An Evolutionarily Conserved Mediator of Plant Disease Resistance Gene Function Is Required for Normal *Arabidopsis* Development

Ben F. Holt III,[1] Douglas C. Boyes,[1,5]
Mats Ellerström,[1,6] Nicholas Siefers,[1,7]
Aaron Wiig,[1,8] Scott Kauffman,[1] Murray R. Grant,[2]
and Jeffery L. Dangl[1,3,4]
[1]Department of Biology
University of North Carolina, Chapel Hill
Chapel Hill, North Carolina 27599
[2]Department of Biological Sciences
Imperial College at Wye
Wye, Ashford TN25 5AH
United Kingdom
[3]Department of Microbiology and Immunology and
Curriculum in Genetics
University of North Carolina, Chapel Hill
Chapel Hill, North Carolina 27599

Summary

Plants recognize many pathogens through the action of a diverse family of proteins called disease resistance (*R*) genes. The *Arabidopsis R* gene *RPM1* encodes resistance to specific *Pseudomonas syringae* strains. We describe an RPM1-interacting protein that is an ortholog of TIP49a, previously shown to interact with the TATA binding protein (TBP) complex and to modulate c-*myc*- and β-catenin-mediated signaling in animals. Reduction of *Arabidopsis TIP49a (AtTIP49a)* mRNA levels results in measurable increases of two *R*-dependent responses without constitutively activating defense responses, suggesting that *AtTIP49a* can act as a negative regulator of at least some *R* functions. Further, *AtTIP49a* is essential for both sporophyte and female gametophyte viability. Thus, regulators of *R* function overlap with essential modulators of plant development.

Introduction

Plants prevent most pathogens from causing disease through a variety of preformed and active defense responses. Genetic dissection of active defense responses has identified a related class of plant resistance (*R*) genes whose protein products are necessary for the direct or indirect recognition of pathogen avirulence (*avr*) gene products (reviewed in Dangl and Jones, 2001). While the simplest interpretation of this genetic model is that the *R* product is a receptor for the *avr* product,

[4]Correspondence: dangl@email.unc.edu
[5]Present address: Paradigm Genetics, 108 Alexander Drive, P.O. Box 14528, Research Triangle Park, North Carolina 27709.
[6]Present address: Botanical Institute, Göteborg University, Box 461, S-405 30 Göteborg, Sweden.
[7]Present address: Department of Biological Sciences, Stanford University, Stanford, California 94305.
[8]Present address: BASF Agricultural Products Center, 26 Davis Drive, P.O. Box 13528, Research Triangle Park, North Carolina 27709.

this has been difficult to generalize experimentally. Numerous downstream consequences from *R-avr* interaction have been described, including cellular ion influxes, an oxidative burst leading to the accumulation of superoxide and hydrogen peroxide, nitric oxide production, the hypersensitive response (HR; a form of programmed cell death), and the production of toxic antimicrobial metabolites (Dangl and Jones, 2001). The specific functional contributions of each downstream response to the *R-avr* interaction are still unclear, but their combined effect is a potent barrier to pathogen invasion.

Five main classes of *R* genes have been isolated, with the majority encoding a carboxy-terminal leucine-rich repeat region (LRR) (Jones, 2001). LRRs are commonly found in proteins responsible for signal recognition and are typically involved in protein-protein and peptide-ligand interactions (Dangl and Jones, 2001). Genetic and functional evidence suggests that the LRR domain determines R protein specificity (Ellis et al., 2000). The largest structural group of R proteins also possesses centrally located nucleotide binding (NB) sites that bind GTP and ATP in other proteins. Hence, these are termed the NB-LRR proteins. There are two structural subclasses of NB-LRR proteins, those that feature a putative *c*oiled-*c*oil domain at the N terminus (CC-NB-LRR) and those that encode a domain with homology to the mammalian *T*oll and *I*nterleukin 1 *r*eceptors at the N terminus (TIR-NB-LRR).

Extensive forward genetic screens looking for loss of HR and/or loss of resistance phenotypes have defined additional loci required for *R* gene-triggered defense responses (Glazebrook, 2001). For example, the TIR-NB-LRR *R* genes identified thus far require a functional *EDS1* (enhanced *d*isease *s*usceptibility; Parker et al., 1996) gene for their disease resistance phenotype, while many, but not all, CC-NB-LRR genes require a functional *NDR1* (*n*on-race-specific *d*isease *r*esistance; Century et al., 1997) gene (Aarts et al., 1998). The current sum of genetic evidence suggests that *R*-mediated responses are not the product of a single, linear signal transduction cascade but rather the product of a network of cellular responses indicative of multiple signaling pathways acting in parallel.

Several explanations, including activation of parallel signaling pathways and lethality, could account for the low number of loci defined in screens for loss of *R* function. For example, analysis of *RPP8* function (resistance to *Peronospora parasitica* isolate Emco5) supports the concept of *R* gene-signaling redundancy. *RPP8* does not require *EDS1* or *NDR1*, but combined mutations in both moderately suppress *RPP8* (McDowell et al., 2000). To date, no genes required for *Arabidopsis* viability have been additionally implicated in *R* gene-mediated disease resistance. However, numerous *Arabidopsis* mutants whose phenotypes alter *R* gene-mediated resistance, basal defense activation, and normal morphological development have been identified (Yu et al., 1998). Nearly all of these mutants feature morphological abnormalities accompanied by constitutive expression of pathogenesis-related (PR) genes, which are downstream markers for defense activation in plants. Mutants thus activated

for defense responses are also generally rendered resistant to normally virulent pathogens. Numerous stresses, including developmental impairment, may activate plant defense. Therefore, caution should be exercised when implicating a direct role in disease resistance for the wild-type product of the genes identified by such mutations. Nevertheless, phenotypes of this mutant class suggest that genes involved in disease resistance may also have important developmental functions. Those with essential functions would not be isolated in most genetic screens. Recently, mutation of the *Arabidopsis MAPK4* gene was demonstrated to both enhance resistance to virulent pathogens and cause developmental abnormalities (Petersen et al., 2000). That a single MAPK protein can function in various contexts (Madhani and Fink, 1998) is an illustrative example of how pleiotropy can confound simple assignment of function in disease-resistance signaling.

