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Over-expression of *TGA5*, which encodes a bZIP transcription factor that interacts with NIM1/NPR1, confers SAR-independent resistance in *Arabidopsis thaliana* to *Peronospora parasitica*

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Summary

The *Arabidopsis thaliana NIM1/NPR1* gene product is required for induction of systemic acquired resistance (SAR) by pathogens, salicylic acid (SA) or synthetic SA analogs. We identified, in a yeast two-hybrid screen, two NIM1/NPR1 interacting proteins, TGA2 and TGA5, which belong to the basic region, leucine zipper (bZIP) family of transcription factors. Both TGA2 and TGA5 strongly interact with NIM1/NPR1 in yeast and *in vitro*, and recognize the *as-1 cis* element found within the promoter of several pathogenesis-related genes, such as *PR-1*. To determine the role TGA2 and TGA5 may play in NIM1/NPR1-mediated disease resistance, we introduced sense and antisense versions of both genes into transgenic Arabidopsis plants. Characterization of *TGA2* transgenic plants revealed that inhibition or overexpression of *TGA2* does not significantly affect *PR-1* expression or induction of SAR after pathogen infection or INA treatment. Surprisingly, all *TGA5*-antisense transgenic plants produced showed increased accumulation of *TGA5* transcripts compared with untransformed control plants, while the *TGA5*-sense lines showed no significant increase in *TGA5* mRNA levels. Interestingly, the high level of *TGA5* mRNA in the antisense lines was accompanied by significant resistance to a highly virulent isolate of the oomycete pathogen *Peronospora parasitica*. Further, resistance was not coupled to accumulation of products from the SAR-linked *PR-1* gene following inoculation with *P. parasitica* or treatment with INA, indicating that these plants express a robust, *PR-1*-independent resistance mechanism. Resistance was retained when a *TGA5*-accumulating line was combined genetically with a *nim1-1* mutation or *nahG* (salicylate hydroxylase) transgene, indicating that resistance in these plants is due to an SA and SAR-independent mechanism.

Keywords: salicylic acid, systemic acquired resistance, transgenic plants, disease resistance, bZIP transcription factor TGA2, TGA5.

Introduction

Systemic acquired resistance (SAR) is one of several critical defense pathways in plants that are induced by pathogen attack. Induction of SAR leads to enhanced and sustained resistance to infection by a wide variety of pathogens (Delaney, 1997; Ryals *et al.*, 1996; Sticher *et al.*, 1997). A key event required for initiation of SAR is the pathogen-induced production and accumulation of salicylic acid (SA) in the plant host. Evidence for this requirement comes from observations that exogenous application of SA to plants induces SAR, and on experiments with transgenic plants that express the bacterial *nahG* gene that encodes salicylate hydroxylase; NahG plants neither accumulate SA nor express SAR (Dempsey *et al.*, 1998; Hunt *et al.*, 1996). To identify regulatory genes that act downstream of SA and control SAR, several groups have conducted genetic screens in *Arabidopsis thaliana* to find mutants unable to respond to the SA signal. These efforts led to identification of at least 12 SA non-responsive mutants, each with mutations in the same gene called *NIM1*, *NPR1* or *SAI1* (Cao *et al.*, 1994; Delaney *et al.*, 1995; Glazebrook *et al.*, 1996; Shah *et al.*, 1997). The failure of these screens to identify other genes required for SA-induction of SAR suggests that the signaling pathway that couples SA accumulation to the onset of resistance is composed of few gene

products, or involves genes that are redundant or required for viability.

The *NIM1/NPR1* gene product has also been reported to play a role in an inducible defense response called induced systemic resistance (ISR), which is activated in Arabidopsis plants after exposure of roots to certain non-pathogenic rhizobacteria (van Loon *et al.*, 1998). Unlike SAR, induction of ISR does not require SA accumulation and is not associated with expression of SAR-associated *PR* genes (Pieterse *et al.*, 1996). However like SAR, ISR induction is dependent upon NIM1/NPR1 function (Pieterse *et al.*, 1998). Thus, SAR and ISR appear to represent distinct defense pathways in Arabidopsis that are likely to differ in their modes of action, yet share a requirement for NIM1/NPR1.

The predicted NIM1/NPR1 protein has a central region that contains multiple ankyrin repeat domains (Cao *et al.*, 1997; Ryals *et al.*, 1997), which in other proteins are known to be involved in protein–protein interactions (Bork, 1993). The ankyrin repeat domains, together with an amino terminal region, show similarity to members of the mammalian IκB family of transcription factor inhibitors, suggesting possible evolutionary homology between these proteins (Ryals *et al.*, 1997). Members of the IκB family interact through ankyrin repeats with the transcriptional activator protein NF-κB, masking its nuclear localization signal and thereby preventing it from entering the nucleus (Baeuerle and Baltimore, 1988; Baeuerle and Baltimore, 1996). Accordingly, many signals that induce NF-κB-regulated genes do so by regulating IκB stability through specific IκB-kinases that phosphorylate the protein, thus triggering its ubiquitination and destruction. The proteolysis of IκB liberates NF-κB, enabling it to enter the nucleus and modulate gene expression (Baldwin, 1996; Mercurio *et al.*, 1997; Woronicz *et al.*, 1997). Like IκB, the function of NIM1/NPR1 may also depend upon its interaction with other proteins through ankyrin repeats, because the *nim1-2*, *npr1-2*, and *npr1-5* mutations disrupt conserved ankyrin repeat residues and also produce a severe phenotype (Cao *et al.*, 1997; Ryals *et al.*, 1997; Shah *et al.*, 1999). However, the plant and animal proteins differ in some respects, because NIM1/NPR1 has been shown to require translocation to the nucleus for its action (Kinkema *et al.*, 2000), and loss-of-function *nim1/npr1* mutations prevent target gene activation, whereas comparable mutations in IκB activate target genes.

