodes a protein with homology to lipases (Falk *et al.*, 1999) and is necessary for resistance mediated by TIR-containing R proteins. In contrast, *NDR1* is required by non-TIR-containing R proteins (Aarts *et al.*, 1998). These two genes define at least two independent signalling pathways involved in *R* gene-mediated plant disease resistance. Two other components essential for *R* gene function have been recently identified. SGT1, a highly conserved component needed for the function of certain SCF (Skp1/Cullin/F-box protein) type E3 ubiquitin ligase complexes, is essential for the function of several *R* genes (Austin *et al.*, 2002; Azevedo *et al.*, 2002). Two highly homologous *SGT1* genes exist in *Arabidopsis*, *SGT1a* and *SGT1b* (Austin *et al.*, 2002). *Nicotiana benthamiana* SGT1 homologues are indispensable for *N*-dependent resistance to TMV; *Rx*-dependent resistance to potato virus X (PVX) and *Pto*-dependent resistance to *P. syringae* carrying *avrPto* (Liu *et al.*, 2002; Peart *et al.*, 2002b). *RAR1* is also required at an early convergent point in the defence pathways mediated by several *R* genes (Muskett *et al.*, 2002; Shirasu *et al.*, 1999; Tornero *et al.*, 2002b). *RAR1* encodes a

protein with two zinc-binding (CHORD) domains and was originally isolated from barley (Shirasu *et al.*, 1999). *RAR1* interacts directly with SGT1 (Azevedo *et al.*, 2002). HSP90 is an abundant cytosolic protein in bacteria and eukaryotes. Association of client proteins with HSP90 complexes has proved to be important for their signal transduction activity (Richter and Buchner, 2001). Mutations in the ATP-binding domain of isoform AtHSP90.2 compromise *RPM1* resistance to *P. syringae* expressing *avrRpm1* (Hubert *et al.*, 2003). Isoform AtHSP90.1 is required for full *RPS2* resistance to *P. syringae* carrying *avrRpt2* (Takahashi *et al.*, 2003). Virus-induced gene silencing (VIGS) of *NbHSP90* resulted in loss of *Pto*-mediated resistance against *P. syringae* harbouring *avrPto*, *Rx*-mediated resistance against PVX and *N*-mediated resistance against TMV (Lu *et al.*, 2003). HSP90 interacts with *RAR1* and SGT1 (Hubert *et al.*, 2003; Takahashi *et al.*, 2003). SGT1 and *RAR1* may function as co-chaperones of HSP90 to assemble or to regulate multi-protein signalling complexes in plant disease resistance (Hubert *et al.*, 2003; Shirasu and Schulze-Lefert, 2003; Takahashi *et al.*, 2003).

The *Arabidopsis* TIR-NB-LRR *R* gene *RPS4* confers resistance to *P. syringae* pv. *tomato* DC3000 strain carrying *avrRps4* (Gassmann *et al.*, 1999). Here, we investigate the physiological responses upon transient expression of *RPS4* and *RPS4* derivatives in tobacco. Overexpression of *R* genes sometimes leads to HR in the absence of the corresponding Avr product. This has been shown for *Pto* in tomato (Tang *et al.*, 1999), *RPS2* in *Arabidopsis* (Tao *et al.*, 2000) and *L* in tobacco (Frost *et al.*, 2004). These data suggest that the signalling cascade can be activated independently of pathogen recognition. However, nothing is known about the mechanisms governing this response, or whether signalling mechanisms are well conserved in different species. Dissection of this mechanism would be facilitated by a high-throughput transient assay, as was used to investigate mammalian NOD2 (Tanabe *et al.*, 2004). Here, we show that transient *RPS4* expression induces an HR-like phenotype in tobacco. This response depends on protein accumulation level, requires EDS1, SGT1 signalling components and heat shock protein HSP90, but does not require the alternative spliced product. In addition, we show that both the TIR and the functional NB domains of *RPS4* are necessary to induce the HR.

Results

Transient expression of RPS4 induces an HR-like cell death phenotype in tobacco

To investigate the expression of *RPS4* protein in tobacco leaves, an HA tag was fused to the C-terminus of *RPS4* (see Experimental procedures). In this construct, *RPS4* is driven by the cauliflower mosaic virus 35S promoter (Figure 1a). An *Agrobacterium*-mediated transient assay (Rivas *et al.*, 2002)

was used to deliver the *RPS4* construct into *N. tabacum* leaves. The expression of *RPS4* leads to a necrotic phenotype (Figure 1b), which is visible 2–3 days after transient transformation. No necrosis was observed with the vector (pBin19g) control attesting that the cell death phenotype is not due to *Agrobacterium* itself or due to the overexpression process. In parallel, transiently expressed *RPS4* protein was monitored. Tissue samples were harvested 2 days after transformation. Total protein was extracted and immunoassayed with an HA antibody. Under these conditions, *RPS4::HA* fusion protein was detected as a strong signal in a Western blot (Figure 1d). In order to rule out an eventual *RPS4* autoactivation caused by the fused HA peptide, we engineered a *35S::RPS4* construct without HA at the C-terminus. The same cell death phenotype was observed in the

transient assay of this construct in *N. tabacum* (data not shown).

The cell death phenotype is reminiscent of a typical HR induced in incompatible interactions or by elicitor treatments (Heath, 2000). Hypersensitive cell death is usually accompanied by the accumulation of fluorescent phenylpropanoid derivatives (phytoalexins) around the pathogen infection sites (Dixon and Paiva, 1995; Dorey *et al.*, 1997). These compounds can be easily visualized under UV light. As shown in Figure 1(c), fluorescent spots appeared under UV light 2 days after *RPS4* expression but not after *GUS* (pBin19g vector) expression. The expression of defence-related genes is usually induced in incompatible interactions (Linthorst, 1991). Transcriptional activation analysis using reverse transcriptase-polymerase chain reaction (RT-PCR) proved that the expression of defence marker genes *BsPR1*, *hin1*, *hsr203J* and *tpoxC1* (Gopalan *et al.*, 1996; Hiraga *et al.*, 2000; Linthorst, 1991; Pontier *et al.*, 1994) was induced concomitantly with *RPS4* expression in tobacco (data not shown). Altogether, these results suggest that the

