MPMI Vol. 22, No. 2, 2009, pp. 157–165. doi:10.1094/MPMI-22-2-0157. *e*-Xtra*

The TIR Domain of TIR-NB-LRR Resistance Proteins Is a Signaling Domain Involved in Cell Death Induction

Michal R. Swiderski, Doris Birker, and Jonathan D. G. Jones

Sainsbury Laboratory, John Innes Centre, Norwich Research Park Colney Lane, Norwich, NR4 7UH, U.K.

Submitted 20 January 2008. Accepted 2 October 2008.

In plants, the TIR (toll interleukin 1 receptor) domain is found almost exclusively in nucleotide-binding (NB) leucine-rich repeat resistance proteins and their truncated homologs, and has been proposed to play a signaling role during resistance responses mediated by TIR containing R proteins. Transient expression in *Nicotiana benthamiana* leaves of "TIR + 80", the RPS4 truncation without the NB-ARC domain, leads to EDS1-, SGT1-, and HSP90-dependent cell death. Transgenic *Arabidopsis* plants expressing the RPS4 TIR+80 from either dexamethasone or estradiol-inducible promoters display inducer-dependent cell death. Cell death is also elicited by transient expression of similarly truncated constructs from two other R proteins, RPP1A and At4g19530, but is not elicited by similar constructs representing RPP2A and RPP2B proteins. Site-directed mutagenesis of the RPS4 TIR domain identified many loss-of-function mutations but also revealed several gain-of function substitutions. Lack of cell death induction by the E160A substitution suggests that amino acids outside of the TIR domain contribute to cell death signaling in addition to the TIR domain itself. This is consistent with previous observations that the TIR domain itself is insufficient to induce cell death upon transient expression.

Additional keywords: apoptosis.

In animal and plant kingdoms, the TIR (Toll interleukin 1 receptor) domain is present in proteins involved in immune responses. In mammals, the TIR domain is an effector module in TOLL-like receptors (TLR), the interleukin-1 (IL-1) receptor family, and small adaptor proteins that link active TLR and IL-1 receptors to downstream signaling components (Takeda and Akira 2005). In plants, the TIR domain has been recognized at the N-terminus of resistance proteins carrying the nucleotide binding (NB)-ARC domain and leucine-rich repeats (LRR). Huge sequence diversity exists among NB-LRR proteins, and they confer resistance to various microbial pathogens, viruses, and invertebrates. The TIR-NB-LRR class of resistance (*R*) genes in the *Arabidopsis* genome is represented by nearly 100 genes. In addition, there are more than 50 genes encoding proteins without either an LRR or NB-LRR portion. Intriguingly, the TIR domain is essentially absent from gramineae genomes (Meyers et al. 2002, 2003). The other subclass of NB-LRR proteins either lacks a recognizable N-terminal motif or carries a coiled-coil (CC) domain at the N-terminus.

The NB-ARC domain comprises a region of extended homology between Apaf1, R proteins, and Ced4 (van der Biezen and Jones 1998a). It has been demonstrated that the NB-ARC domain from both the I2 resistance protein and Apaf1 exhibits ATPase activity in vitro (Riedl et al. 2005; Tameling et al. 2002, 2006). This implies that an energy-dependent conformational change in R proteins is crucial for their activity.

The LRR domain mediates protein–protein and protein–ligand interactions and is involved in the specific recognition of pathogen-derived elicitors (Dangl and Jones 2001; Dodds et al. 2006; Hammond-Kosack and Jones 1997). The recognition of avirulence proteins is, at least in some instances, indirect and consistent with the "guard" hypothesis (van der Biezen and Jones 1998b; Dangl and Jones 2001). Proteolytic removal of RIN4 by the *Pseudomonas syringae* effector, AvrRpt2, activates RPS2, inducing defense and cell death (Axtell and Staskawicz 2003; Mackey et al. 2003). Similarly, the cleavage of PBS1 kinase by AvrPphB protease is essential for activation of the RPS5 resistance protein (Shao et al. 2003). Conceivably, many CONSTITUTIVE pathogenesis-related (*PR*) gene expressor (*cpr*) mutations might be located in genes encoding putative "guardees." For example, a mutation in the *BON1* gene which encodes a copine synthase induces defense responses by activating the SNC1 TIR-NB-LRR R protein (Yang and Hua 2004).

Activation of R proteins is likely to lead to a conformational change, as has been documented for the CC-type R proteins Rx and Bs2 (Leister et al. 2005; Moffett et al. 2002). In the presence of coat protein from *Potato virus X*, interactions between CC, NB, and LRR domains of Rx are disrupted (Moffett et. al. 2002; Rairdan and Moffet 2006). Similar intramolecular interactions have not been observed for TIR containing R proteins, although it has been reported that the N protein oligomerizes in the presence of its cognate elicitor (Mestre and Baulcombe 2006). Genetic requirements for the signal transduction pathways initiated by CC and TIR R proteins only partially overlap. Although the CC class seems to be dependent on NDR1, the TIR class is dependent on EDS1 (Aarts et al. 1998; Falk et al. 1999). Two factors known to be involved in protein folding, SGT1 and HSP90, are shared, at least partially, by both pathways (Azevedo et al. 2002; Bieri et al. 2004, Hubert et al. 2003; Takahashi et al. 2003; Tornero et al. 2002b; van Bentem et al. 2005).

It has been reported that elevated expression of *R* genes in plants induces constitutive immunity and that the transient expression of *R* genes may induce spontaneous cell death (Leister and Katagiri 2000; Oldroyd and Staskawicz 1998; Zhang et al. 2004). It seems that the TIR domain plays a crucial role in a

Current address for D. Birker: Max Planck Institute for Plant Breeding Research Carl-von-Linné-Weg 10, 50829 Köln, Germany.