To identify proteins that interact with NIM1/NPR1, we and others have carried out yeast two-hybrid screens using NIM1/NPR1 as a bait for screening libraries of Arabidopsis cDNAs (Chien *et al.*, 1991). We identified two basic region, leucine zipper (bZIP) (Landschulz *et al.*, 1988) transcription factors, TGA2 and TGA5, which strongly interact with NIM1/NPR1. The TGA class of bZIP transcription factors were named for their ability to recognize and bind tandem repeats of a *cis*-element within the Cauliflower Mosaic Virus (CaMV) 35S promoter called activation sequence-1 (*as-1*), which contains a TGACG motif (Bouchez *et al.*, 1989; Lam and Chua, 1989; Lam and Lam, 1995), and is also found within the promoter of the SAR marker gene *PR-1* (Lebel *et al.*, 1998). We confirmed in mobility shift assays that both TGA2 and TGA5 bind normal but not mutant *as-1* elements, consistent with a possible role in regulating defense-related genes. Other groups have reported similar results in two-hybrid screens using NIM1/NPR1 as bait. Zhang *et al.* (1999) isolated TGA2 and TGA6 as NIM1/NPR1 interactors, and Zhou *et al.* (2000) assessed the relative strength of the interactions between NIM1/NPR1 and six members of the TGA family. These groups and Després *et al.* (2000) showed variable strength interactions between different TGA family members and the *as-1* element. Therefore, current evidence for a role of TGA factors in NIM1/NPR1-mediated responses consists of observations that the proteins interact in yeast and *in vitro*, and demonstrations that the TGA factors interact with variable affinity with the *as-1* element within the *PR-1* promoter.

To assess whether the factors found in our work and that of Zhang *et al.* (1999) play an important role in defense within plant cells, we constructed transgenic Arabidopsis plants that contain sense or antisense versions of either TGA2 or TGA5 cDNAs. We describe the phenotype of the transgenic lines with respect to their *PR-1* expression after treatment with biotic and chemical inducers, and their susceptibility to *P. parasitica* infection. Our results suggest a possible role for TGA5 in resistance to *P. parasitica*, independent of *PR-1* induction or SAR.

Results

NIM1/NPR1 interacts with TGA2 and TGA5 in a yeast two-hybrid system

To perform yeast two-hybrid screens to identify NIM1/NPR1-interacting proteins, the full length *NIM1/NPR1* gene was fused to the Gal4 DNA-binding domain in the bait plasmid pBI880 (Kohalmi *et al.*, 1995), which was transformed into yeast strain YRG-2 containing the His3 selectable and LacZ screenable markers under control of GAL4-inducible promoters. A prey plasmid library containing Arabidopsis cDNAs cloned into vector pBI771 produces fusion proteins carrying the Gal4 transcriptional activation domain (Kohalmi *et al.*, 1995). The prey library was transformed into the bait plasmid-containing strain to screen for NIM1/NPR1-interacting proteins. Approximately 960 000 yeast transformants were screened for ability to grow on histidine-deficient (His-) media. Two clones that grew on His- media, and also showed strong activation of β-galactosidase were isolated (Figure 1). Follow-up experiments showed that activation of the two reporter genes required the simultaneous presence of both the NIM1/NPR1 bait