Arabidopsis RPM1 (resistance to P*seudomonas syringae* pv m*aculicola*) encodes a CC-NB-LRR class protein that specifies recognition of *Pseudomonas syringae* strains expressing either the *avrRpm1* or *avrB* avirulence genes (Grant et al., 1995). *P. syringae* (*Ps*) pathogens carrying *avrRpm1* or *avrB* are recognized in planta by RPM1, resulting in a rapid HR (typically 3–5 hr post inoculation) and essentially no pathogen reproduction or plant disease symptoms. In the absence of RPM1, pathogen effector proteins, such as AvrRpm1, can act as virulence factors (reviewed in Nimchuk et al., 2001). RPM1 is a peripheral plasma membrane protein (Boyes et al., 1998). Both AvrRpm1 and AvrB are also localized to the plasma membrane by fatty acylation following expression in the plant cell, and this localization is required for the function of these two type III effector proteins (Nimchuk et al., 2000).

In this paper, we describe the isolation and genetic characterization of the *Arabidopsis* gene *AtTIP49a*. We have previously referred to this gene as *RIN1* (*RPM1 interactor 1*) but have adopted the animal nomenclature to emphasize its close homology to TIP49a. TIP49a was initially identified as a factor associated with the TATA binding protein (TBP) complex (see below). We demonstrate that AtTIP49a interacts with both the RPM1 (CC-NB-LRR) and RPP5 (TIR-NB-LRR) proteins. Reduction of *AtTIP49a* mRNA levels results in measurable increases of *RPP5* and *RPP2* function, suggesting that *AtTIP49a* can act as a negative regulator of at least some *R*-dependent responses. Plants with reduced *AtTIP49a* mRNA levels have morphological defects, yet they do not constitutively express pathogenesis-related (PR) genes or express elevated resistance against virulent pathogens. Thus, reduction of *AtTIP49a* function does not lead to constitutive, pleiotropic defense activation. We further demonstrate that *AtTIP49a* is essential for both sporophyte and female gametophyte viability. These results provide the first evidence that regulators of NB-LRR function can overlap with essential modulators of plant development.

Results

Arabidopsis RPM1 and RPP5 Interact with AtTIP49a in the Yeast Two-Hybrid Assay
To identify proteins that interact with RPM1, we performed a yeast two-hybrid screen with full-length RPM1.

Approximately 5.5×10^6 colonies from a pathogen-induced cDNA library were screened, and no interactions were found. Western analysis suggested that full-length RPM1 proteins were made in relatively low abundance in yeast (data not shown). We therefore made bait constructs with shorter regions of RPM1 and screened an additional $\sim2.0 \times 10^6$ colonies with a bait containing RPM1 amino acids (aa) 55–341 (pEG10; Figure 1A). *Arabidopsis* clone L29 was found to consistently and strongly activate both reporter genes. Because L29 encodes an ortholog of the animal protein TIP49a (see below), we refer to it hereafter as *AtTIP49a*. We assayed other regions of RPM1 and demonstrated that a CC-NB bait (pEG7; Figure 1A) also interacted with AtTIP49a. Two additional baits, containing both the CC and NB domains (pEG8 and 9; Figure 1A) and, hence, the interacting region of RPM1 defined by pEG7 and pEG10, did not interact with AtTIP49a. One possibility for the lack of interaction with these baits is that the presence of the CC and NB domains together, in certain contexts, masks the interaction domain of RPM1.

We addressed whether other R proteins could also interact with AtTIP49a. We tested a series of roughly corresponding baits from *RPP5* (resistance to *Peronospora parasitica* isolate Noco2; Parker et al., 1997) and *RPS2* (resistance to *Pseudomonas syringae* with *avrRpt2*; Bent et al., 1994; Mindrinos et al., 1994) for interaction with AtTIP49a. Figure 1B illustrates that two of the RPP5 baits interacted strongly with AtTIP49a. As with RPM1, these baits correspond to the NH$_2$ region of the protein. No RPS2 baits interacted with AtTIP49a.

AtTIP49a Interacts with RPM1 In Vitro
To confirm the AtTIP49a/RPM1 yeast two-hybrid interaction, we performed an in vitro pull-down assay. A fusion protein between glutathione s-transferase (GST) and the AtTIP49a peptide (aa 31–458) was purified and bound to glutathione Sepharose 4B. Glutathione Sepharose matrix containing either GST-AtTIP49a or GST alone (negative binding control) was incubated with in vitro-transcribed and -translated RPM1-Myc proteins (either full-length RPM1-Myc protein or an RPM1-aa1-177-Myc peptide). The results presented in Figure 1C demonstrate that GST-AtTIP49a enriches binding to both RPM1-Myc proteins compared to GST alone. The absence of an AtTIP49a interaction with full-length RPM1 in the yeast two-hybrid assay may reflect both reduced transcription/translation in yeast or complex structural requirements necessary for the interaction.

AtTIP49a Is One Member of a Highly Conserved Gene Pair in Eukaryotes and an Archaebacteria
AtTIP49a (TAIR chromosomal locus At5g22330; http://www.arabidopsis.org) was sequenced, and its deduced translational product was compared to other proteins by BLAST and protein motif analysis (see Supplemental Data for protein comparisons and other TIP49 information). The deduced AtTIP49a protein has two nucleotide binding sites (Walker A and B motifs; Traut, 1994) and two nuclear localization signal sequences (Yoneda, 2000). *AtTIP49a* is highly conserved in all Eukaryotes examined and is also found in an Archaebacteria. *Arabidopsis* has two *AtTIP49a* homologs (*AtTIP49b1* and
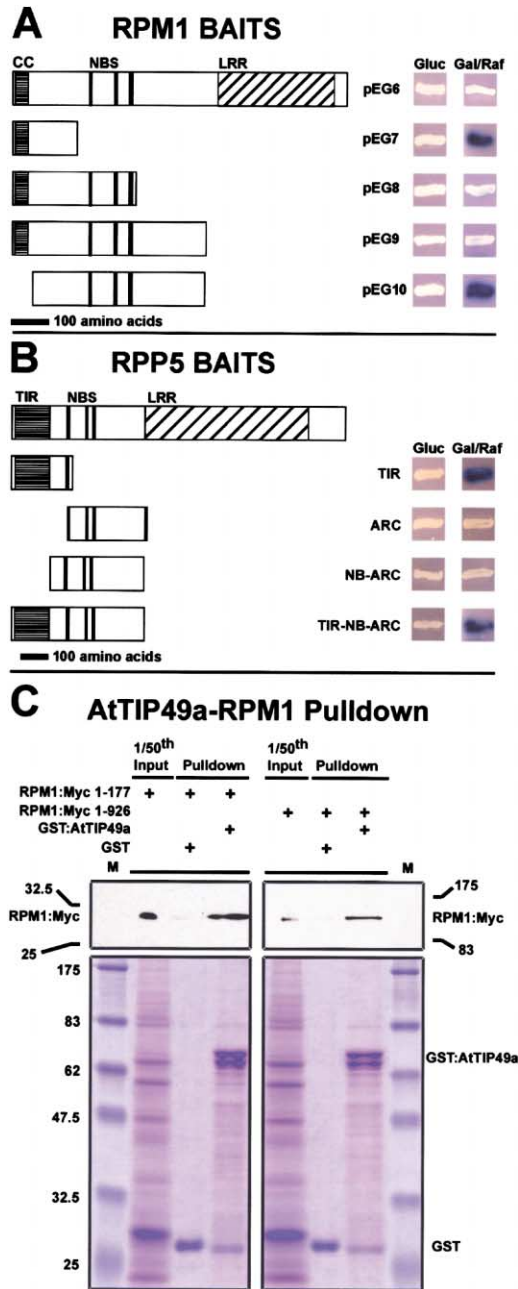
Figure 1. AtTIP49a Interacts with RPM1 and RPP5