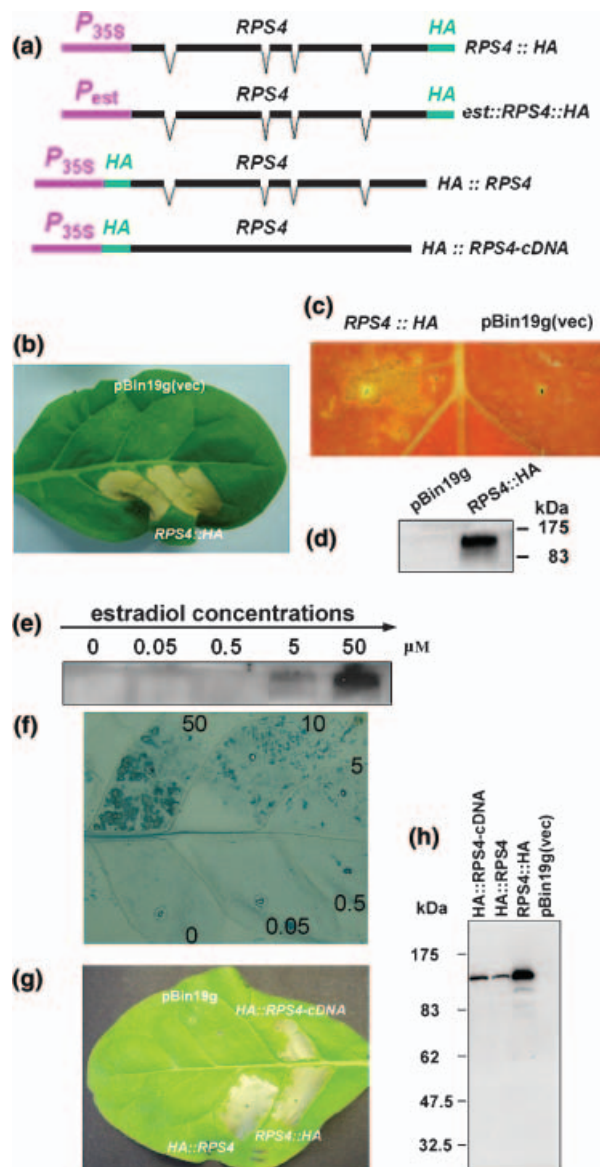


Figure 1. Expression of *RPS4* induces an AvrRps4-independent HR in *Nicotiana tabacum*.

(a) Schematic diagram of the *RPS4* constructs. *P*_{35S}, 35S promoter of cauliflower mosaic virus; *RPS4*, full-length *RPS4* genomic DNA or cDNA; *HA*, HA tag; *P*_{estr}, estradiol-inducible promoter; *RPS4::HA*, the construct controlled by the 35S promoter with an HA tag fused to the C-terminus of *RPS4* encoded by its full-length genomic DNA; *est::RPS4::HA*, the construct controlled by the estradiol promoter with an HA tag fused to the C-terminus of *RPS4* encoded by its full-length genomic DNA; *HA::RPS4*, the construct controlled by the 35S promoter with an HA tag fused to the N-terminus of *RPS4* encoded by its full-length genomic DNA; *HA::RPS4-cDNA*, the construct controlled by the 35S promoter with an HA tag fused to the N-terminus of *RPS4* encoded by its full-length cDNA. Black bar: *RPS4* coding sequence. V-shaped curve: introns.

Panels (b), (c) and (d) show the hypersensitive response (HR) induced by *RPS4::HA*.

(b) *RPS4*-induced HR phenotype. Tobacco leaves were infiltrated with *Agrobacterium* harbouring *RPS4::HA* or pBin19g (vector). Phenotypes were recorded at 4 dpi.

(c) Accumulation of phenylpropanoid compounds during the course of HR. The picture was taken under UV light at 3 dpi.

(d) Immunodetection of *RPS4* protein. Protein samples (2 dpi) were immunoassayed with an anti-HA antibody. Estimated *RPS4* size is 138 kDa.

Panels (e) and (f) show the strength of HR correlated with *RPS4* protein level. Tobacco leaves were infiltrated with *Agrobacterium* containing the estradiol-inducible *RPS4* construct (*est::RPS4::HA*). Two days later, leaves were treated with either water (0) or 0.05, 0.5, 5, 10, 50 μM estradiol.

(e) Estradiol dose-dependent *RPS4* expression. Protein samples were harvested 8 h after estradiol treatment. *RPS4::HA* protein was detected with an anti-HA antibody.

(f) Induced cell death corresponding to estradiol dose-dependent *RPS4* expression level. Dead cells were stained *in situ* by trypan blue. The leaf was stained at 48 hpi after estradiol treatment.

Panels (g) and (h) show the requirement for alternative splicing in the *RPS4*-triggered AvrRps4-independent HR in *N. tabacum*.

(g) HR phenotype induced by *RPS4* genomic DNA or cDNA. Phenotypes were recorded at 4 dpi after *Agrobacterium* infiltration. pBin19g is the empty vector as negative control.

(h) Immunodetection of *RPS4* protein. Immunodetection was performed with an anti-HA antibody. No truncated *RPS4* proteins can be detected corresponding to *HA::RPS4* alternatively spliced transcripts. Protein markers are given on the left in kDa.

expression of *RPS4* in tobacco activates both defence-related responses and cell death, independently of AvrRps4 recognition.

Correlation between the strength of HR and RPS4 protein level

Transient expression of *RPS4* driven by the 35S promoter results in readily detectable *RPS4* protein (Figure 1d). To compare this protein expression level with the endogenous *RPS4* expression level, a C-terminal HA-tagged *RPS4* driven by its native promoter was constructed. This *RPS4* derivative was also transiently delivered into tobacco leaves. Protein samples were harvested 1–6 days after transformation. However, no apparent cell death phenotype was observed and no *RPS4* protein was detected (data not shown). To further test whether the extent of cell death induced by *RPS4* is dependent on protein level, we made an *RPS4* construct under the control of an estradiol-inducible promoter system (Zuo *et al.*, 2000) (Figure 1a). Following transient delivery of this construct, we treated the leaves with a series of estradiol concentrations. As shown in Figure 1(e), *RPS4* protein became detectable in a Western blot after 5 μM estradiol treatment and accumulated to high levels at 50 μM estradiol. Trypan blue staining was used to detect the presence of dead cells after application of different concentrations of estradiol. After 50 μM estradiol treatment, a strong HR can be observed (Figure 1f). The HR is also visible, although more weakly after 5 and 10 μM of estradiol treatment. These results suggest that there is a strict correlation between the extent of cell death and the amount of *RPS4* protein.