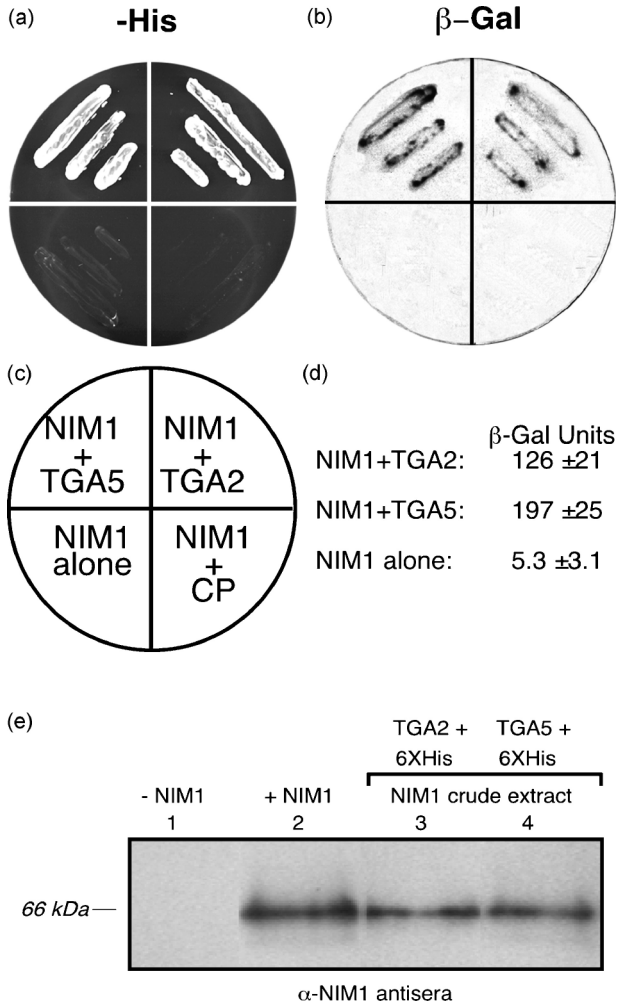


Figure 1. *In vivo* and *in vitro* interaction of NIM1/NPR1 with TGA2 and TGA5. Yeast clones containing both a NIM1/NPR1 bait and TGA2 or TGA5 prey fusion proteins are able to grow on minimal media lacking histidine (a), and show activation of a β-galactosidase reporter in an X-Gal filter assay (b). Clones containing NIM1/NPR1 alone, or expressing the bait plus an arbitrary CP (SV40 viral coat protein) prey do not show activation of His3 or β-galactosidase reporters (a–c). Quantification of the efficiency of interaction was assessed by measuring β-galactosidase activity in yeast cells containing the NIM1/NPR1 bait alone or together with TGA2 or TGA5 prey plasmids (d). NIM1/NPR1 interacts *in vitro* with TGA2 and TGA5 (e). Ni-NTA resin columns containing immobilized histidine-tagged TGA2 or TGA5 proteins were incubated with NIM1/NPR1 protein, extensively washed, and eluted to free column-bound proteins that were then examined by western analysis with an anti-NIM1/NPR1 polyclonal antiserum (see Experimental procedures). No signal is seen in the absence of NIM1/NPR1 protein (lane 1), but is evident in the eluate from Ni-NTA columns containing the his-tagged TGA2 (lane 3) or TGA5 (lane 4) proteins, indicating *in vitro* interaction between these proteins. Lane 2 is a positive control containing NIM1/NPR1 protein added alone to the gel.

plasmid and either of the two isolated prey plasmids (Figure 1). Isolation and sequencing of the cDNA inserts from the two NIM1/NPR1-interacting clones revealed that they encoded partial length members of a subclass of bZIP transcription factors previously identified as AtHBP-1b and OBF-5 (Kawata *et al.*, 1992; Lam and Lam, 1995; Zhang *et al.*, 1993). Because both bind TGA elements within the promoters of target genes, they have also been called TGA2 and TGA5 (Lam and Lam, 1995), respectively, a nomenclature we will use here. Full length TGA2 and TGA5 cDNAs were subsequently isolated by PCR from a cDNA library, cloned into pBI771, and retested in the two-hybrid assay for interaction with the NIM1/NPR1 bait. We found similar strong binding in both the full length and partial TGA factor prey constructs.

Other groups have used yeast two-hybrid screens to identify NIM1/NPR1-interacting proteins or used the method to directly test TGA family transcription factors for interaction. Our results are similar to those of others, although the specific family members implicated differ. We found strong interaction between NIM1/NPR1 and TGA2 and TGA5. NIM1/NPR1 was found by Zhang *et al.* (1999) to interact with TGA2 and TGA6, while Zhou *et al.* (2000) found strong interaction with TGA2 and an N-terminal truncated form of TGA3, and weaker interaction with TGA5 and TGA6. Després *et al.* (2000) confirmed the interaction between TGA2 and NIM1/NPR1 and found an additional TGA factor not previously described.

NIM1/NPR1 interacts with TGA2 and TGA5 in vitro

Interactions observed in yeast two-hybrid assays were confirmed *in vitro* by co-purification assays between NIM1/NPR1 and TGA2 or TGA5. TGA2 and TGA5 genes were fused to a 6xHis tag in vector pET21A + to permit affinity purification of the fusion protein. NIM1/NPR1 protein was produced in insect cells, which were lysed to produce an extract containing the plant protein. His-tagged TGA2 and TGA5 proteins were immobilized on Ni-NTA columns, which were then loaded with the insect cell extract. After multiple washings, the columns were eluted and the eluates fractionated by SDS-PAGE. The gel was blotted and the presence of NIM1/NPR1 protein was detected using a polyclonal antibody raised against the C-terminal region of NIM1/NPR1 (Figure 1e). NIM1/NPR1 protein was retained only on columns containing the bound TGA2 or TGA5, indicating a strong *in vitro* interaction between NIM1/NPR1 and the His-tagged TGA2 or TGA5 proteins.

TGA2 and TGA5 bind to the promoter region of PR-1

Because many pathogenesis-related (PR) proteins, particularly PR-1, are highly induced upon SAR activation (Uknes *et al.*, 1993), and because the minus strand of the *PR-1 -640* to *-645* promoter region contains a perfectly conserved *as-1* element, we tested whether TGA2 or TGA5 may interact with this region of the *PR-1* promoter using electrophoretic mobility shift (EMS) assays. This region of the *PR-1* promoter had been shown to be essential for activation of the promoter by INA (Lebel *et al.*, 1998). EMS assays using a

probe that contains the two tandem *as-1* elements show that TGA2 and TGA5 bind strongly to this element (unpublished data). The interaction between TGA factors and the DNA element is specific, because no binding was observed using a mutant version of the *as-1* element probe that contained four substitutions in residues critical for binding bZIP transcription factors (Lam and Lam, 1995).