(A and B) Yeast two-hybrid β-galactosidase plate assays. Columns under Gluc (glucose) and Gal/Raf (galactose/raffinose) depict yeast containing pJG4-5/AtTIP49a and the indicated pEG202/RPM1 or RPP5 construct on X-Gal plates. The absence of β-galactosidase activation on the Gluc plates is a control showing that autoactivation does not occur; blue color on Gal/Raf plates indicates a positive interaction. All tests were repeated at least twice.

(A) RPM1 baits tested for interaction with AtTIP49a. RPM1 baits were composed of the following amino acids: pEG6, full-length aa 1–926; pEG7, aa 1–176; pEG8, aa 1–341; pEG9, aa 1–534; pEG10, aa 55–341.

(B) RPP5 baits tested for interaction with AtTIP49a. The RPP5 baits were composed of the following amino acids: TIR, aa 1–223; ARC, aa 226–531; NB-ARC, aa 161–518; TIR-NB-ARC, aa 1–518. These baits were previously described (van der Biezen et al., 2000).

(C) In vitro pull-down assay between AtTIP49a and RPM1. Top panels represent anti-myc exposed Western blots; lower panels are

AtTIP49b2; TAIR chromosomal loci At3g49830 and At5g67630, respectively) that encode proteins ~45% identical to AtTIP49a. Due to their high level of identity (83%), *AtTIP49b1* and *AtTIP49b2* appear to be derived from a recent duplication. All other eukaryotic species have paired orthologs to *AtTIP49a* and *AtTIP49b1/2*. Protein pairs within a given organism are approximately 40% identical. Interspecific comparison between any two AtTIP49a or AtTIP49b1/2 orthologs reveals ~65% identity (see Supplemental Table S1). Thus, orthologs are more related than any pair within a species, strongly suggesting that this small gene family is the product of an ancient duplication, some sequence divergence, and subsequent evolutionary conservation.

We obtained a full-length *Arabidopsis AtTIP49b1* clone and tested it for interaction with R proteins in the yeast two-hybrid system. AtTIP49b1 also interacted with the RPM1 bait pEG7 but did not interact with pEG10 or any of the RPP5 baits (data not shown). Two groups recently used the yeast two-hybrid system to demonstrate interaction between the mammalian orthologs of AtTIP49a and AtTIP49b1/2 (alternatively called TIP49a/b in rat and ECP-54/-51 in human, respectively; Kanemaki et al., 1999; Salzer et al., 1999). We confirmed this two-hybrid interaction for *Arabidopsis* AtTIP49a and AtTIP49b1 (data not shown). Thus, AtTIP49b1 and AtTIP49b2 may also be involved in pathogen responses.

Reduction of *AtTIP49a* Impairs *Arabidopsis* Meristem Establishment

An insertion in *AtTIP49a* was lethal (see below), so we used antisense suppression to generate viable reduction of function alleles (*tip49as*). Aberrant meristem development phenotypes were apparent in the first generation of plants. Plants ranging in phenotypic severity were selected, and stable nonsegregating *tip49as* lines were established.

The strongest *tip49as* alleles germinated normally and had largely wild-type cotyledons, with rare cotyledon enlargement and cupping compared to Col-0 at 7–10 days post germination (Figure 2A). The nearly wild-type morphologies at this developmental stage were important for subsequent pathology tests with *P. parasitica*. At the next major developmental stage, when most wild-type plants had obvious first true leaves, most *tip49as* plants halted growth (Figure 2B). In some cases, one or both of the first true leaves emerged in a timely fashion but were typically misshapen with uneven margins, cupping, and twisting. For the strongest *tip49as* alleles, ~20%–30% of the plants died at this stage, while others exhibited subsequent aberrant meristem development, eventual flower formation, and set seed. The surviving plants typically made small rosettes, and some of the leaves had defects, such as uneven margins (Figures 2C and 2D). We chose a line with no obvious phenotypes (*tip49as7*) and three lines with visible morphological defects (*tip49as11*, *14*, and *18*) for RNA analysis. The RNA

Coomassie-stained gels from the same extracts and are equivalent to one-fifth the loading of the Western blots. Positions of size markers (M) are noted in KDa to the sides of Western blots and Coomassie gels.

blot in Figure 2E demonstrates that the morphological and meristem defects correlated with strongly reduced levels of *AtTIP49a* mRNA in 20-day-old plants. These data suggest a requirement for *AtTIP49a* in meristem development.

AtTIP49a Negatively Regulates *RPP5*- and *RPP2*- Mediated Disease Resistance

Since AtTIP49a interacts with RPP5 in yeast two-hybrid assays, we tested the effects of *tip49as* alleles on resistance to *Peronospora parasitica* (*Pp*) isolates. This system has the advantage, compared to *P. syringae* (described below), that single infection sites can be observed and subtle mutant phenotypes can be quantified (Holub et al., 1994; Morel and Dangl, 1999). We increased our ability to detect subtle loss or gain of resistance phenotypes by using two dose-sensitized assays for *R* function. First, *RPP5*-mediated resistance against *Pp* isolate Noco2 is semidominant (Parker et al., 1993). *RPP5/rpp5* heterozygotes support hyphal growth on cotyledons and allow some sporulation, albeit much reduced compared to fully susceptible *rpp5/rpp5* plants. Col-0(*rpp5*) transgenic for *RPP5* (Col-0::*RPP5*; Parker et al., 1997) are fully resistant to Noco2, and resistance is manifested as small HR sites with no hyphal growth. We designed a dose-sensitive experiment to test whether a line strongly suppressed for *AtTIP49a* mRNA accumulation would also enhance or suppress *RPP5* function. *tip49as11* was crossed to Col::*RPP5* and F1 seeds were collected (genotype *tip49as11/−, RPP5/−*). As hemizygous controls for the experiment, Col-0(*rpp5*) was crossed to both *tip49as11* (yielding *tip49as11/−, −/−)* and Col::*RPP5* (yielding *−/−, RPP5/−*). Following *Pp* Noco2 infection and trypan blue staining, various interaction classes, defined in Figure 3A, were counted for each cotyledon, as displayed in Figure 3B. The strong *tip49as11* allele enhanced *RPP5* function. For example, greater than 60% of the *RPP5/−, tip49as11/−* double hemizygous plants had no symptoms compared to less than 30% of the *RPP5/−* hemizygotes (all cotyledons were heavily inoculated). Conversely, *RPP5/−* cotyledons were much more likely to contain the free hyphae associated with higher susceptibility than the *RPP5/−, tip49as11/−* double hemizygotes. Additionally, this enhanced resistance to Noco2 is *RPP5* dependent. Cotyledons hemizygous for *tip49as11* and null for *rpp5* are fully susceptible. Greater than 80% of the *tip49as11/−, rpp5/rpp5* cotyledons had free hyphae, and ~60% had greater than five sporangiophores (similar to results for wild-type Col-0(*rpp5*); data not shown). These data suggest that *AtTIP49a* functions as an *R*-dependent negative regulator of disease resistance.