Corresponding author: J. D. G. Jones; Fax: (0044) (0) 1603 450011; E-mail: jonathan.jones@sainsbury-laboratory.ac.uk

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cell death signaling pathway (Weaver et al. 2006; Zhang et al. 2004). Indeed, transient expression in tobacco leaves of the flax L10 TIR domain with extra C-terminal amino acids induces cell death (Frost et al. 2004). However, the role of the TIR domain is still poorly understood.

Here, we show that the NB-ARC domain is not necessary for induction of cell death caused by an expression of truncated derivatives of TIR resistance proteins. Expression of truncations containing the TIR domain with a short C-terminal fragment directly following it is sufficient to cause cell death. We analyze the TIR domain of the RPS4 protein by site-directed mutagenesis and characterize several gain-of-function substitutions.

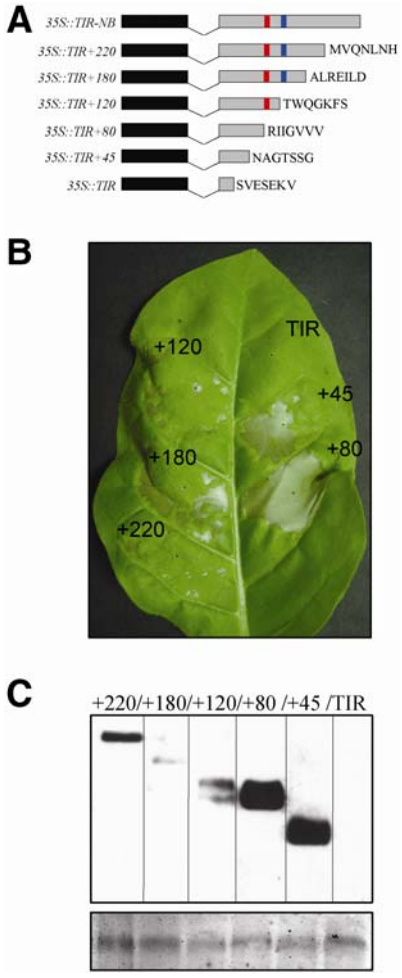

Fig. 1. Truncated derivatives of *RPS4* without nucleotide-binding (NB)-ARC domain induce cell death upon transient expression in *Nicotiana tabacum* leaves. **A,** Schematic representation of the deletion alleles of RPS4 used in B. TIR (toll interleukin 1 receptor)-NB-ARC (*35S::TIR-NB*) provides a comparison with other constructs. The TIR domain is shown as a black box. Expression of all derivatives is driven by the 35S promoter of *Cauliflower mosaic virus*. Red and blue boxes mark Walker A and Walker B motifs present in the NB-ARC domain, respectively. Letters at the end of boxes represent amino acids at which particular construct ends. **B,** Derivatives of *RPS4* as in A were delivered into different segments of a tobacco leaf via *Agrobacterium*. Cell death was observed 36 to 48 h later. Picture was taken 4 days postinfiltration. Construct expressing the TIR domain did not induce cell death. **C,** Expression of constructs delivered into tobacco leaf. Samples were taken 40 to 48 h after infiltration. The membrane was probed with an anti-hemagglutinin-HRP conjugated antibody. The TIR construct could not be detected. Equal protein loading was checked by staining membrane with Ponceau S (bottom panel). Visualized bands represent the large subunit of rubisco. All experiments were repeated a minimum of four times and produced a similar pattern of responses.

RESULTS

The NB-ARC domain of RPS4 is not necessary for induction of cell death.

Previous data suggest that the TIR domain of RPS4 is important for induction of cell death when RPS4 is transiently expressed in tobacco leaves (Zhang et al. 2004). However, this inference was based on the absence of cell death induction by *RPS4* derivatives lacking their TIR domain. To investigate more directly a role for the TIR domain of RPS4, a series of constructs were made, deleted through the NB-ARC domain (Fig. 1A). The TIR+80 and TIR+45 constructs terminated before the NB motif. A single hemagglutinin (HA) tag was incorporated at the 3′ end of each truncated construct. Transient *Agrobacterium* spp.-mediated expression of these truncations in tobacco leaves allowed us to evaluate cell death induction. Surprisingly, all constructs except the one which encoded only