Alternatively spliced RPS4 product is not required for the AvrRps4-independent HR in tobacco

Alternative splicing is crucial to defence responses mediated by TIR domain-containing R proteins (Jordan *et al.*, 2002). Intron retention is one mechanism of generating alternatively spliced variants. Analysis of the *RPS4* DNA sequence has shown that introns 1, 2 and 3 contain in-frame stop codons. Therefore, we tested whether alternative splicing of *RPS4* is involved in the AvrRps4-independent HR in tobacco. First, a full-length *RPS4* cDNA was cloned (see Experimental procedures) and fused to an N-terminal HA tag to enable protein detection. Secondly, the cDNA construct was delivered into tobacco leaves using the transient assay described above. The *RPS4* cDNA triggers an HR that is indistinguishable from that triggered by the genomic DNA (Figure 1g). The removal of the introns did not affect the HR. The alternative transcripts of a TIR-NB-LRR R gene encode truncated TIR-NB proteins (Jordan *et al.*, 2002). To test this, *RPS4* genomic DNA was fused to an N-terminal HA tag and transiently expressed in tobacco in parallel with the N-terminally tagged cDNA clone. Protein samples were then immunoas-

ayed using an anti-HA antibody. Immunoanalysis could detect only one mature protein corresponding to the full-length *RPS4* (Figure 1h). No other truncated protein products from the alternatively spliced transcripts were detected. Thus, at the level of detection for protein in Figure 1(h), alternatively spliced *RPS4* products do not seem to be required for the AvrRps4-independent HR in tobacco.

The TIR and NB domains of RPS4 are necessary for the HR phenotype

RPS4 contains a TIR domain, an NB domain, a C-terminal LRR and a CNL domain (Dodds *et al.*, 2001; Gassmann *et al.*, 1999). The TIR domain of R proteins is thought to interact with downstream signalling components (Feys and Parker, 2000). As shown for Apaf1, the NB domain could mediate the activation of the N-terminal signalling domain (van der Biezen and Jones, 1998). The LRRs may also facilitate the interaction of R proteins with other proteins that participate in defence signal transduction (Bent, 1996; Dangl and Jones, 2001). To investigate the roles of these different domains in *RPS4*-mediated HR in tobacco, a series of deletion constructs were made using the full-length *RPS4* genomic DNA as template (see Experimental procedures). In order to monitor the protein expression levels, all the derivatives were HA-tagged at the C terminus (Figure 2a). Transient expression of derivatives harbouring both the TIR and the NB domains led to a cell death phenotype similar to that conditioned by the full-length protein. However, this was not the case for the TIR domain alone, which did not trigger HR. In addition, the LRR and CNL domains, expressed separately or in combination, did not induce HR either (data not shown).

To check whether the absence of HR was due to the absence of protein, an immunoanalysis was performed using an anti-HA antibody. Tissue samples were harvested before the appearance of necrosis. In most cases, the different truncated proteins could be detected even in the absence of cell death. Interestingly, the proteins corresponding to the three derivatives that do not possess the LRR and CNL domains were not detected (Figure 2). One possible explanation is that these two domains are involved in *RPS4* protein stabilization. Overall, these results suggest that the TIR and NB domains are both necessary and sufficient for the *RPS4*-induced HR in tobacco. As the TIR-NB derivative alone was active, we concluded that the LRR and CNL domains are, in contrast, dispensable for the HR.

A point mutation in the P-loop motif of RPS4 abolishes the HR phenotype

Deletion analyses showed that the NB domain of *RPS4* is likely to be involved in HR induction. The NB domain of R proteins contains consensus kinase 1a (P-loop), kinase 2 and

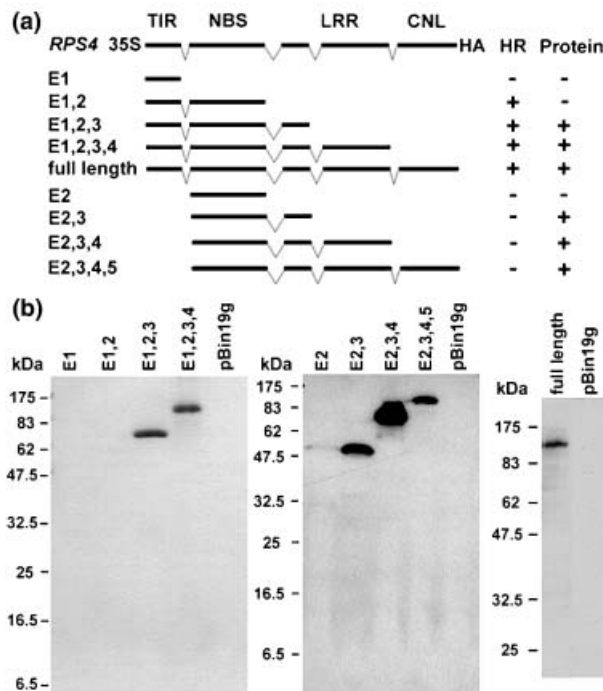


Figure 2. RPS4 domain deletion analysis.

(a) At the top of the panel, different domains of RPS4 corresponding to the coding sequence are schematically shown. The left panel shows the N-terminal and C-terminal deletion constructs used in the transient assay. All of them are driven by the 35S promoter and have a C-terminal HA tag. HR phenotypes were recorded at 3 dpi after *Agrobacterium* infiltration. The hypersensitive response (HR) phenotypes are described as presence (+) or absence (-) of cell death. The transiently expressed RPS4 derivative proteins were immunoassayed with an anti-HA antibody (b). The immunodetection results are summarized as detectable (+) or undetectable (-) for the protein products with predicted size.