For the second sensitized screen, we assayed the action of *RPP2* (resistance against *Pp* isolate Cala2; Holub et al., 1994) in Col-0. *RPP2* is a relatively weak *R* gene, as indicated in Figure 4A by microscopic examination of Cala2-infected cotyledons stained with trypan blue (TB; stains dead cells and *Pp* structures). Col-0 cotyledons normally support limited hyphal growth, and the weak *RPP2* function in this genetic background is manifested as multicellular HR surrounding the expanding hyphae, termed trailing necrosis (Figure 3A).

Figure 2. Reduction of *AtTIP49a* mRNA Accumulation Results in Morphological Abnormalities

(A–D) Morphology and developmental time course of *tip49as11*.
(A) Seven-day-old seedlings.
(B) Two-week-old plants.
(C) Three-week-old plants.
(D) The flowering *tip49as11* plant shown here is healthier/larger than most.
(E) *AtTIP49a* RNA blot of four *tip49as* lines. The probe detected only endogenous *AtTIP49a* mRNA.
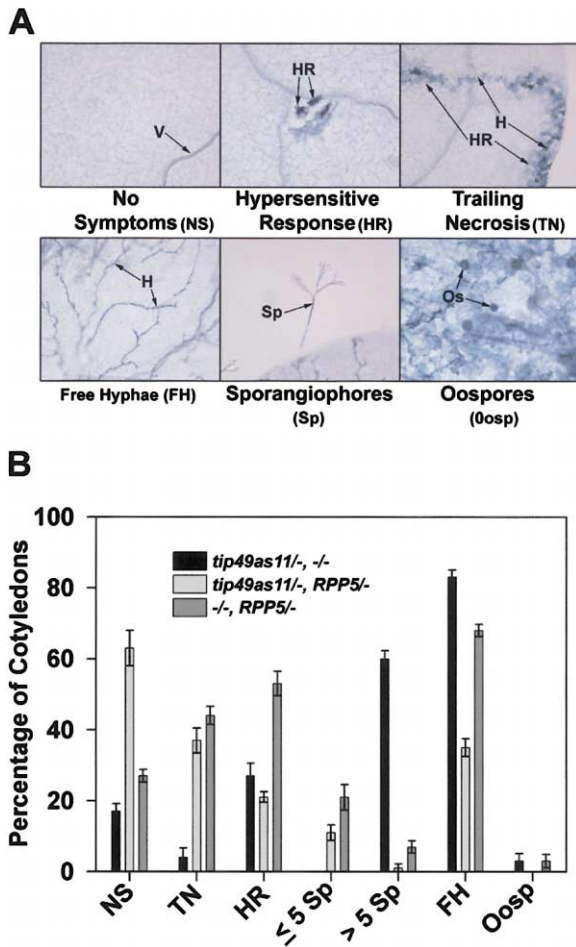
Figure 3. Suppression of *AtTIP49a* mRNA Accumulation Enhances RPP5-Mediated Resistance in a Sensitized Genetic Background

(A) Pathogen growth classes. Each cotyledon was scored blind as having (in decreasing order of resistance) no symptoms, HR, trailing necrosis, less than or equal to five sporangiophores per cotyledon, greater than five sporangiophores, free hyphae, and oospores. Cotyledons were scored for the presence or absence of each class and could have more than one class (i.e., the same cotyledon could have an HR interaction site and a separate trailing necrosis interaction site and would be scored as positive for both).

(B) Percentage of cotyledons showing each category of *Pp* growth. Values represent the mean for three replicates (±SE). At least 30 cotyledons were examined per replicate. This experiment was repeated with similar results. H, hyphae; HR, hypersensitive response; Os, oosporangia; Sp, sporangiophore; V, vein.

Col-0 *tip49as* alleles were quantitatively compared to Col-0 (*RPP2*) and Landsberg (La-*er*; *rpp2*) controls (Figure 4B). We observed a clear shift toward greater resistance in the strongest *tip49as* lines (*tip49as11*, *14*, and *18*), as measured by the percentage of cotyledons exhibiting no pathogen growth. As predicted from its level of *AtTIP49a* mRNA suppression, *tip49as7* responded like wild-type. These data demonstrate that *AtTIP49a* suppression reduces the growth of the *Pp* isolate Cala2 and suggest that *AtTIP49a* also negatively regulates *RPP2* function. Because *RPP2* was not cloned, we were unable to test whether its product interacts with AtTIP49a.
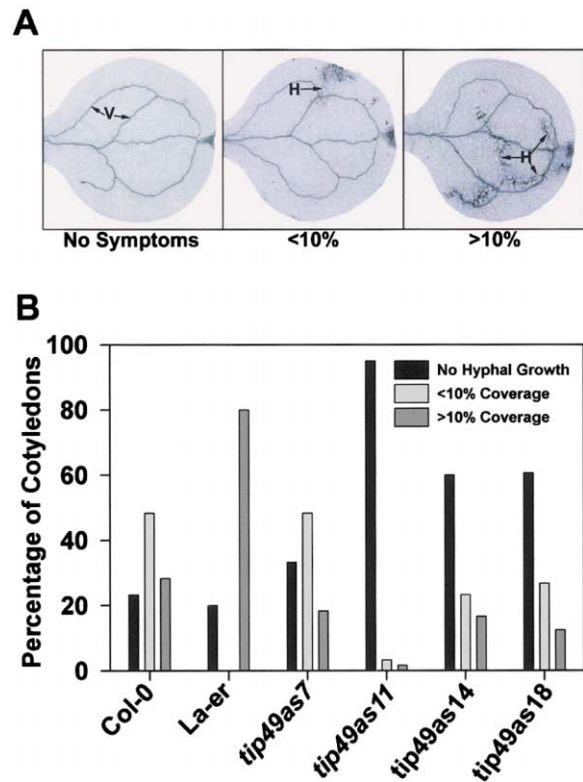

Figure 4. *RPP2* Function Is Enhanced in Several *tip49as* Lines

(A) Pathogen growth classes. Each cotyledon was scored blind as having no symptoms (no growth of the pathogen, with or without an HR), <10% hyphal coverage, or >10% coverage.