The leucine zipper domain of bZIP transcription factors may allow dimerization with other bZIP factors to form hetero or homodimers (Landschulz *et al.*, 1988). To test if heterodimerization occurs between TGA2 and TGA5 in binding to the *PR-1* promoter *as-1* element probe, other EMS assays were conducted using combinations of TGA2 and TGA5. No additional mobility class was seen for the probe, indicating that heterodimerization did not occur between TGA2 and TGA5 (data not shown). To test whether NIM1/NPR1 can modify the interaction between TGA factors and the *as-1* element, additional studies were performed by combining NIM1/NPR1 with either TGA2, TGA5, or both in EMS assays. The presence of NIM1/NPR1 protein did not alter the concentration or migration rate of the probe complex (data not shown), indicating that TGA2 and TGA5 are equally capable by themselves to bind the *as-1* element probe.

Transgenic plants containing sense or antisense TGA2 *and* TGA5 *genes and quantitation of* TGA2 *and* TGA5 *transcript levels*

The *Agrobacterium tumefaciens* floral dip method (Clough and Bent, 1998) was used to transform Arabidopsis ecotype Wassilewskija (Ws-0) plants with constructs containing a dual 35S CaMV promoter driving either sense or antisense *TGA2* or *TGA5* cDNAs. For each of the four constructs, multiple independent transgenic lines were identified, promoted to subsequent generations and homozygous T2 lines and their (T3) progeny selected for further study. Independently transformed T3 homozygous plant lines were examined to determine the levels of *TGA2* and *TGA5* mRNA in these plants, using gene-specific reverse transcriptase (RT)-PCR designed to discriminate between different members of the TGA factor family. The RT-PCR assays amplified at the same time a β-*tubulin* mRNA internal standard. To enhance sensitivity, RT-PCR reaction products were separated by DNA gel electrophoresis and visualized by Southern hybridization using probes corresponding to *TGA2*, *TGA5* or β-*tubulin* genes. Plants containing the *TGA2*-sense construct showed high levels of *TGA2* transcript accumulation, as expected (Figure 2a), whereas lines carrying the *TGA2*-antisense construct showed low levels of *TGA2* transcripts comparable with untransformed wild-type Arabidopsis plants (Figure 2a). Because subsequent experiments would involve inoculations with *P. parasitica* or treatments with INA, we also examined *TGA2* expression

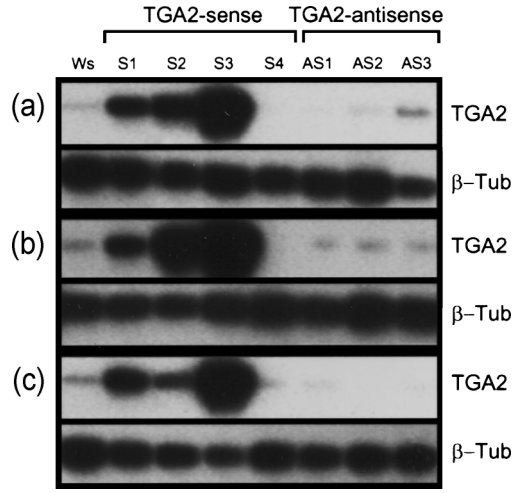


Figure 2. *TGA2* mRNA accumulation in sense or antisense-*TGA2* transgenic plants.
To assess *TGA2* mRNA levels in transgenic plants, RT-PCR was performed using gene-specific primers in conjunction with amplification of β-*tubulin* as an internal standard. PCR products were separated on an agarose gel, Southern blotted, and hybridized to radioactive probes corresponding to *TGA2* and β-*tubulin* . The autoradiogram shown shows similar amounts of β-*tubulin* product, which allows assessment of relative *TGA2* mRNA levels in nontransformed (Ws-0) plants and in the *TGA2*-sense and antisense plants, as indicated. To determine whether induction of resistance affects *TGA2* mRNA levels, samples were collected from naïve untreated plants (a), and plants 3 days after exposure to a virulent *P. parasitica* isolate (b), or INA (c).

in the transgenic lines after these treatments (Figures 2b,c), and saw no significant changes in *TGA2* mRNA abundance.

Unlike the *TGA2* transgenic plants, none of the *TGA5*-sense lines showed an apparent increase in *TGA5* transcript levels compared to wild-type plants (Figure 3a), whereas the *TGA5*-antisense lines showed a surprising and large increase in *TGA5* transcript accumulation, particularly in lines AS15 and AS16 (Figure 3b). In repeated RT-PCR assays, the level of TGA5 transcripts were consistently 10-fold greater than in non-transformed wild-type plants. A fragment containing the CaMV 35S promoter-*TGA5* gene fragment was amplified by PCR from all antisense *TGA5* lines and sequenced to confirm the antisense orientation of the *TGA5* cDNA transgene (data not shown). Additional RT-PCRs were performed on first strand cDNA using primers specific for sense versus antisense TGA5 RNA to confirm that *TGA5* mRNA levels were elevated in the transgenic *TGA5*-antisense lines. No significant *TGA5* antisense RNA accumulation was detected in the antisense lines, showing that the RT-PCR detected *TGA5* mRNA (data not shown).