(b) Immunodetection of all the RPS4 derivative proteins. The proteins encoded by all the derivatives shown in (a) were immunoassayed in a Western blot with an anti-HA antibody. Protein markers are given in kDa.

kinase 3a motifs common to a large variety of NB proteins (Traut, 1994). In the P-loop motif, several residues are highly conserved among the NB-LRR R proteins (Meyers *et al.*, 1999). Among them, Lys 188 in RPS2 is essential for RPS2 function (Tao *et al.*, 2000). In RPS4, this corresponding residue is Lys 242. We mutagenized this lysine either to alanine (K242A) or to glutamine (K242Q). Transient expression of these two mutants did not trigger an HR, in contrast to the wild type (Figure 3a). The RPS4 mutant proteins can still be detected in a Western blot (Figure 3b) and the protein accumulation level of these mutants is higher than that of the wild type. This difference is probably related to the presence or absence of HR. Altogether these results indicate that P-loop point mutants completely abolish RPS4-induced HR in tobacco and that the loss of HR induction is not due to the non-expression of these mutants or the instability of the mutant proteins. Therefore, the NB domain with an active P-loop motif is required for RPS4 activity in tobacco.

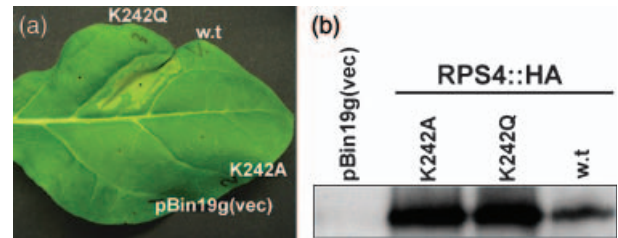


Figure 3. A P-loop point mutation abolishes the RPS4-induced hypersensitive response (HR) phenotype.

Nicotiana tabacum leaves were infiltrated with *Agrobacterium* containing RPS4::HA (w.t.), P-loop mutants K242Q or K242A, or pBin19g (vector) constructs.

(a) Phenotypes resulting from the expression of wild type and mutant RPS4. HR phenotype was recorded at 4 dpi.

(b) Immunodetection of RPS4 proteins. Immunodetection was performed with an anti-HA antibody.

RPS4-triggered cell death in tobacco is dependent on EDS1, SGT1 and HSP90

In *Arabidopsis*, RPS4, like other TIR-NB-LRR proteins, requires the presence of EDS1, but not NDR1 (Aarts *et al.*, 1998). In order to test the involvement of EDS1 in the RPS4-triggered AvrRps4-independent HR in tobacco, VIGS (Peart *et al.*, 2002a) was used. *Nicotiana benthamiana* plants were silenced for *NbEDS1* by an inoculation with *Agrobacterium* harbouring TRV:EDS1. Control *N. benthamiana* plants were inoculated with *Agrobacterium* carrying a TRV empty vector (TRV:00). RPS4 was then transiently expressed in the upper leaves of silenced *N. benthamiana* plants. The HR phenotype was assessed at 7 days post-inoculation (dpi). Figure 4(a) and Figure S1(a) show that RPS4 expression confers an HR phenotype in the TRV:00 silenced control plants similar to, but somewhat weaker than, that in *N. tabacum*. The HR phenotype was completely abolished in the *NbEDS1*-silenced plants (Figure 4b and Figure S1b). This indicates that the absence of *NbEDS1* expression compromises the HR resulting from RPS4 expression, and that *NbEDS1* is required for RPS4-triggered HR in tobacco.

Homologues of yeast SGT1, a component of certain Skp1-Cullin-F box protein (SCF) ubiquitin ligase complexes, are required for disease resistance in plants mediated by NB-LRR R proteins (Austin *et al.*, 2002; Azevedo *et al.*, 2002; Tor *et al.*, 2002). In *N. benthamiana*, there are two SGT1 isoforms, *NbSGT1.1* and *NbSGT1.2* (Peart *et al.*, 2002b), and they play an important role in the TIR-NB-LRR N-mediated resistance to TMV (Liu *et al.*, 2002; Peart *et al.*, 2002b). To investigate the role of SGT1 in the RPS4-induced response in tobacco, VIGS of SGT1 homologues in *N. benthamiana* was performed using TRV:SGT1. In *NbSGT1*-silenced plants, the RPS4-induced HR was compromised (Figure 4c and Figure S1c), indicating the requirement of SGT1 for this HR in tobacco.

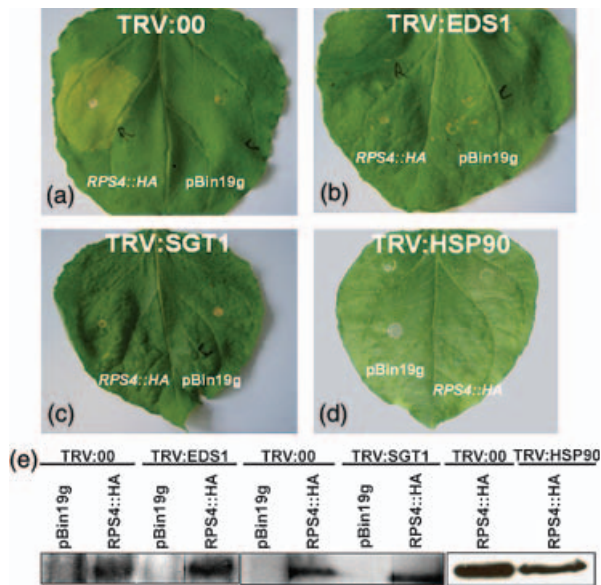


Figure 4. EDS1, SGT1 and HSP90 are required for *RPS4*-induced hypersensitive response (HR) in *Nicotiana benthamiana*. TRV:00 (a, empty vector), TRV:EDS1 (b), TRV:SGT1 (c) or TRV:HSP90 (d)-silenced *N. benthamiana* leaves were infiltrated with *Agrobacterium* containing *RPS4::HA* (right half) or pBin19g (vector, left half) construct. The HR phenotype was recorded at 7 dpi. (e) Immunodetection of *RPS4* proteins from the plants mentioned in (a), (b), (c) and (d). Protein samples (2 dpi) were immunoassayed with an anti-HA antibody.