(B) Percentage of cotyledons showing each growth class. Col-0 (*RPP2/RPP2*) and La-*er* (*rpp2/rpp2*) are controls for resistance and susceptibility to Cala2. *tip49as* constructs were transformed into Col-0. Note from Figure 3 that *tip49as7* has nearly wild-type levels of *AtTIP49a* mRNA expression, while *tip49as* lines 11, 14, and 18 are all strongly reduced for expression. Approximately 60 cotyledons were examined in each line. This experiment was repeated twice with similar results. H, hyphae; V, vein.

The *tip49as* Phenotypes Are Not the Result of Constitutive Defense Activation

Because perturbation of unrelated metabolic pathways can potentially induce defense gene activation (reviewed in Dangl et al., 2000), we tested several *P. parasitica* isolates (Noco2, Emco5, and Ahco2; all virulent on Col-0) on various *tip49as* homozygous lines. *tip49as* had no measurable effect on growth of any of these isolates (data not shown). We extended this phenotypic data by following the accumulation of *PR1* mRNA (a commonly used marker for defense activation; Uknes et al., 1993) in *tip49as11* and wild-type plants inoculated with *P. parasitica* Cala2. We matched the time points used for the *P. parasitica* pathology tests (see Figure 4) and tissue collections for mRNA blotting. The results presented in Figure 5A clearly show that *tip49as11* plants do not constitutively express *PR1* and that the timing of *PR1* transcriptional activation following infection is similar to wild-type plants. However, *PR1* is more strongly expressed following pathogen challenge in the *tip49as* plants. Thus, one function of *AtTIP49a* is to suppress *R*-dependent activation of defense responses.
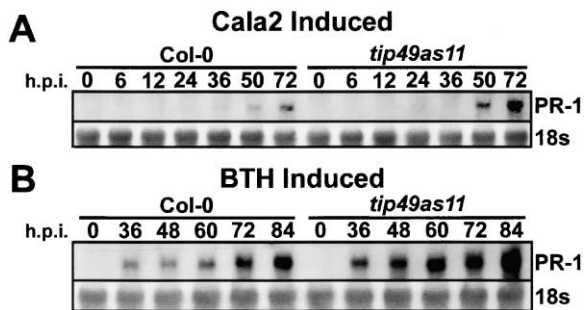
A
Cala2 Induced

B
BTH Induced

Figure 5. Suppression of *AtTIP49a* mRNA Expression in Cotyledons Does Not Result in Constitutive Activation of Defense-Related Genes

(A) *PR1* RNA blot on Cala2-challenged, 10-day-old plants. Entire aboveground plants were harvested at the indicated time points. The *PR1* probe was generated as described previously. The defense-related genes *PR5* and *PDF1.2* were expressed normally (data not shown). The 18s gene serves as an internal loading control.
(B) *PR1* RNA blot on BTH-challenged, 10-day-old plants. Plants/RNA treated as in (A).

This is in agreement with the pathology data presented in Figures 4 and 5. We further examined this enhanced defense response by measuring *PR1* mRNA accumulation following application of the salicylic acid (SA) functional analog benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl-ester benzothiodiazole (BTH; Görlach et al., 1996) to *tip49as11* plants. Figure 5B shows that *PR1* mRNA accumulated with the same time course in *tip49as11* and Col-0 cotyledons (no significant accumulation is seen in either case before 36 hr; data not shown) and that *PR1* accumulates to higher levels in the *tip49as11* plants. We observed similar results in adult plants, with strong increases in PR1 expression levels and faster timing of induction following BTH treatment (data not shown). Because our *tip49as* lines were still fully susceptible to virulent pathogens and did not ectopically express *PR1*, we infer that they were not constitutively activated for disease resistance.

Reduction of *AtTip49a* Does Not Significantly Affect *RPM1* Function

We tested *tip49as* for alterations in HR and disease resistance following challenge with the bacterial pathogen *Pseudomonas syringae*. We used strains DC3000 (virulent on Col-0) and DC3000(*avrRpm1*) (avirulent on Col-0 due to *RPM1*-mediated resistance; Debener et al., 1991). At standard inoculum levels of DC3000(*avrRpm1*), both Col-0 and *tip49as* (lines *11*, *14*, and *18*) leaves displayed a typical HR at approximately 5 hr post infiltration (data not shown). Leaves inoculated with DC3000 alone displayed no HR and began showing disease symptoms at approximately 24 hr post inoculation. There were no observable differences in either disease symptoms or in planta growth of the either bacterial strain between Col-0 and the *tip49as* lines (data not shown). Because *RPM1*-mediated resistance limits bacterial growth over 5 days to <10-fold, it is likely that our results reflect an inherent inability to measure enhancement of the already very strong *RPM1*-mediated response.

AtTip49a Is Essential for *Arabidopsis* Development During Both Female Gametophyte and Sporophyte Development

Plants homozygous for an *AtTIP49a* T-DNA insertion, *tip49*^{T}, located near the 3′ end of the open reading frame (see Supplemental Data) could not be recovered (>250 plants from several *tip49*^{T}/*AtTIP49a* selfings were examined by PCR). We therefore concluded that, for the standard growth conditions tested, *AtTIP49a* is an essential gene in *Arabidopsis*. *AtTIP49a*/*tip49*^{T} plants were allowed to self-pollinate, and the segregation of kanamycin resistance to kanamycin susceptibility (K^S) was scored. The ratio of K^R to K^S plants (497:445, respectively) was close to 1:1 (X^2 = 2.88; p > 0.05). This segregation pattern is indicative of gametophyte lethality (Howden et al., 1998). We therefore made reciprocal crosses between PCR-selected *AtTIP49a*/*tip49*^{T} heterozygotes and wild-type plants. If transmission of the *rin*^{T} allele was unaffected, 50% of the F1 plants were expected to be Kan^R. When *AtTIP49a*/*tip49*^{T} plants were used as the pollen (male gamete) donors, 49.7% (109 K^R:110 K^S) of the resulting F1 plants were Kan^R. Thus, functional *AtTIP49a* is not necessary for pollen transmission. In the reciprocal cross, using *AtTIP49a*/*tip49*^{T} plants as the pollen recipients, 25% (52 K^R:160 K^S) of the F1 plants were Kan^R. Therefore, female gametophyte lethality largely explains the difference between the expected and actual results for the segregation of Kan^R in self-pollinated *AtTIP49a*/*tip49*^{T} plants. Because there is clearly some *tip49*^{T} transmission through female gametophytes, the complete absence of *tip49*^{T} homozygous plants demonstrates a sporophyte requirement for *AtTIP49a* function. Dissection of twenty fully expanded, green siliques revealed that approximately half of the expected embryos never developed and consisted of bare funiculi (data not shown). Collectively, these observations establish a critical requirement for *AtTIP49a* in both female gametophyte development and early seedling development.