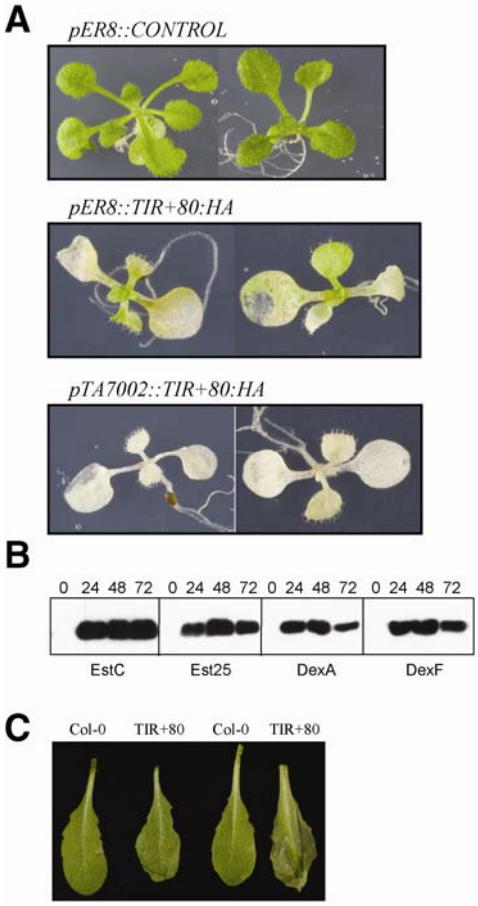

Fig. 2. TIR+80 induces cell death in *Arabidopsis* seedlings. **A,** *pER8::TIR+80* or *pTA7002:TIR+80* transgenic seedlings of *Arabidopsis* Col-0 were transferred after 10 to 14 days to fresh medium supplemented with either 40 μM estradiol or dexamethasone. The top pictures represent the control with unrelated fragment cloned into pER8 vector. Pictures in the middle represent seedlings after 5 days on estradiol-supplemented medium. Bottom pictures present seedlings expressing TIR+80 from pTA7002 vector. Pictures were taken 5 days after transplantation to induction medium. Control transgenic lines expressing FLAG22 peptide did not show any symptoms. **B,** Expression of TIR+80 after treatment with estradiol (Est) or dexamethasone (Dex). Proteins were extracted from seedlings at 0, 24, 48, and 72 h after induction. Est and Dex names below represent independent transgenic lines transformed with estradiol- or dexamethasone-inducible construct, respectively. **C,** *pTA7002::TIR+80* transgenic *Arabidopsis* exhibit cell death after infiltration of the dexamethasone (right side of a leaf). Cell death is visualized as collapsed leaf tissue. Picture taken 24 h after the dexamethasone infiltration. Similar results were obtained in three independent repeats.

the TIR domain of RPS4 and a very short C-terminal extension (TIR) were able to cause cell death (Fig. 1B). This validated our previous conclusion that the TIR domain is indeed an effector domain for cell death signaling. Nearly all respective proteins were detected, though steady state accumulation of the individual proteins differed substantially (Fig. 1C). The inactive TIR construct produced an undetectable level of protein while the TIR+80 construct produced a high amount of protein.

To investigate whether *Arabidopsis* is also prone to cell death mediated by the expression of TIR+80, the corresponding fragment was cloned into pTA7002 and pER8 vectors in which gene expression is controlled by the dexamethasone- or estradiol-inducible promoters, respectively (Aoyama and Chua 1997; Zuo et al. 2000). Both *pTA7002::TIR+80:HA* and *pER8::TIR+80:HA* were stably transformed into *Arabidopsis* and transgenic plants were tested for cell death upon induction. Young seedlings from several independent transgenic lines, grown on solid Murashige-Skoog (MS) medium in petri dishes, were transferred to fresh medium supplemented with either 40 μM dexamethasone or estradiol. Three days later, most seedlings started to bleach and, after 5 days, most of them were dead (Fig. 2A). Similar data were obtained with both sets of transgenic lines, although the dexamethasone-inducible lines (*pTA7002::TIR+80:HA*) developed cell death faster than *pER8::TIR+80:HA* lines. Western blot analysis of protein extracts form both estradiol- and dexamethasone-challenged seedlings revealed that TIR+80:HA indeed accumulated in induced plants (Fig. 2B).

In addition, as an independent test to show that TIR+80 induces cell death in *Arabidopsis,* the leaves of *pTA7002:: TIR+80:HA* transgenic lines were infiltrated with dexamethasone solution. Cell death was apparent 9 h later and, 17 h after infiltration, the whole leaf was usually dead (Fig. 2C).

Cell death induced by TIR+80 is *EDS1*, *SGT1*, and *HSP90* dependent.

We then tested whether the TIR+80-mediated cell death was dependent on *EDS1*, *SGT1*, or *HSP90*. All three genes have been shown to be important in pathogen-induced cell death and plant disease resistance (Jones and Takemoto 2004). *SGT1*, *EDS1*, and *HSP90* were silenced individually in *Nicotiana benthamiana* using virus-induced gene silencing (VIGS) (Zhang et al. 2004) and RPS4 TIR domain-dependent cell death was transiently induced in these plants. To assay cell death, the dexamethasone-inducible construct was used because it gave more reproducible results. In wild-type plants and TRV-00-silenced plants, cell death usually occurred 24 h after inducer application but, in *EDS1*-, *SGT1*-, and *HSP90*-silenced plants, cell death either did not develop or was notably diminished compared with control plants (Fig. 3A). Similar results were obtained with the estradiol-inducible TIR+80 construct (not shown). Sampling protein extracts with an anti-HA antibody showed that the TIR+80 protein accumulated equally well in *SGT1*-, *EDS1*-, *HSP90*-, and TRV-00-silenced plants (Fig. 3B).

Truncated derivatives of two other TIR-NB-LRR proteins induce cell death.

We were interested to establish whether the cell-death-inducing property of TIR+80/TIR+45 was unique to the RPS4 protein or if homologous fragments from other TIR-NB-LRR genes could also cause cell death upon transient expression. TIR+80 fragments from *RPP1A*, *N*, and TIR+45 fragments from four genes from the *RPP2* locus (*At4g19500*, *At4g19510*, *At4g19520*, and *At4g19530*) were constructed and tested via an *Agrobacterium* sp. expression system for their cell-death-inducing activity. *At4g19500* and *At4g19510* represent *RPP2A* and *2B*, respectively (Sinapidou et al. 2004). Two constructs of the RPP1A TIR+80 fragment were prepared, with and without the N-terminal hydrophobic transmembrane peptide (Botella et al. 1998; Weaver et al. 2006). All fragments were fused to the single HA epitope. Both RPP1A constructs (–H and +H) and the At4g19530 construct induced confluent cell death in the area of *Agrobacterium* sp. infiltration. The fragment from the *N* gene induced only a weak, spotty cell death, whereas others were not active at all (Fig. 4A). Immunoprobing of protein

Fig. 3. Expression of the *RPS4 TIR+80* derivative leads to *EDS1-, SGT1-*, and *HSP90*-dependent cell death. **A,** *Nicotiana benthamiana* plants were silenced using virus-induced gene silencing with indicated viral constructs. The dexamethasone-inducible TIR+80 derivative (*pTA7002::TIR+80:HA),* its two gain-of-function forms, or empty vector (EV) were delivered into plant cells via *Agrobacterium* spp. Two days later, dexamethasone was gently infiltrated into same areas on leaves. Cell death was scored 48 h later. Photographs recorded 96 h after dexamethasone infiltration. Numbers indicate the proportion of tested leaves that developed cell death in three independent experiments. A maximum of two leaves per silenced plant were tested. **B,** Expression of TIR+80 after induction with dexamethasone. Samples were taken 24 h after dexamethasone infiltration and protein blots probed with an anti-hemagglutinin-HRP conjugated antibody. Bottom panel shows Ponceau S staining of the membrane as a check of equal loading. Visible bands represent the large subunit of Rubisco.

samples taken from these plants indicated that the lack of cell death induced by the TIR+45 derivative of At4g19520 and greatly diminished cell death caused by expression of N+80 might be due to a much lower amount of protein accumulation compared with fragments from either RPS4 or TIR+80 fragments from other R proteins (Fig. 4C through E). TIR+45 fragments from At4g19500 and At4g19510 accumulate well; thus, lack of cell death induced by these proteins is most likely due to intrinsic properties of these proteins rather than due to insufficient expression level.