Recent studies indicate that HSP90 is also involved in disease resistance. The regulatory or chaperone function of HSP90 is required for the full function of *RPM1*, *RPS2*, *Pto*, *Rx* and *N* (Hubert *et al.*, 2003; Lu *et al.*, 2003; Takahashi *et al.*, 2003). HSP90 was therefore tested for its involvement in *RPS4*-induced HR in tobacco. We silenced *HSP90* in *N. benthamiana* using TRV:8–240 (Lu *et al.*, 2003) and then transiently expressed *RPS4* in both the silenced and TRV:00 control plants. Silencing of *HSP90* compromised the *RPS4*-induced HR (Figure 4d and Figure S1d), indicating that HSP90 also plays some role(s) in this cell death event. However, *Rx*-mediated resistance against PVX was not affected by this VIGS clone, but it was reduced by TRV:9-037-1 or TRV:10-186 (Lu *et al.*, 2003). This could mean that *RPS4* and *Rx* require different HSP90 isoforms. As reported, *RPM1* requires *AtHSP90.1* (Hubert *et al.*, 2003), but *RPS2* requires *AtHSP90.2* (Takahashi *et al.*, 2003). The precise physical function(s) of HSP90 in plant disease resistance remain unclear, but are likely to involve chaperoning the assembly of a functional R protein complex (Schulze-Lefert, 2004). Our data do not rule out a differential quantitative requirement between R proteins for HSP90s, in which partial reduction in the HSP90 pool by silencing a subset of *HSP90* genes will block the proper folding of some, but not all, R protein complexes.

Conceivably, VIGS could compromise *RPS4*-triggered HR through a severe effect on *RPS4* transient expression. To rule

out this possibility, *RPS4* protein expression in silenced plants was analysed. Figure 4(e) shows that *RPS4* still expresses well in the *EDS1*-, *SGT1*- or *HSP90*-silenced plants. VIGS did not severely affect the *Agrobacterium*-mediated transient *RPS4* expression. The silencing of *EDS1*, *SGT1* or *HSP90* genes did not affect the stability of *RPS4* protein, in plant materials sampled before cell death started, compared with the samples from the TRV:00-treated plants. Therefore, the disappearance of HR in the silenced plants above is not due to the absence of *RPS4* protein, but due to the regulatory or chaperone function(s) of these three components in the cell death-signalling pathway.

Discussion

Expression of R genes activates defence mechanisms

Transient expression of *RPS4* in tobacco provokes an HR even in the absence of AvrRps4. In *Arabidopsis*, transient expression of *RPS2* in the absence of AvrRpt2 also leads to an HR (Tao *et al.*, 2000), and this phenomenon has been called an R gene 'overdose effect'. The HR triggered by *RPS4* correlates with the accumulation of phytoalexins and with *PR* gene induction, and requires EDS1. This suggests that this HR is an authentic defence-related response and thus a valid model to investigate R protein function.

In some cases, R gene overexpression leads to the induction of defence responses in the absence of cell death. Indeed, some transgenic tomato plants that carry extra copies of the CC-NB-LRR R gene *Prf* display a SAR phenotype without any HR-like or micro-HR symptoms (Oldroyd and Staskawicz, 1998). Similarly, elevated expression of *At4g16890*, a TIR-NB-LRR R-like gene, induces defence responses without cell death and impairs plant growth even in the absence of pathogens (Stokes *et al.*, 2002). Conceivably, below a signalling threshold correlated with R gene expression levels, defence responses are stimulated, without any cell death, unless expression levels exceed this threshold. Alternatively, two different pathways could exist, one leading to cell death and the other to defence-related gene induction. Depending on the R gene systems, one or the other (or both) pathways will be activated. We favour the former explanation for our results. R proteins are normally found in a complex with a 'guarded' protein; while sequestered in this complex, they are unable to activate defence responses (Dangl and Jones, 2001). Following Avr recognition, a change in the R protein/'guardee' complex would initiate signal transduction. Overexpression of R genes could lead to an excess of the R protein relative to the 'guardee'. R protein molecules that are not negatively regulated by a 'guardee' may then activate the downstream events. Interestingly, although transient overexpression of *RPS4* in tobacco induces a strong HR, we did not observe lethal phenotypes in *35S::RPS4::HA* transgenic *Arabidopsis*

plants in which the RPS4 protein accumulation level is comparable with that in tobacco transiently expressing *35S::RPS4::HA* (Figure S2). This is consistent with negative regulator(s) of RPS4 function that are present in the *Arabidopsis* tested, but absent or ineffective on RPS4 in the tobacco species tested.

Domains of RPS4 required to trigger HR

Domain deletion analyses indicate that the TIR domain of RPS4 plays an indispensable role in the induction of RPS4-mediated HR in tobacco. Similarly, removal of the TIR domain of N abolished the signal transduction leading to resistance to TMV (Dinesh-Kumar *et al.*, 2000). Deletion of the TIR domain of IL-1R in human cells leads to loss of IL-1R-mediated cellular response to its ligand, IL-1 (Leung *et al.*, 1994). Mutations in the TIR domains of human TLR1 and TLR2 also result in disruption of the signal transduction leading to host defence (Xu *et al.*, 2000). Thus, the requirement for a functional TIR domain seems well conserved across species of different kingdoms. A principal function of TIR domains is to mediate homotypic or heterotypic protein–protein interactions in signal transduction (Kopp and Medzhitov, 1999; Xu *et al.*, 2000). However, this has not yet been verified for plant proteins containing TIR domains. The N-terminal CC domain of R proteins seems essential for their function (Tao *et al.*, 2000) perhaps also through similar protein–protein interactions. The N-termini of these two subclasses of proteins recruit other proteins through protein–protein interactions and the TIR and CC domains serve analogous roles in these proteins (Jebanathirajah *et al.*, 2002). Interestingly, recent data demonstrate that CC and NB-LRR domains of Rx protein can act in *trans* to activate defence responses (Moffett *et al.*, 2002), suggesting that intra-molecular interactions are likely to be essential for R protein function. In our experiment, co-delivery of TIR and TIR-deficient RPS4 constructs in *N. tabacum* did not restore the loss of HR (data not shown). This could be due to the instability of RPS4 TIR domain protein, because this protein could not be detected in Western blots. Thus, we could not investigate the intra-molecular interaction between the TIR domain and the other domains of RPS4 protein by co-immunoprecipitation.