PR-1 *expression levels of in the* TGA2 *and* TGA5 *transgenic lines*

To determine if *TGA2* and *TGA5* sense or antisense lines were altered in their defense-gene expression phenotype, we used RNA gel blot analysis to examine *PR-1* gene

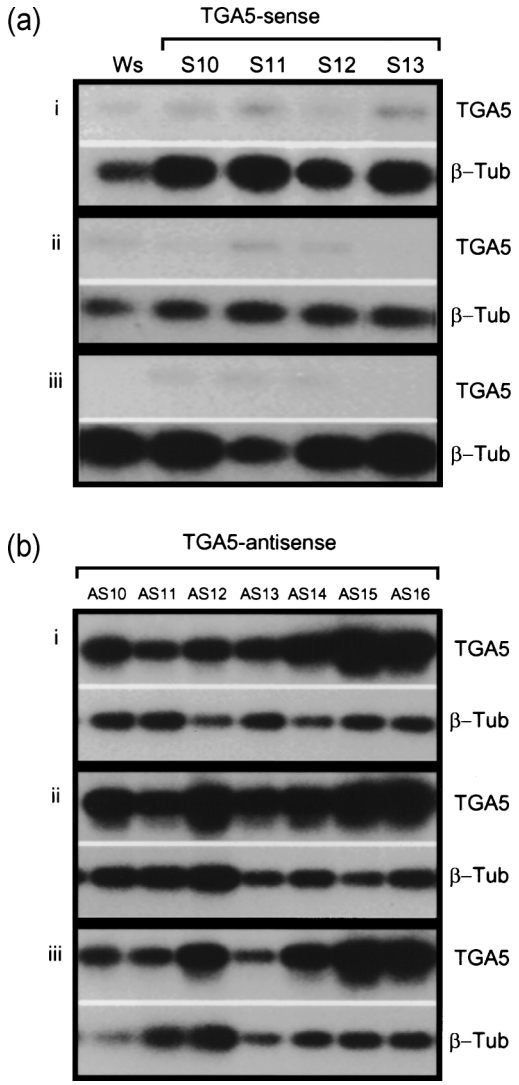


Figure 3. *TGA5* mRNA accumulation in sense or antisense-*TGA5* transgenic plants.
TGA5 mRNA levels were examined in transgenic plants using RT-PCR as described in Figure 2 with gene-specific TGA5 primers. The Southern blotted gel was hybridized with probes corresponding to *TGA5* and β-*tubulin*. Similar amounts of β-tubulin product were amplified, allowing assessment of the relative amounts of TGA5 mRNA in untransformed (Ws) plants and in the *TGA5*-sense (a) and antisense (b) plants, as indicated. The data in panels a and b were collected together, so the Ws control is appropriate for both. To determine whether induction of resistance affects *TGA5* mRNA levels, samples were collected from naïve untreated plants (i), and plants 3 days after exposure to a virulent *P. parasitica* isolate (ii) or treatment with INA (iii).

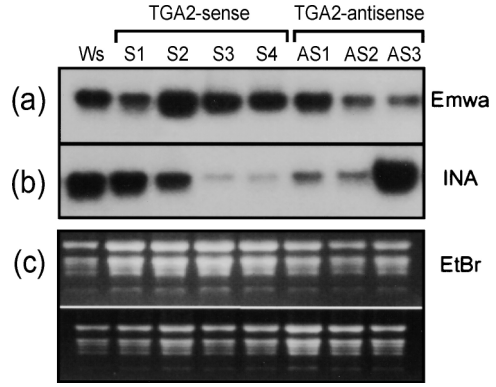


Figure 4. *PR-1* mRNA accumulation in *TGA2*-sense and antisense transformed plants after pathogen exposure or INA treatment.
RNA was collected from wild type (Ws), *TGA2*-sense (S1-S4) and antisense (AS1-AS3) plants 3 days after exposure to the virulent *P. parasitica* isolate Emwa1 (a) or treatment with INA (b). RNA was separated on denaturing gels in the presence of ethidium bromide (EtBr), photographed to assess equal loading (c, corresponds to samples in a and b), and examined by RNA gel blot analysis. A radioactive *PR-1* cDNA probe was hybridized to the membrane, which was washed and examined by autoradiography.

expression responses after infection with the virulent *P. parasitica* isolate Emwa1 or following treatment with INA. We had already established that none of the transgenic lines showed elevated *PR-1* accumulation in the absence of induction (data not shown). Leaf tissues were collected 3 days after inoculation (dai) with a *P. parasitica* conidial suspension, and examined for *PR-1* mRNA accumulation. *TGA2* sense and antisense lines showed approximately normal *PR-1* induction similar to wild type after *P. parasitica* infection (Figure 4a).

Two *TGA2*-antisense lines, AS2 and AS3, showed slightly reduced *PR-1* levels. Most *TGA5*-sense lines also were similar to wild type in *PR-1* expression 3 dai, except line S11, which exhibited reduced *PR-1* RNA accumulation (Figure 5a). Of the seven *TGA5*-antisense lines tested, the