Mutagenesis of the RPS4 TIR domain.

To further characterize the RPS4 TIR domain, we introduced 32 mutations into the TIR+80 fragment. Amino acids chosen for site-directed mutagenesis were based on their conservation in an amino acid sequence alignment of plant TIR domains and, in most instances, were hydrophilic (Fig. 5; Supplementary Fig. 3; Table 1). Secondary structure prediction suggested a similar arrangement of β-sheets and α-helices in plant TIR domains as in TIR domains from IL-1R and TLR1, although the β-strand E is uncertain (Fig. 5). For some positions, multiple substitutions were constructed. These mutated TIR+80 fragments were tested for their ability to induce cell death. Cell death was visually evaluated based on the size of the infiltrated area that collapsed and the timing of cell death. Routinely, leaves were checked 24, 36, and 48 h after *Agrobacterium* sp. infiltration. Both Table 1 and Supplementary Figure 1 summarize our observations of the cell death elicited by the mutated derivatives of TIR+80.

All mutated proteins were detected, except mutations E111K and E134K, and most of the substitutions accumulated to a level comparable with wild-type TIR+80 (Fig. 6). We noticed that several mutations, notably substitutions E111K and E134K, induced a faster and much stronger cell death response than the control (Table 1). The gain-of-function character of these mutations was additionally revealed by an *Agrobacterium* dilution and ion leakage assays. In the dilution assay, the *Agrobacterium* sp. harboring a construct was diluted to different concentrations and infiltrated into tobacco leaves. *Agrobacterium* strains carrying gain-of-function mutations in TIR+80 always induced cell death at the concentration 5 to 25 times lower than wild-type TIR+80. Comparisons of conductivity measurements taken from leaf samples expressing the gain-of-function mutant versions of TIR+80, an inactive control (TIR), a wild-type TIR+80, and one mutant version of TIR+80 which does not change cell death activity are shown in Figure 7. All gain-of-function mutations produced 1.3 to 2 times higher values than the wild-type TIR+80.

Two gain-of-function mutant forms of TIR+80, W84A, and D136E137NQ were cloned into the pTA7002 vector and tested in *N. benthamiana* plants to see whether the cell death they induced was still dependent on *EDS1, SGT1*, and *HSP90*. Indeed, cell death elicited by expression of these mutant forms was dependent on all of these genetic components (Fig. 3A).

DISCUSSION

Previous data have suggested that the TIR domain of TIR-NB-LRR resistance proteins is involved in initiating a signal transduction pathway leading to apoptosis (Frost et al. 2004; Weaver et al.2006; Zhang et al. 2004). Here, we show that transient expression RPS4 derivatives without NB-ARC domain (TIR+45/TIR+80) in either tobacco leaves or *Arabidopsis* stable transgenic lines causes cell death. Expression of "NB-ARC-less" constructs of RPP1A or At4g19530 proteins also led to autonecrosis in tobacco leaves. Not all of the TIR+45 homologous constructs are capable of cell death induction. In some instances, this might be due to a low amount of protein accumulation, exemplified by At4g19520 and *N*. However, the *RPP2B* (*At4g19510*) TIR+45 construct did not induce cell death despite a relatively high level of protein accumulation. *Arabidopsis* Col-0 *RPP2B* requires *RPP2A* (*At4g19500*) in order to confer resistance against *Hyaloperonospora parasitica* race cala2 (Sinapidou et al. 2004). The lack of cell death