Yeast *TIP49a* Deletion Alleles Are Lethal, and *Arabidopsis AtTIP49a* Rescues Yeast Viability

The yeast ortholog of *AtTIP49a* (*YDR190C*) in the yeast strain YEF473 was replaced by homologous recombination with an HIS3 selectable marker. As previously reported (Qiu et al., 1998), null mutants were inviable (data not shown). To date, no biological function beyond its essential nature has been ascribed to *YDR190C* in yeast. We cloned *Arabidopsis AtTIP49a* behind a galactose-inducible promotor in the vector pGAL316 and examined whether this construct could rescue the lethal deletion of *YDR190C*. Diploid yeast cells heterozygous for *YDR190C* replacement were transformed with either pGAL316 alone or pGAL316/*AtTIP49a* and sporulated. Under galactose induction, only pGAL316/*AtTIP49a* rescued the lethal phenotype of the YDR190C deletion (data not shown).

Discussion

R proteins and their cellular partners are presumed to transduce signals, leading to multifaceted defense responses that halt pathogen growth. To understand

these signaling pathways, identification of R protein partners and elucidation of their functions is essential. In addition to the difficulties posed to genetic screens by overlapping and/or essential defense-related functions, proteins that play quantitative roles in either enhancing or suppressing defense responses are difficult to isolate in forward genetic screens.

We demonstrated that *AtTIP49a* is both an essential gene and that it acts genetically as a negative regulator of two *R* functions. Quantifying this suppression required a sensitized genetic background. Our use of a sensitized genetic screen is comparable to similar experiments, assaying very different cellular functions, with the *Drosophila* orthologs of *AtTIP49a/AtTIP49b1/2* (*dTIP49a* and *dTIP49b*). As with *AtTIP49a*, deletions of *dTIP49a* (Pontin) or *dTIP49b* (Reptin) are lethal. This finding limited Bauer et al. (2000) to examining *dTIP49a* and *dTIP49b* deletion heterozygotes, and these lacked obvious phenotypes. Importantly, dTIP49a and dTIP49b interacted with β-catenin (the human ortholog of the *Drosophila* Arm protein). These authors subsequently examined the effects of lowering *dTIP49a* or *TIP49b* dosage in flies with increased and decreased levels of signaling-competent Armadillo (Arm) protein. In these sensitized genetic backgrounds, they elegantly demonstrated that dTIP49a and dTIP49b act antagonistically as positive and negative regulators of Arm signaling, respectively.

In addition to our demonstration of *AtTIP49a*-mediated suppression of resistance in an *RPP5*-sensitized genetic background, we noted a *tip49as* effect on the weak resistance gene *RPP2*. We were unable to identify additional pathology phenotypes in either the *AtTIP49a/ tip49*[T] heterozygotes or *tip49as* lines in otherwise wild-type backgrounds. Several possible explanations for this lack of pathology phenotype are possible. (1) *AtTIP49a* has a minor involvement in resistance that is not measurable given our current quantification techniques. (2) *AtTIP49a* is important in negative regulation of *R* function, but the AtTIP49a protein remaining in heterozygous and antisense plants is sufficient to mediate these functions. (3) *AtTIP49a* is not involved in resistance. The deregulation of PR1 accumulation in *tip49as* plants following pathogen and BTH induction coupled with the interaction between AtTIP49a and two R proteins (RPM1 and RPP5) suggests that the third explanation is not likely and that *AtTIP49a* might play an active role in defense responses. Our data comparing wild-type *rpp5* plants to *RPP5*/− hemizygous plants strongly suggests that *AtTIP49a* acts as a negative regulator of *R* gene-mediated disease resistance. The lack of an RPM1-associated phenotype may be due to the strength of the *RPM1-avrRpm1* interaction. This interaction results in a rapid HR (<5 hr) and very limited pathogen growth (Dangl et al., 1992b), suggesting that it may not be possible to measure a further enhancement in *tip49as* lines. It is important to note that all of the *tip49as* lines still have detectable, although strongly reduced, levels of *AtTIP49a* mRNA. Because of the additional, essential function of *AtTIP49a*, it is possible that its role in resistance is greater than we can measure phenotypically.

Mammalian TIP49a was initially identified in rat nuclear extracts via its association with the TATA binding protein (TBP) complex (Kanemaki et al., 1997). Subsequently, TIP49a and its homolog, TIP49b (Kanemaki et al., 1999), have been implicated in diverse transcription-related functions. Based on their postulated cellular roles, TIP49a/b have variably been called RUVBL1 (no b homolog identified), Pontin52/Reptin52, NMP238 (no b homolog), ECP-54/-51, and TIP49/TIP48 (Bauer et al., 1998; Holzmann et al., 1998; Qiu et al., 1998, Salzer et al., 1999; Wood et al., 2000, respectively). TIP49a and TIP49b express opposing orientation helicase activity in vitro (Kanemaki et al., 1999; Makino et al., 1999). Both proteins interact with mammalian c-*myc* (Wood et al., 2000). Dominant-negative mutations in the Walker B nucleotide binding motif of TIP49a inhibit c-*myc*-mediated oncogenic transformation (Wood et al., 2000). TIP49a and TIP49b also interact with β-catenin and have opposing regulatory effects on transcriptional regulation of β-catenin-sensitive reporter genes (Bauer et al., 2000; Bauer et al., 1998; Wood et al., 1996).