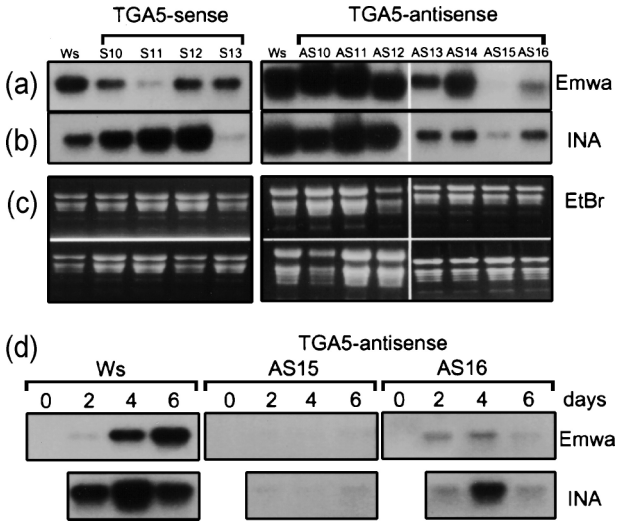
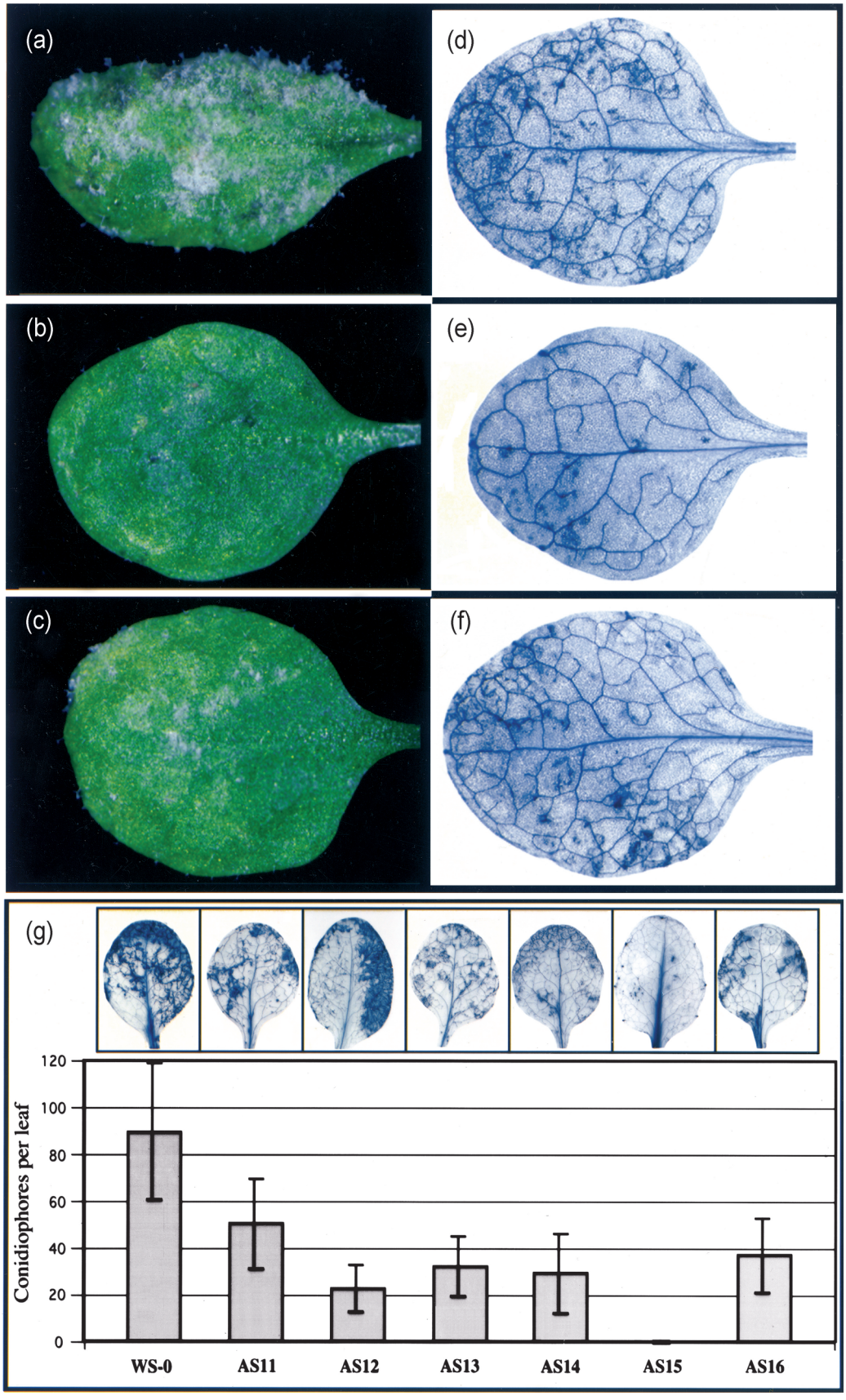


Figure 5. *PR-1* mRNA accumulation in *TGA5*-sense and antisense transformed plants after pathogen exposure or INA treatment.
RNA was collected from wild-type (Ws), *TGA5*-sense and antisense plants as indicated 3 days after exposure to a virulent *P. parasitica* isolate (a) or treatment with INA (b). RNA gel blot analysis and hybridization to a *PR-1* probe was as described in Figure 4. Autoradiograms are shown above pictures of the corresponding ethidium bromide stained gels (c). The kinetics of *PR-1* RNA accumulation were examined (d, autoradiograms) 0, 2, 4 and 6 days after Emwa1 or INA treatment, as indicated, in untransformed wild-type plants (Ws), and in *TGA5*-antisense lines AS15 and AS16.


(a)
(d)
(b)
(e)
(c)
(f)
(g)
Conidiophores per leaf
120
100
80
60
40
20
0
WS-0
AS11
AS12
AS13
AS14
AS15
AS16

two that accumulated especially high levels of *TGA5* mRNA (AS15 and AS16; Figure 3b) also showed reduced induction of *PR-1* by *P. parasitica* (Figure 5a). To examine *PR-1* induction in greater detail in AS15 and AS16 plants, tissues were collected from leaves 2, 4, and 6 dai, and subjected to RNA blot analysis; both lines showed substantial suppression of *PR-1* accumulation (Figure 5d).