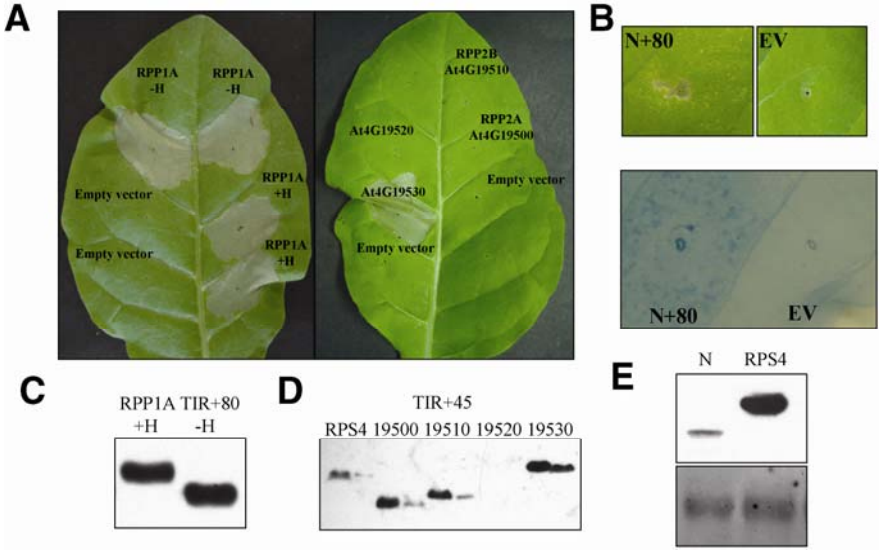

Fig. 4. Expression of TIR+80/TIR+45 constructs from various resistance proteins induces cell death. **A,** Tobacco leaves were infiltrated with *Agrobacterium* spp. carrying marked TIR+80 (RPP1A; left picture) or TIR+45 constructs (right picture). RPP1A and At4g19530 derivatives induced cell death. Pictures were taken 3 to 4 days postinfiltration. **B,** Expression of the N TIR+80 fragment in tobacco leaves induces weak cell death. Lower picture shows the trypan-blue-stained tobacco leaf to visualize dead cells. EV = empty vector control. **C,** Expression of RPP1A TIR+80:HA with (+H) and without (–H) hydrophobic N-terminal fragment. Protein blot was probed with anti-hemagglutinin (HA)-HRP conjugated antibody. **D,** Western blot showing the lack of expression of the At4g19520 TIR+45 fragment. All other proteins were detected with an anti-HA-HRP conjugated antibody. Two sets of samples, collected 24 h (weaker signal) and 48 h (stronger signal) after *Agrobacterium* infiltration, loaded on the gel in D. **E,** N TIR+80 accumulates to a substantially lower amount then RPS4 TIR+80. Lower panel shows Ponceau S staining of membrane as an equal protein-loading control. Visualized bands represent the large subunit of rubisco. Expressed proteins were detected as in C and D.

induced by RPP2B might be due to a unique mode of cooperation between these proteins to provide immunity. Our data are consistent with the idea that the function of the NB-ARC domain in R proteins might be to provide an energy-dependent conformational change in order to expose the N-terminus for recruitment of components required for cell death signaling. P-loop-dependent conformational change of the CC containing R protein Rx has previously been inferred (Moffett et al. 2002). Mutations in the ATP-binding motif of many resistance proteins abolish their function and lead to plant susceptibility to corresponding pathogens (Dinesh-Kumar et al. 2000; Tao et al. 2000; Tornero et al. 2002a). An energy-driven conformational change of APAF1, stimulated by binding cytochrome C to WD40 repeats, allows N-terminal CARD domains to self-interact, a key step in apoptosome assembly (Hu et al. 1999). The intact nucleotide binding motif in the tobacco resistance protein N is necessary for its oligomerization, occurring in the presence of the viral elicitor P50 (Mestre and Baulcombe 2006). How exactly the activities of R proteins are negatively regulated is not clear. Transient expression of *RPS2*, *RPS4*, or *RPP1A* in *N. benthamiana* leaves causes cell death, indicating that either at least a portion of the accumulated protein is in an active conformation or additional components are necessary to block activity of these proteins (Day et al. 2005; Weaver et al. 2006; Zhang et al. 2004). Indeed, co-expression of RIN4 with RPS2 in *N. benthamiana* leaves abolishes RPS2-induced cell death (Day et al. 2005).

Plant signal transduction pathways leading to cell death during resistance responses remain mysterious. Signaling initiated by TIR R proteins is compromised by mutations in the *EDS1* gene (Aarts et al. 1998; Falk et al. 1999). EDS1 resembles lipases and interacts with itself and with other lipase-like proteins, PAD4 and SAG101 (Feys et al. 2001, 2005). EDS1 also controls cell death elicited by transient expression of RPS4 and its truncated derivatives (Zhang et al. 2004). Dependence of the TIR+80-mediated cell death on EDS1 indicates that such a cell death mirrors that observed during pathogen ingress rather than being due to nonspecific interference of TIR+80 with cellular metabolism or cell integrity. The TIR+80-induced cell death is also dependent on SGT1 and HSP90. This is consistent with findings which suggest an additional role for SGT1 in plant resistance other than only to cotrol the abundance of R proteins in the cell or their folding (Holt et al. 2005). Indeed, interactions of SGT1 with components of the putative proteasome lid prompted speculation that SGT1 participates in the removal of negative regulators of apoptosis (Azevedo et al. 2002; Holt et al. 2005). Identification of F-box and U-box proteins as necessary components of signaling pathways in plant disease resistance and cell death promotion is consistent with this view, although the targets degraded by these proteins are unknown (González-Lamothe et al. 2006; Suk Kim and Delaney 2002; Yang et al. 2006).

The crystal structure of the TIR domain from the TLR1, TLR2, and IL-1RAPL shows a similar basic structure, one that