Point mutations in the NB domain of N interfere with its function and behave like dominant negative mutations (Dinesh-Kumar *et al.*, 2000). Directed mutagenesis in *Arabidopsis* has shown that the NB domain of RPS2 is critical for its function (Tao *et al.*, 2000). Mutations in the NB domain of RPM1 also eliminate RPM1 activation (Tornero *et al.*, 2002a). Our domain deletion results are consistent with the key role of the NB domain in R protein activation and emphasize its implication in the overdose phenotype. The P-loop motif is very conserved in the NB domain and seems to play a critical role in its function

(Aravind *et al.*, 1999). We found that a point mutation in the Lys 242 of RPS4 leads to a complete abolition of the HR in tobacco. The mutation of the corresponding lysine in RPS2 and N also completely blocks their function (Dinesh-Kumar *et al.*, 2000; Tao *et al.*, 2000). Interestingly, a corresponding amino acid change also affects Apaf1 function by preventing ATP binding, the subsequent procaspase activation and cell death (Li *et al.*, 1997). In the case of Rx, the effective interaction between CC and NB-LRR domains is dependent on a wild type P-loop motif (Moffett *et al.*, 2002). The mechanism of how the NB domain contributes to R protein activation is unknown. However, as shown in mammalian systems, the NB domain could mediate the activation of the N-terminal signalling domain through nucleotide triphosphate binding and hydrolysis. Consistent with this model, it has been recently shown that the tomato R proteins I-2 and Mi-1 are functional ATP-binding proteins and have ATPase activity (Tameling *et al.*, 2002). Altogether these data suggest a well-conserved R protein mechanism involving a fully active NB domain.

Deletion of the LRR and/or CNL domains of RPS4 did not influence the HR induction. This suggests that these domains of RPS4 are not involved in downstream signalling. Similarly, the entire LRR is dispensable for the RPS2 overexpression phenotype (Tao *et al.*, 2000). There is compelling evidence that LRRs of NB-LRR proteins determine R gene specificity (Dodds *et al.*, 2001; Jia *et al.*, 2000) rather than having a specific role in signalling. A current model suggests that the LRR domain of R proteins may serve as the binding domain for ligands, which could be the target of Avr proteins (Bonas and Lahaye, 2002; Dangl and Jones, 2001).

Significance of alternative splicing of RPS4 for triggering HR

In this study, we have shown that alternative splicing may not be required for the RPS4-induced AvrRps4-independent HR in tobacco. However, alternative RPS4 transcripts are essential for its AvrRps4-specific resistance in *Arabidopsis* (Zhang and Gassmann, 2003). We hypothesize that requirement for the alternatively spliced transcripts might be dependent on the presence of an 'off-on' switch to regulate the activity of R proteins. Expression of RPS4 induced an HR in tobacco but not in *Arabidopsis* (data not shown). This suggests that a negative regulator might exist in *Arabidopsis* but not in tobacco. In *Arabidopsis*, RPS4 is in the 'off' state. When pathogens attack plants, AvrRps4 is recognized and the proportion of RPS4 alternatively spliced variants may be somehow altered to potentiate the RPS4 function. In tobacco, due to the absence of this negative regulator, RPS4 is already in the 'on' state and does not need the alternatively spliced transcript(s) to turn on its function switch. This hypothesis could explain the contradiction described above. Alternative splicing could be important either at the

non-coding RNA level (Eddy, 2001) or at the corresponding truncated protein level (Iwami *et al.*, 2000). The majority of spliced *R* gene variants found to date were not shown to be translated into polypeptides (Dinesh-Kumar and Baker, 2000; Jordan *et al.*, 2002; Zhang and Gassmann, 2003) suggesting that the function of the alternatively spliced transcript(s) could be at the RNA level. However, transgenic RLD with intron-deficient Ler *RPS4* did not show full resistance to *P. syringae* carrying *avrRps4* (Zhang and Gassmann, 2003). If the regulatory function was solely at the RNA level, the RLD *rps4* alternative transcript(s) should complement its function due to the high homology (>99%) between Ler and RLD *RPS4* alleles. It is therefore more likely that the truncated protein(s) translated from the alternatively spliced transcript(s) are important. Full-length and truncated protein(s) may need to interact for full resistance (Jordan *et al.*, 2002).

Requirement of signalling components for RPS4-triggered HR

To understand the HR, it is crucial to know whether it is caused solely by an alteration of the cellular metabolism induced by *R*-gene overexpression or whether it corresponds to a physiological mechanism involving other essential genes in the defence signalling pathway. Ectopically expressed genes with diverse functions from a variety of sources can cause lesion formation in plants, suggesting that metabolic perturbation may also result in cell death. For example, elevated expression of *Arabidopsis AtMEK4* and *AtMEK5*, encoding two closely related MAPK kinases, leads to HR-like cell death (Ren *et al.*, 2002). Our data suggest that in the case of *RPS4*, the cell death process in tobacco is dependent on known and essential signalling components for *RPS4* function in *Arabidopsis*. We found that in *NbEDS1*-silenced *N. benthamiana*, *RPS4*-induced HR is completely abolished. In contrast, *NbNDR1* silencing has no effect on the HR phenotype (data not shown). It is well established that in *Arabidopsis*, the *RPS4*-mediated transduction pathway requires *EDS1* (Aarts *et al.*, 1998). Our data suggest that in tobacco, the *RPS4*-induced HR also requires *NbEDS1*. Interestingly, the *N. benthamiana* *NbEDS1* homologue is involved in *N* gene-mediated resistance but is not required for Pto (a protein kinase) or Rx (a CC-NB-LRR protein) signalling pathways (Peart *et al.*, 2002a). These authors concluded that recruitment of *EDS1* by TIR-NB-LRR proteins is evolutionarily conserved between dicotyledonous plant species. Our results confirm this model and prove that the *EDS1* signalling pathway can also be activated by heterologous TIR-NB-LRR *R* gene expression. The fact that *NbNDR1* is not involved in the *RPS4*-mediated HR in tobacco confirms the specificity of implicated signalling components.