To test responses to INA, transgenic plants were sprayed with the SA analog, and examined 3 days later for *PR-1* RNA accumulation. *TGA2*-sense lines S3, S4 and *TGA2*-antisense lines AS1 and AS2 showed greatly reduced *PR-1* accumulation compared with wild type (Figure 4b), indicating a reduction in INA sensitivity, although these same lines had an approximately normal response to *P. parasitica* elicitation (Figure 4a). Most *TGA5*-sense lines showed normal *PR-1* induction by INA, although line S13 failed to show a response (Figure 5b). *TGA5*-antisense lines AS15 and AS16 showed greatly reduced *PR-1* induction after treatment with INA (Figure 5b,d), similar to its lack of responsiveness to the pathogen elicitor.

Resistance of TGA5-*antisense lines to* Peronospora parasitica

To assess whether *TGA2* and *TGA5* sense and antisense lines had an altered defense phenotype, we inoculated homozygous T3 plants with the virulent *P. parasitica* isolate Emwa1. No difference from wild type was observed in susceptibility to Emwa1 for any of the *TGA2* transgenic lines or the *TGA5*-sense lines, with all showing abundant sporulation 1 week after inoculation (not shown). However, the *TGA5*-antisense lines showed significant resistance to infection by *P. parasitica*, as indicated by reduced hyphal growth and sporulation. Resistance was most pronounced in the AS15 and AS16 lines (Figure 6), which also accumulated the largest amounts of *TGA5* mRNA and showed the strongest inhibition of *PR-1* induction by *P. parasitica* or INA. AS12, AS13 and AS14 lines also showed significant resistance to *P. parasitica* (Figure 6g), while accumulating both *TGA5* and *PR-1* mRNAs (Figures 3 and 5), suggesting that *TGA5* accumulation is not linked to *PR-1* suppression in these lines. All lines tested showed reduced hyphal development and conidiophore production compared with wild-type controls (Figure 6g), indicating that the resistance phenotype is a general property of these lines. Resistance in AS15 and AS16 was accompanied by induction of small areas of plant cell death, similar to that observed in Arabidopsis plants expressing *R*-gene mediated or INA-induced resistance against *P. parasitica* (unpublished results).

Because resistance to *P. parasitica* in the *TGA5*-antisense lines was not correlated with induction of *PR-1*, we tested whether or not resistance in the most resistant AS15 line was independent of SAR by crossing this line to *nim1-1* mutants and to *NahG* transgenic plants. F_3 progeny homozygous for the AS15 antisense construct and the *nim1-1* mutation or the *nahG* transgene were then tested for resistance to *P. parasitica* isolate Emwa1. Seven days after inoculation, leaves from the *nim1-1* and *NahG* parental plants showed massive hyphal and conidiophore development, while AS15 *nim1-1* and AS15 *NahG* plants showed little or no signs of pathogen structures, similar to the parental AS15 line (Figure 7). These results show that resistance in AS15 was not due to activation of SAR. Similar to the *AS15* line, resistance to *P. parasitica* in the AS15 *nim1-1* and the AS15 *NahG* plants was associated with necrotic lesions that were, however, more pronounced than in the *AS15* line.

Figure 6. *TGA5*-antisense lines are resistant to infection by *P. parasitica*. Living leaves (a–c) and lactophenol trypan blue (LPTB) stained leaves (d–f) from untransformed wild type plants (a, d), *TGA5* mRNA over-accumulating plants AS15 (b, e), and AS16 (c, f) after inoculation with *P. parasitica* isolate Emwa1. Photographs of living and LPTB-stained leaves were taken 7 days following infection with *P. parasitica* Emwa1. All *TGA5*-antisense lines were examined and found to display resistance to Emwa1 (g). Each transgenic line is shown 3 days after Emwa1 infection, and the mean number of conidiophores produced per leaf at day 7 plotted with standard deviation. The bar chart value for the AS15 line is near zero, as the vast majority of leaves examined showed no conidiophores.

Discussion

In this study, we examined the phenotype of transgenic plants that accumulate mRNA corresponding to either TGA2 or TGA5, members of the TGA subclass of bZIP transcription factors that have been found to interact with NIM1/NPR1. This, and a number of other studies have shown interaction in yeast and *in vitro* between TGA factors and NIM1/NPR1, and have shown the binding of the factors to *cis* elements within the promoter of the SAR-associated gene *PR-1* (Després *et al.*, 2000; Zhang *et al.*, 1999; Zhou *et al.*, 2000; this work). These groups found strong interaction between NIM1/NPR1 and TGA2, and also observed TGA3, TGA5, TGA6 and TGA7 to interact with differing affinities to NIM1/NPR1, although not all studies agree as to which factors bind most strongly.

bZIP transcription factors play many roles in plants, including those unrelated to defense gene regulation, such as control of hypocotyl elongation and floral development (Cheong *et al.*, 1998; Chuang *et al.*, 1999). bZIP factors may form homodimers, or may heterodimerize with other bZIP proteins or to other classes of transcription factors to positively or negatively regulate diverse types of genes (Singh, 1998). TGA factors bind *as-1*-like *cis* elements found in the promoters of a number of genes including Arabidopsis *PR-1* (Lebel *et al.*, 1998), and salicylic acid can enhance the transcriptional activity of *as-1* (Jupin and Chua, 1996; Xiang *et al.*, 1996), suggesting a possible role for TGA factors in SAR regulation. The finding that NIM1/NPR1 interacts with TGA factors thus led to tests that confirmed the interaction of these factors with *as-1* (Després *et al.*,