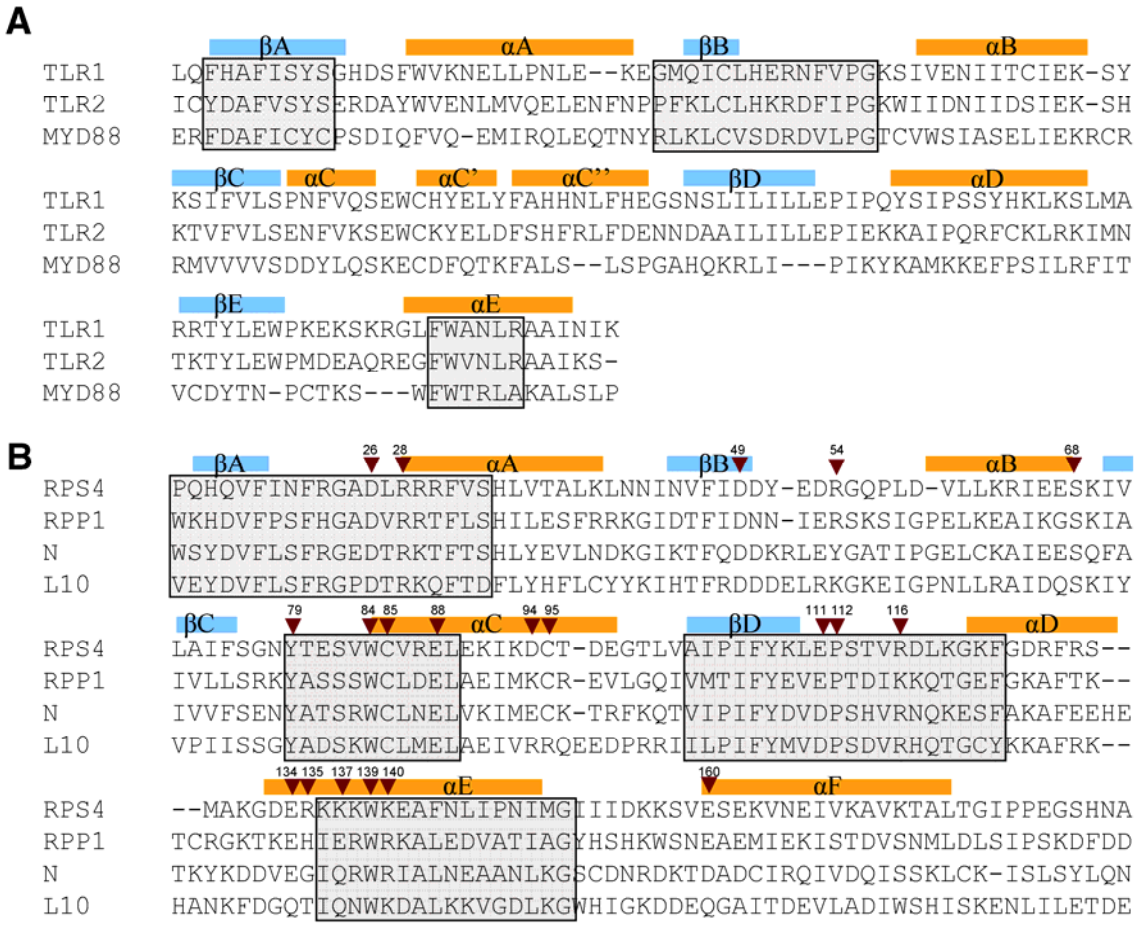

Fig. 5. Amino acid alignment of TIR (toll interleukin 1 receptor) domain sequences. **A,** Alignment of TIR domains from TLR1, TLR2, and MYD88. Blue and orange bars represent β-sheets and α-helices according to Xu and associates (2000). **B,** Alignment of plant TIR domains from several resistance proteins. Blue and orange bars represent β-sheets and α-helices according to Xu and associates (2000). Secondary structures were predicted by numerous programs online. Predicted helix adjacent to the C-terminus of αE was named αF. Note the lack of βE. Arrowheads indicate amino acids that were substituted and numbers above them mark position in the RPS4 TIR domain. Light-gray boxes mark conserved regions in animal TIR domains as defined by Slack and associates (2000) or in plant TIR domain as defined by Meyers and associates (2002).

is represented by a five-stranded β-sheet surrounded by α-helices (Xu et al. 2000). Different surfaces of the TLR TIR domain have been implicated in dimerization and binding of either Myd88 or Mal adaptors (Dunne et al. 2003; Xu et al. 2000). Many mutations that affect signaling from the interleukin receptor IL1-R or TLR interfere with binding of adaptor proteins (Takeda and Akira 2005). The secondary structure of the plant TIR domain resembles the one in TLR, although it seems that β-strand E may be missing. The molecular consequences of such a difference are unknown. At this moment, it is unclear whether the RPS4 TIR domain is sufficient to signal cell death because the construct expressing the TIR domain only does not produce a detectable amount of protein (Zhang et al. 2004). However, we have not observed cell death elicitation by expression of only the TIR domain from either N or RPP1A (Mestre and Baulcombe 2006; Weaver et al. 2006). This suggests that a part of the sequence between the TIR domain and the NB motif is also important for signaling with the TIR domain itself. The requirement of C-terminal sequence for TIR-domain-mediated cell death signaling is corroborated by the E160A substitution which abolishes cell death even though the protein still accumulates well (Fig. 5). These results suggest that this predicted α-helix forms a functional signaling entity with the TIR domain itself.

An amino acid alignment of plant TIR domains has revealed that nearly one-third of residues display a high level of conservation. Three regions (box1, box2, and box3) which have been shown to be important for TLR and IL1-R signaling display a high degree of identity in plant TIR domains. More detailed analyses of plant TIR domains have revealed the presence of four rather then three conserved regions, named TIR1-4 (Meyers et al. 2002, 2003). It has been suggested that these boxes play an important role in homotypic interactions between TIR domains (Slack et al. 2000). Interactions between the TIR domain of TLR and Myd88 or MAL are crucial for recruitment of IRAKs and TRAF6 into an activated receptor complex (Wu and Arron 2003). It seems also

Table 1. List of mutations introduced individually into the TIR domain of RPS4 and their impact on cell death[a]

Position of mutation	Strength of cell death
TIR80 (control)	++
D26A	–
R28E	–
D49A	–
D49D50/AA	+
D50E52D53/NQN	–
R54N	–
S68A	–
Y79A	–
Y79F	–
W84A	**++++**
C85A	–
E88A	–
D94N	++
C95A	++
D97E98/QN	**++++**
D97E98/KK	++
E111K	**++++**
P112A	–
P112K	–
S113T114/EE	–
R116A	–
M129A130Δ	–
E134A	**++++**
E134K	**++++**
R135E	–
D133E134/NQ	**++++**
K137E	–
K136K137K138Δ	–
W139A	–
W139F	–
K140N	–
E160A	–

[a] Strength of the cell death was visually assessed by comparing the onset of cell death and an extent of collapsed tissue between mutated forms of TIR+80 and the wild type. The following scoring system was used: lack of cell death, –; cell death similar to wild type, ++; cell death weaker then wild type +; and cell death stronger then wild type, ++++. Mutations showing increased cell death activity are in bold. Double or triple substitutions are separated from the original amino acid sequence by forward slash (/); Δ indicates deletion of amino acids.