Transcription of genes involved in both the c-*myc* and β-catenin pathways appears to involve the recruitment of factors involving chromatin rearrangement, such as histone deacetylases (reviewed in Elad and Volpin, 1991; Hecht and Kemler, 2000, respectively). This suggests that the helicase activities of TIP49a/b may modulate transcription through modification of chromatin structure. The presence of nuclear localization signals in AtTIP49a and its mammalian orthologs supports a role in transcription, although these proteins have been shown to have both nuclear and cytoplasmic distributions (Hawley et al., 2001; Woods et al., 1988). Immunofluorescence microscopy and protein blots on cytosolic fractions confirm the nonnuclear localization of a small fraction of cellular hTIP49a (Holzmann et al., 1998; Makino et al., 1998; Salzer et al., 1999). Our preliminary localization of *Arabidopsis* AtTIP49a mirrors the mammalian data (M.E. and J.L.D., unpublished data). That AtTIP49a complements the TIP49a deletion in yeast further strengthens the argument that both localization and function are conserved across species. The potential for dual localization is important because RPM1 is associated with the plasma membrane (Boyes et al., 1998), while both RPP5 and RPP2, which have not been localized, are predicted to be cytoplasmic. We cannot exclude the possibility that the AtTIP49a/R protein interaction takes place in a variety of cellular compartments and that AtTIP49a action is subsequently nuclear.

In all species examined to date, *TIP49a* deletions conferred lethal phenotypes (Qiu et al., 1998, Kanemaki, et al., 1999, Bauer, et al., 2000). We carefully examined the effects of significantly lowering levels of TIP49a. We demonstrate that reduction of *AtTIP49a* mRNA levels leads to severe morphological and developmental defects in *Arabidopsis*. These defects include an inability or delay in establishing the meristem, leaf development abnormalities, such as incomplete margins, and female gametophyte lethality. Even though we have shown that *AtTIP49a* partially complements *TIP49a* deletions in yeast, these developmental processes are plant life cycle specific. This suggests that AtTIP49a retained basic functions during evolution but has also been recruited for novel, plant-specific processes. Notably, TIP49a has been shown to bind and activate plasminogen (Hawley

et al., 2001), but this interaction is dependent on a carboxy-terminal lysine residue in TIP49a that does not exist in AtTIP49a. This observation further suggests that functional divergence between animal TIP49a and *Arabidopsis* AtTIP49a is plausible.

In addition to AtTIP49a, two additional proteins, RIN4 and RAP1, have recently been identified as RPM1 interactors in yeast and subsequently shown to have essential functions in plant development (Mackey et al., 2002; M. Torres and M.R.G., unpublished data). It is possible that the developmental functions of AtTIP49a and other RPM1 interactors are completely unrelated to their role in pathology. In this regard, the diversity of putative TIP49 interactors in animal systems, including factors such as plasminogen (Hawley et al., 2001), stomatin (Salzer et al., 1999), c-Myc (Wood et al., 2000), and β-catenin (Bauer et al., 1998), has prompted the suggestion that TIP49a/b "moonlight" as participants in a variety of cellular machines (Hawley et al., 2001). Factors such as cell type, localization, and complex formation allow moonlighting proteins to serve multiple, distinct cellular functions (Jeffery, 1999).

Nevertheless, it also remains possible that the AtTIP49a functions in plant development and pathology are intertwined. The so-called "guard hypothesis" suggests that R proteins protect cellular targets of bacterial virulence factors, and this idea may provide a framework for the AtTIP49a/R protein interaction (Dangl and Jones, 2001; van der Biezen and Jones, 1998). It is possible that the proteins or complexes guarded by R proteins are involved in crucial metabolic functions in the cell rather than being directly or solely involved in plant defense. In this scenario, AtTIP49a is part of an RPM1-, RPP5-, or RPP2-containing protein complex that is targeted by pathogen virulence factors. Reduction of AtTIP49a might destabilize this complex and thus lower the threshold for *R* gene signaling. Our observation of enhanced *PR1* expression in the *tip49as* lines supports this notion. The enhanced RPP2 and RPP5 function we observe might be a consequence of this lowered threshold rather than direct biochemical regulation of *R* gene signaling by AtTIP49a. Lethality resulting from reducing or eliminating R protein interactors might suggest that assembly of these specific complexes is essential for crucial plant functions and/or that lack of complex formation leads to constitutive activation of R protein signaling.

Our current definition of genes mediating *R* function follows from the identification of mutants with strong loss-of-function pathology phenotypes. With the completion of the *Arabidopsis* genome sequence and the accompanying new techniques and tools, such as expression arrays and the ability to screen for point mutations (TILLING; McCallum et al., 2000), we can now look more carefully for weak and intermediate mutant phenotypes. It is reasonable to expect that many proteins associated with a given R protein will contribute in an additive or synergistic manner to the overall resistance response. Some, like AtTIP49a, will also have essential functions. We will need to further develop genetic tools, like sensitized screens, and biochemical tools to unravel disease resistance in *Arabidopsis*.

Experimental Procedures

Plant Cultivation, Agrobacterium Transformation, and Herbicide Resistance

Seeds and plants were grown on a mixture of four parts Promix (Premier Horticulture, Red Hill, PA), two parts sand, and one part vermiculite. Plants were cultivated in growth chambers under a 9 hr light/15 hr dark regime at 24°C daytime, 20°C nighttime, and 60% constant relative humidity. Basta (glufosinate-ammonium) selection was performed as previously described (Altman et al., 1992). The *tip49as* and *AtTIP49a* complementation constructs were transformed into the Agrobacterium GV3101 by electroporation. Agrobacterium containing the appropriate constructs was used for transformation into plants as previously described (Bechtold et al., 1993). The *AtTIP49a* T-DNA insertion line was isolated from a population of approximately 11,000 plants generated by the Thomas Jack lab, available from the *Arabidopsis* Biological Resource Center at Ohio State University. An *AtTIP49a*-specific primer and a T-DNA-specific primer were used to identify the T-DNA insertion by PCR amplification and DNA blotting as described (Campisi et al., 1999).

Yeast Strains and Methods

For the yeast two-hybrid library, Col-0 plants were infiltrated with DC3000(*avrRpm1*), and tissue samples were taken at 0.5 hr intervals from 0 to 3.5 hr post inoculation. Poly(A)$^+$ RNA was isolated using DynaBeads following the manufacturers instructions (Dynal, Oslo, Norway). A cDNA synthesis kit (Stratagene, La Jolla, CA) was used to prepare 5 μg of this RNA (>500 bp) for insertion into the yeast library vector pJG4-5 (Gyurius et al., 1996). A primary library of 6 × 10^6 transformants was generated by electroporation into MC1061 and, following amplification, was purified by CsCl centrifugation. The RPM1 and RPP5 yeast two-hybrid baits were cloned into the pEG202 vector (Gyurius et al., 1996). All RPM1 and RPP5 baits were expressed and localized to the nucleus as determined by repression of the pJK101 *lacZ* reporter in the yeast strain EGY48 (Finley and Brent, 1996). AtTIP49a was initially isolated as an RPM1 interactor using pEG10 as described in the Results section. Subsequent tests examining the AtTIP49a interaction with other RPM1 and RPP5 baits were done by directly cotransforming both bait and prey into competent EGY48 cells prepared by the Frozen-EZ Yeast Transformation II Kit (Zymo Research, Orange, CA). Several unrelated baits (e.g., pCDC42 and pDAD) did not interact with AtTIP49a. All screening, retesting of positive clones, and plasmid isolations from yeast were done as described (Ausubel et al., 1987).