Tao *et al.* (2000) suggested that the overdose effect could be associated with non-specific inhibition of *de novo* protein synthesis, or induction of massive, non-specific protein

degradation. Interestingly, using a VIGS strategy we found that *RPS4*-induced HR is completely abolished in the *NbSGT1*-silenced *N. benthamiana*. SGT1 is known as a key protein in the function of certain SCF complexes for ubiquitin-mediated proteolysis in yeast (Kitagawa *et al.*, 1999). Our result concerning SGT1 is also consistent with its essential role in R protein-mediated resistance (Austin *et al.*, 2002; Azevedo *et al.*, 2002; Liu *et al.*, 2002; Peart *et al.*, 2002b). Recent data suggest that SGT1 is also involved in non-host resistance (Peart *et al.*, 2002b). According to genetic studies, *RPS4*-mediated resistance is *SGT1b*-independent in *Arabidopsis* (Austin *et al.*, 2002). Nothing is known about the other allele, *SGT1a*, in *Arabidopsis*. If these two isoforms have redundant or overlapping function, knocking out one gene may not affect its function. In this case, a double mutant is necessary. However, the *SGT1a* and *SGT1b* double mutant is lethal in *Arabidopsis* (K. Shirasu, The Sainsbury Laboratory, John Innes Center, Norwich, UK, personal communication). So, it is difficult to demonstrate the role of *SGT1* in the *RPS4* signalling pathway in *Arabidopsis*. In our VIGS experiment, we may have silenced both of the *SGT1* isoforms *NtSGT1.1* and *NtSGT1.2* because these two isoforms are 98% identical in the region cloned for silencing (Peart *et al.*, 2002b), and the HR was compromised. This proved that SGT1 is required for the *RPS4*-induced cell death in tobacco. An alternative possibility is that *AvrRps4*-independent cell death triggered by *RPS4* in tobacco requires SGT1 whereas *AvrRps4*-dependent disease resistance in *Arabidopsis* does not. However, this interpretation seems to be less likely. Levels of *RPS4* protein were comparable in *SGT1*-silenced and non-silenced plants (Figure 4e), suggesting that SGT1 was not required for *RPS4* protein accumulation. In contrast, Moffett *et al.* (2002) only detected Rx in *SGT1*-silenced plants, because it did not accumulate to the detectable level before cell death.

HSP90 has been recently identified as a new component required for R protein function in plants (Hubert *et al.*, 2003; Lu *et al.*, 2003; Takahashi *et al.*, 2003). We show here that *NbHSP90* is required for *RPS4*-induced HR in tobacco. How is HSP90 involved in R protein function? HSP90 is well known for its role in assembling protein complexes and regulating the activity of signal transduction proteins (Richter and Buchner, 2001). Hubert *et al.* (2003) showed that RPM1 is a client protein of HSP90 and *hsp90.2* mutant severely affects RPM1 accumulation. Lu *et al.* (2003) showed that VIGS of *HSP90* resulted in reduction of Rx protein level. This implies that HSP90 could facilitate the assembly of a functional R protein signalling complex. Our data show a slight reduction in the *RPS4* protein level in *HSP90*-silenced plants, which may be due to the attenuation of the transient transformation efficiency, as GUS protein level was also reduced at a similar ratio in the *HSP90*-silenced plants (data not shown). The *HSP90* VIGS clone we used (TRV:8-240) is from the less conserved 3' coding sequence and the adjacent

UTR region (Lu *et al.*, 2003). It compromised *RPS4*-induced HR, but does not affect *Rx* resistance to PVX. HSP90 also affects *Rx* function because silencing *HSP90* by two other clones TRV:9-037-1 or TRV:10-186, which carry the most conserved central region of *HSP90*, compromised *Rx* resistance to PVX (Lu *et al.*, 2003). In the current database, there are at least 11 different *HSP90* sequences from *N. benthamiana* (Kanzaki *et al.*, 2003; Liu *et al.*, 2004; Lu *et al.*, 2003). Therefore, clone TRV:8-240 possibly silences less isoforms than clone TRV:9-037 and TRV:10-186. This indicates that *RPS4*-induced HR may require different *NbHSP90* members from or have a higher quantitative requirement for HSP90 function compared with *Rx* resistance. Similarly, *RPM1* requires *AtHSP90.2* (Hubert *et al.*, 2003), but *RPS2* requires another isoform *AtHSP90.1* (Takahashi *et al.*, 2003).

Our analysis suggests that transient *RPS4* expression in *Nicotiana* species is a valid model for defence activation by R proteins, and we will be using it for further analysis of R protein mechanisms.

Experimental procedures

Plasmid construction

All the *RPS4* sequences in this study were amplified by PCR using genomic *RPS4* (Gassmann *et al.*, 1999) as the template. *Clal* and *Bam*HI restriction sites were introduced in the forward and reverse primers respectively. Flanking by 5' *Clal* and 3' *Bam*HI, these amplified PCR fragments were digested and cloned into pBin19g binary vector containing a *35S::GUS::HA* cassette at the same sites to generate C-terminal HA-fused *RPS4* derivatives driven by 35S promoter unless indicated. The primers used in this work are as the following: P1: 5' AGAATCGATGGAGACATCATCTATTTCCA C 3'; P2: 5' CGCGGATCCGCGCCTTTTCTTGCTAATGATGATG 3'; P3: 5' CGCGGATCCGCGCATTTTATTTTGAATACATTGATT 3'; P4: 5' CGCGGATCCGCGCTTGTACCCCTCCCAAAGTT 3'; P5: 5' CGCGGATCCGCGCCATTGTAGCGTTTCCGAG 3'; P6: 5' TGGATCGATGAAAGCTGCCAATGTTAGAGG 3'; P7: 5' GAGGGATCCCTTGTACCCCTCCCAAAGT 3'; P8: 5' CGCGGATCCCAATTGTAGCGTTTCCGAG 3'; P9: 5' ACTGGATCCGAAATCTTAACCGTGTGCATG 3'; P10: 5' CCATCGATGGACACCATGCTTAAGGTG 3'. For brevity, the description of these fusion constructs will be available on request.