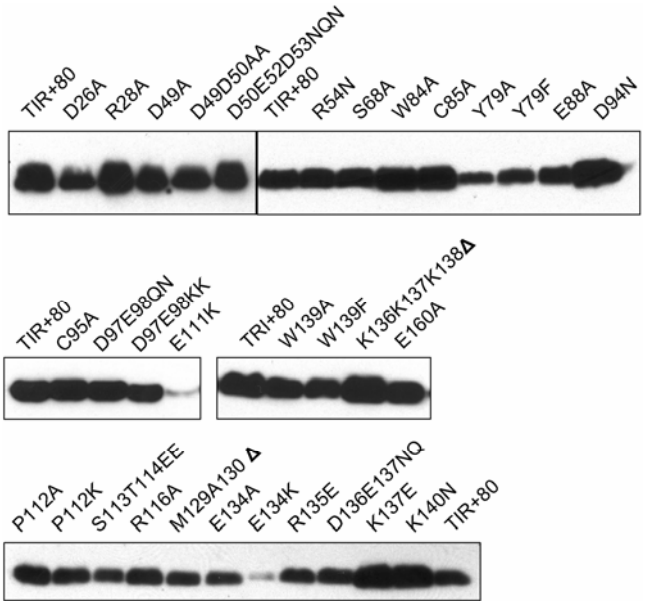

Fig. 6. Expression of mutant forms of the RPS4 TIR+80. Substituted forms of the TIR+80 fragment were delivered into a tobacco leaf by *Agrobacterium* spp. Leaf samples were taken 40 h after the *Agrobacterium* spp. infiltration or prior the uniform tissue collapse (cell death) of the infiltrated area. Expressed proteins were visualized by probing the blot with an anti-hemagglutinin-HRP conjugate. Most of the substitutions were expressed to a level similar to wild-type TIR+80. Low amounts of E111K and E134K were reproducibly observed and correlated with strong cell death elicitation by these mutant forms. Equal protein loading was confirmed by staining membranes with the Ponceau S dye (not shown).

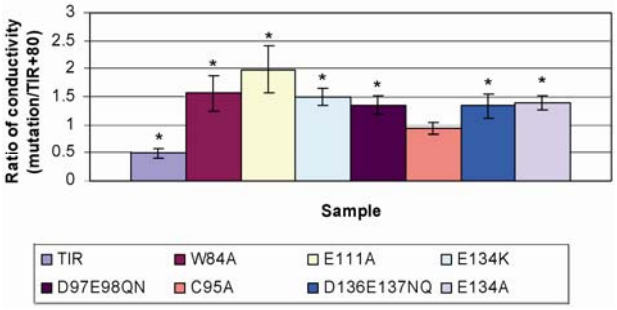

Fig. 7. Several substitutions in the RPS4 TIR (toll interleukin 1 receptor) domain exhibit gain of function phenotype. *Agrobacterium* spp. harboring corresponding mutated derivatives of TIR+80 were infiltrated in half of a tobacco leaf. Second half of the same leaf was infiltrated with the *Agrobacterium* strain carrying the wild-type TIR+80 fragment. Sample and control leaf disks were taken from symmetrical position on the same leaf and washed, and conductivity of the water measured 2 h later. Shown are means of conductivity ratios sample:wild type TIR+80 of at least six independent measurements. Error bars represent the standard deviation. Star above bars indicates statistically significant differences at $P < 0.03$. C95A did not change activity (ratio ≈ 1). TIR is an inactive control (ratio < 1).

that interactions between TIR domains are essential for receptor dimerization driven by the recognition of elicitor (Takeda and Akira 2005), a feature also observed for the N protein (Mestre and Baulcombe 2006). Our attempts to co-immunoprecipitate HA- and Myc-tagged TIR+80s from either RPS4 or RPP1A were unsuccessful (not shown). This might be due to extra C-terminal sequence present in the TIR+80 protein or an instability of such a complex. We cannot rule out the possibility that the RPS4 TIR domain interacts with the TIR domain of other R proteins upon activation.

Site-directed mutagenesis of the RPS4 TIR domain followed by transient cell death assays have revealed that substitution of conserved amino acids in TIR1, TIR2, TIR3, and TIR4 boxes led to loss of function. Substitutions occupying a homologous position in N and RPS4 produced similar results. (Dinesh-Kumar et al. 2000; Mestre and Baulcombe 2006). Such similarities emphasize the universal character of cell death initiation by TIR R proteins.

Interestingly, a few amino acid substitutions potentiated cell death activity of TIR+80. These gain of function substitutions are located in conserved boxes TIR2 (W84A) and TIR3 (E111K) and in loosely conserved sequences between boxes TIR2-3 (D97E98/NQ) and TIR3-4 (E134A and E134K). It is not clear how these mutations may elevate the activity of TIR+80. We hypothesize that the conformational change in the TIR domain exerted by such substitutions increases interactions with an unknown factor that is required for initiation of cell death. Indeed, box TIR4 is located in the αE helix and this helix has been suggested to be engaged in protein–protein interactions (Radons et al. 2003). The sequence of this helix shows a low level of identity between plant TIR domains but its distinct feature is the accumulation of positively and negatively charged residues. Conceivably, the conformation of this region might determine the active or inactive status of the R protein to signal cell death. Such a hypothesis might be addressed in the future by obtaining the crystal structure of the RPS4 TIR domain and corresponding gain-of-function mutant forms and might be helped by the identification of proteins that interact with the TIR domain.

MATERIALS AND METHODS

Construct preparation.

Deletion constructs of *RPS4* and TIR+80 or TIR+45 homologous sequences from other genes were prepared by amplification of corresponding fragments using polymerase chain reaction (PCR). Primers used for amplification were tailed with appropriate restriction sites. PCR products were then digested with corresponding restriction enzymes and cloned into *Cla*I/*Bam*HI sites of the pBIN19:HA binary vector (Zhang et al. 2004). For cloning into pER8 or pTA7002 vectors, the TIR+80:HA of *RPS4* was amplified with *Xho*I and *Nhe*I sites at the 5′ and 3′ end, respectively, and cloned into *Xho*I/*Spe*I sites of vectors. All primer sequences used in the preparation of constructs will be provided upon request.