Deletions of the *Saccharomyces cerevisiae* ortholog of *AtTIP49a* (*YDR190C*) were done by homologous recombination as described previously (Baudin et al., 1993). Yeast colonies putatively lacking *YDR190C* were confirmed by PCR analysis. To test for *AtTIP49a* rescue of the *YDR190C* deletion, we created pGAL316 and pGAL316/*AtTIP49a*. PFU polymerase (Stratagene) was used to amplify the full-length ORF of *AtTIP49a* from pBS(KS)/*AtTIP49a* (isolation of full-length *AtTIP49a* is described in DNA Manipulations). The latter construct allowed galactose inducibility of *AtTIP49a* in yeast cells. pGAL316 and pGAL316/AtTIP49a were transformed into chemically competent YEF473 diploid cells heterozygous for the YDR190C deletion and selected on plates lacking uracil (URA3 marker derived from pRS316). Sporulation and subsequent tetrad analysis were performed according to standard protocols.

Pathogen Culture, Inoculations, and Imaging

The *Pp* isolates Ahco2 and Noco2 were propagated weekly on the genetically susceptible ecotype Col-0. Cala2 and Emco5 were propagated on La-*er* and Wassilewskija (Ws-0), respectively (Dangl et al., 1992a). To determine the growth of *Pp* on *tip49as* plants, *Pp* conidiospores were suspended in water (5 × 10^4 spores/ml) and sprayed onto 10-day-old cotyledons. Inoculated plants were kept under a sealed lid to achieve high relative humidity at 19°C under an 8 hr light period (100–160 μE m^{-2} s^{-1}). *Pp* growth was determined 7 days post challenge by both visual observation with a dissecting microscope for sporangiophore counts and trypan blue staining for hyphal growth (Koch and Slusarenko, 1990). Trypan blue-stained leaves were visualized by light microscope (Nikon Eclipse E800, Melville, NY). Images of *Pp* infected leaves were captured with a

digital camera mounted to the microscope (Spot camera and Spot capture software version 2.1.2; Diagnostic Instruments, Sterling Heights, MI).

Determinations of *Pst* growth were performed by dip assay (Tornero and Dangl, 2002) on ~2.5-week-old preflowering plants. HR tests were done as described previously (Dangl et al., 1992b). The strains DC3000(empty vector) and DC3000(*avrRpm1*) were maintained on KB media plates containing kanamycin (30 μg/ml) and rifampicin (100 μ/ml).

DNA/RNA Manipulations

AtTIP49a was subcloned into pBS(KS) (Stratagene, La Jolla, CA) using the EcoRI and XhoI restriction sites, and sequencing revealed that it was missing the first exon (aa 1–28). This DNA was used as a probe on an *Arabidopsis* genomic phage library using standard procedures. A genomic clone containing the 5′ end of AtTIP49a, including approximately 500 bp of 5′ untranslated region and the first exon (90 bp), was used to make a predicted full-length clone without introns. This same 5′ region was amplified by PCR and cloned in reverse orientation behind the constitutive 35S promoter in binary vector p35S-Bar (a modification of pGPTV-BAR plasmid by S. Kjemtrup and J.L.D., unpublished data). A 6 kb EcoRV restriction fragment containing *AtTIP49a* was isolated from the P1 clone MWD9, cloned into the binary vector pBAR1 (pGPTV-BAR containing modified pBS cloning site; B.F.H., D.C.B., and J.L.D., unpublished data), and used to complement *tip49^T^*. The *AtTIP49b1* full-length clone was isolated from our yeast two-hybrid library using the EST clone R65101 as a hybridization probe.

For analysis of RNA, tissue was collected from 10-day-old cotyledons or 3-week-old preflowering plants and ground by mortar and pestle in liquid nitrogen. RNA was extracted with Trizol (GibcoBRL, Grand Island, NY). Approximately 15 μg of total RNA was loaded per lane in denaturing gels. RNA was transferred to Hybond-N membranes (Amersham Pharmacia Biotech, Buckinghamshire, England) and hybridization was performed in ULTRAhyb (Ambion, Austin, TX) at 45°C per manufacturers directions. All probes were labeled with α-ATP using reagents provided in the Prime-It II random primer labeling kit (Stratagene).

Protein Manipulations

AtTIP49a (aa 31–458) was cloned into the vector pGEX4T-1, and GST-AtTIP49a was purified per manufacturers directions (Amersham Pharmacia Biotech, Piscataway, NJ). Myc epitope-tagged RPM1 in both full-length (aa 1–926) and short N-terminal form (aa 1–177) were cloned into pET14b (Novagene, Madison, WI). In vitro RPM1-Myc proteins were produced using the Promega (Madison, WI) TNT Quick Coupled Transcription/Translation System. GST and GST-AtTIP49a proteins coupled to glutathione Sepharose 4B beads were incubated with these RPM1-Myc proteins for ~2 hr in 200 μL TEDM buffer (80 mM Tris-HCl [pH 7.5], 4 mM EDTA, 20 mM MgCl₂, 4 mM DTT, and 1× protease inhibitor cocktail [product number P9599; Sigma, St. Louis, MO]) at 4°C. Following incubation, the Sepharose beads and bound proteins were washed five times at 4°C with 1 ml high-stringency wash buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5 mM EDTA, 0.5% Triton X-100, 0.5 mM DTT, and 1× protease inhibitor cocktail). Each wash was allowed to incubate with constant, gentle mixing for 10 min before pelleting the Sepharose at 12,000 rpm in a 4°C microcentrifuge and repeating. Following the final wash, the Sepharose pellet was resuspended in ~70 μL 3× SDS loading buffer and run on standard 10% SDS-PAGE gels. The Western blot and Coomassie gels were performed by standard methods.

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Erratum

An Evolutionarily Conserved Mediator of Plant Disease Resistance Gene Function Is Required for Normal *Arabidopsis* Development

In this article (Developmental Cell *2*, 807–817, June, 2002), the title to the legend of Figure 4 contains an error. The correct title is given below:

Figure 4. *RPP2* Function Is Enhanced in Several *tip49as* Lines

Ben F. Holt III, Douglas C. Boyes, Mats Ellerström, Nicholas Siefers, Aaron Wiig, Scott Kauffman, Murray R. Grant, and Jeffery L. Dangl