The two P-loop point mutants were generated using overlapping PCR, in which two amplified fragments with an intended mutation in the overlapping region are joined together by PCR (Horton *et al.*, 1989, 1990). The primer pairs used to create mutations are as follows (substituted nucleotides are underlined).

242K to A P36: 5' GATGCCCGGAATTGGTGCAACCACACTCCTGAAAG 3'; P37: 5' CTTTCAGGAGTGTGGTGCACCCAATTCCGGGCATC 3'.

242K to Q P38: 5' GATGCCCGGAATTGGTCAAACCACACTCCTGAAAG 3'; P39: 5' CTTTCAGGAGTGTGGTTTCACCAATTCCGGGCATC 3'.

Pfu DNA polymerase was used for amplification and all the PCR-generated constructs were verified by DNA sequencing.

The estradiol::*RPS4*::*HA* construction was made by inserting a *Clal*-*Sall* fragment and an *Sall*-*Xba*I fragment from *35S::RPS4::HA* into a modified pER8 vector (Zuo *et al.*, 2000) at *Bst*BI and *Spe*I sites.

The recombinant *Clal*-*Sall* and *Sall*-*Xba*I fragments is the full-length *RPS4::HA* sequence.

cDNA cloning

Total RNA was extracted from *Arabidopsis* leaves using Tri-Reagent (Sigma, Steinheim, Germany) and single-strand cDNA was prepared from 2 µg of total RNA with Expand reverse transcriptase (Roche, Roche, Mannheim, Germany) and oligo (dT) primer. Full-length cDNA of *RPS4* was amplified by PCR with P1 and P15 (5' ACTGGATCCCTCAGAAATCTTAACCGTGTGC 3') using Pfu polymerase (Promega, Madison, WI, USA) and cloned into pBlue-script II. The cDNA fragment was verified by DNA sequencing.

Transient assay in *N. tabacum* and *N. benthamiana*

Overnight bacterial cultures of *A. tumefaciens* strain GV3101 containing the plasmid of interest were harvested by centrifugation. Cells were re-suspended in induction buffer (10 mM Mes, pH 5.6, 10 mM MgCl₂ and 150 µM acetosyringone) to an OD₆₀₀ of 0.5 and incubated for 2 h at room temperature. The agrobacteria were then hand-infiltrated into 4-week-old *N. tabacum* or silenced *N. benthamiana* leaves with a 1-ml needleless syringe. Tissue samples were harvested for protein analysis at 2 dpi (unless indicated), frozen immediately in liquid nitrogen, and stored at -80°C.

Protein analysis

Tobacco leaves were ground in liquid nitrogen. Total protein was extracted in 2 volumes of extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 2 mM DTT, 1% Triton, 2% polyvinylpyrrolidone and Roche complete protein inhibitor tablets). The protein concentration was determined using a Bradford protein assay kit with BSA as the standard. 2x loading buffer (20% glycerol, 4% SDS, 30 mM Tris-HCl, pH 6.8, 300 mM DTT and 1% bromophenol blue) was added to an equal volume of the extracted protein samples. Protein samples (40 µg) were separated on a 7.5–12.5% SDS-polyacrylamide gel (Laemmli, 1970) and were transferred onto nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, UK) by wet electroblotting (Mini-Protean II system; Bio-Rad, Hemel Hempstead, UK). For immunodetection, after overnight incubation with 1:5000 diluted primary HA monoclonal antibody (3F10, Roche), the membrane was incubated with 1:5000 diluted horseradish peroxidase-conjugated anti-rat secondary antibody (Sigma) for 1 h. Specific protein bands were visualized with the ECL Plus kit (Amersham Pharmacia Biotech).

Cell death assays

Dead cells were stained with trypan blue according to Koch and Slusarenko (1990).

Virus-induced gene silencing

Using a tobacco rattle virus vector, VIGS of the *N. benthamiana* homologues *EDS1*, *SGT1* and *HSP90* was performed as described by Peart *et al.* (2002a,b) and Lu *et al.* (2003). Briefly, 14-day-old *N. benthamiana* plants were infiltrated with *Agrobacterium* carrying TRV:EDS1, TRV:SGT1 or TRV:8-240(HSP90) to silence *EDS1*, *SGT1* and *HSP90* (Ratcliff *et al.*, 2001). After 3–4 weeks, the upper leaves of these plants were used for *Agrobacterium*-mediated transient assay experiments.

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Supplementary material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2201/TPJ2201sm.htm>

Figure S1. *EDS1*, *SGT1* and *HSP90* are required for *RPS4*-induced HR in *Nicotiana benthamiana*.

TRV:00 (a, empty vector), TRV:*EDS1* (b), TRV:*SGT1* (c) or TRV:*HSP90* (d) silenced *N. benthamiana* leaves were infiltrated with *Agrobacterium* containing the following constructs: 1, full-length *RPS4::HA* (see Figure 1a); 2, truncated *RPS4* E1,2 (see Figure 2a); 3, *Pto* and *avrPto*; 4, pBin19g. The leaves were stained by trypan blue at 7 dpi. The hypersensitive response (HR) triggered by full-length *RPS4* and truncated *RPS4* E1,2 are compromised in *EDS1*-, *SGT1*- and *HSP90*-silenced plants. The HR triggered by *Pto* and *avrPto* does not require *EDS1*, as an internal control; this HR is weakly suppressed by virus-induced gene silencing of *HSP90* using the TRV:8-820 clone (Lu et al., 2003).

Figure S2. Comparison of *RPS4* protein accumulation level in *Nicotiana tabacum* and *Arabidopsis thaliana*.

Protein samples from *N. tabacum* transiently expressing *RPS4::HA* (see Figure 1a) and from *RPS4::HA* transgenic *A. thaliana* were immunoassayed with an anti-*HA* antibody. The top panel shows the detection of *RPS4::HA* protein in a Western blot, the bottom panel shows the Ponceau S staining of ribulose-1,5 bisphosphate carboxylase/oxygenase (Rubisco) for equal loading in each lane. Lanes 1 and 2 are two different batches of samples from *N. tabacum*. Lanes 3, 4, 5 and 6 are the samples from two different plants of two different transgenic *A. thaliana* lines.

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