***Agrobacterium* spp. transient assay, silencing, and trypan blue staining.**

The transient expression of constructs was done essentially as described in (Zhang et al. 2004) but *Agrobacterium* spp. were suspended in the ASB buffer (5 mM $MgCl_2$; 5 mM morpholineethanesulfonic acid (MES), pH 5.6; and 100 μM acetosyringone). Silencing of *SGT1*, *EDS1*, and *HSP90* was performed as reported earlier (Zhang et al. 2004). A maximum of two leaves per silenced plant were tested. Trypan blue staining was done according to Zhang and associates (2004).

***Arabidopsis* stable transformation.**

Arabidopsis stable transformations were done by following the standard approach (Clough and Bent 1998). Transgenic lines were selected on solid MS medium with hygromycin at a concentration of 40 μg/ml.

Ion leakage measurements and *Agrobacterium* spp. dilution assay.

To measure the ion leakage caused by the progressing cell death, *Agrobacterium* strains carrying corresponding constructs were suspended in ASB (2 mM $MgCl_2$ and 2 mM MES, pH 5.6) and infiltrated into half of a tobacco leaf. The other half was infiltrated with the reference construct (*35S::TIR+80:HA*). At 36 to 48 h postinfiltration, two leaf disks (1.5 cm^2) were taken per leaf sector, briefly washed in 50 ml of water, and put into 2 ml of water with an addition of Silwet-L77 (0.0005%) and vacuum infiltrated for 2 min. The vacuum was gently released and leaf disks were visually inspected for uniform infiltration. Reference samples were taken always from symmetrical sectors of the same leaf to minimize the variation due to the position on a leaf from which samples were punched. Conductivity of the solution was measured after infiltration (time 0) and 2 h later. Conductivity was recorded with a conductivity meter. For the *Agrobacterium* dilution assay, strains harboring corresponding constructs were diluted in ASB buffer to optical densities 0.1, 0.025, 0.00625, and 0.00155. Diluted suspensions of *Agrobacterium* spp. were hand infiltrated into individual panels of a tobacco leaf. The other half of the same leaf was infiltrated with an *Agrobacterium* strain carrying wild-type TIR+80 diluted to the same density.

Protein analysis.

Leaf tissue samples were collected 48 h after the *Agrobacterium* sp. infiltration or just prior to cell death occurring. Leaf disks (0.7 cm in diameter, two disks per sample) were ground in liquid nitrogen in an Eppendorf tube and 100 μl of the loading buffer (120 mM Tris-HCl, pH 6.8; 3% sodium dodecyl sulfate [SDS]; 100 mM dithiothreitol; 12% glycerol; and Bromophenol blue at 0.04 mg/ml) was added. Samples were briefly vortexed and incubated for 5 min at 95°C. Samples were centrifuged for 5 min and 10 to 15 μl of supernatant was loaded on 12% SDS/polyacrylamide protein gel. After electrophoresis, proteins were blotted onto an ECL nitrocellulose membrane (Amersham-Pharmacia, Piscataway, NJ, U.S.A.). Expressed proteins were visualized by appropriate HRP conjugated antibodies (HA, clone 3F10; Roche) and Supersignal West Pico (or Femto or a mix of both of them) Chemiluminescent Substrates (Pierce, Rockford, IL, U.S.A.). Antibodies were diluted according to the manufacturer's recommendations.

Site-directed mutagenesis.

The cDNA fragment of RPS4 TIR+80 was prepared by PCR using the RPS4 full-length cDNA as a template (Zhang et al. 2004). Primers used in the PCR contained *Cla*I and *Bam*HI restriction sites, respectively. The amplified fragment was cloned into pGEMT-easy (Promega Corp., Madison, WI, U.S.A.) and served as a template for an in vitro mutagenesis. Mutations in the RPS4 TIR+80 were introduced following instructions to QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.). Substituted forms of TIR+80 were recloned into pBin19:HA. The sequences of all primers used will be available upon request.

Dexamethasone and estradiol treatment.

Estradiol and dexamethasone were dissolved in ethanol and spread on the surface of solid MS medium to a final concentration of the inducer in total volume of medium (25 ml) of 40

μM. Plates were left open until the ethanol evaporated and kept at room temperature at least 24 h to facilitate diffusion of chemicals throughout the medium. Two-week-old seedlings grown on the solid MS medium supplemented with hygromycin were transplanted onto a fresh plate with inducers and grown further until visual symptoms appeared (2 to 5 days). To analyze protein expression, seedlings were collected 24, 48, and 72 h after transplantation on the expression induction medium. For infiltrations, dexamethasone was diluted in water to the final concentration of 40 μM.

Sequence alignment.

Alignment of amino acid sequences of TIR domains was done using ClustalX (Thomson et al. 1997). Alignment was manually corrected and displayed in GeneDoc (Nicholas et al. 1997). Secondary structure predictions were conducted by various programs accessible online.

ACKNOWLEDGMENTS

We thank P. Mestre for providing cDNA of the *N* gene, M. Smoker for reading and correcting the manuscript, and K. Sohn for discussion and advice on ion leakage assay. This work was supported by the Biotechnology and Biological Science Research Council grant P15093. The work in the Sainsbury Laboratory is supported by the Gatsby Charitable Foundation.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Advanced Protein Secondary Structure Prediction server: www.imtech.res.in/raghava/apssp2

Jpred 3 server: www.compbio.dundee.ac.uk/~www-jpred

National Resource for Biomedical Supercomputing (NRBSC) website: www.psc.edu/biomed/genedoc

PredictProtein server: www.predictprotein.org