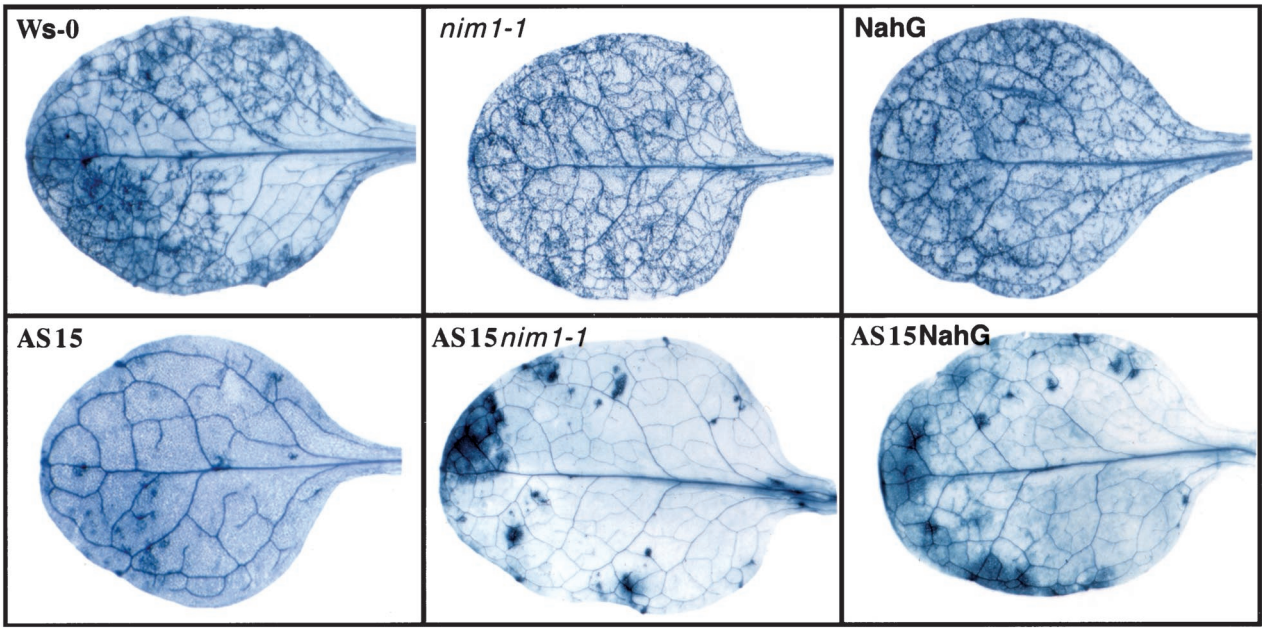


Figure 7. *P. parasitica* resistance in the *TGA5*-antisense line AS15 is independent of NIM1 and is expressed in NahG plants.
Plant genotypes are indicated in each panel. Leaves were stained with lactophenol trypan blue 7 days after inoculation with *P. parasitica* isolate Emwa1. Top row shows the susceptible wild type (Ws-0), *nim1-1* mutant and NahG transgenic line (all derived from Ws-0 accession). Bottom row shows that resistance to *P. parasitica* in the TGA5-antisense line AS15 is expressed in a *nim1-1* background, and when combined with the salicylate hydroxylase (*nahG*) transgene.

2000; Zhang *et al.*, 1999; Zhou *et al.*, 2000; this work), previously described by others (Lam and Lam, 1995; references therein). Using EMS assays, the later work also showed that TGA2 and TGA3 bind specifically to SA-responsive positive and negative regulatory elements within the *PR-1* promoter (Després *et al.*, 2000; Lebel *et al.*, 1998; Zhou *et al.*, 2000), and that the binding of TGA2 to the positive element *LS7* is enhanced by presence of NIM1/NPR1 protein (Després *et al.*, 2000).

The interaction of TGA factors with NIM1/NPR1 and their ability to bind *as-1*-like elements within SAR-associated gene promoters has suggested that certain TGA factors may be involved with NIM1/NPR1 in regulating induced resistance. However, conflicting conclusions have been reached in different laboratories with respect to which TGA factors bind most efficiently to NIM1/NPR1. Further, although TGA2 was found to bind the *LS7* positive regulatory region defined by Lebel *et al.* (1998), it also was observed to bind the negative regulatory region *LS5* in the *PR-1* promoter (Després *et al.*, 2000). Finally, although extracts from SA-treated Arabidopsis plants displayed enhanced *as-1* binding activity, extracts from *nim1/npr1* plants showed enhanced binding activity whether or not the plants had been treated with SA (Després *et al.*, 2000). Because SA treatment induces *PR-1* in wild-type but not *nim1/npr1* plants (Després *et al.*, 2000), these observations indicate that *as-1* binding activity does not correlate with expression of the SAR marker gene *PR-1*.

Because of the limitations to inferring biological function from yeast two-hybrid data or EMS assays, we believed that to assess whether TGA factors play a role in regulating induced resistance would require testing plants in which the levels of these factors were manipulated relative to wild-type plants. Therefore, we created transgenic Arabidopsis plants that contained sense or antisense oriented *TGA2* and *TGA5* genes. These plants were examined to assess *TGA2* and *TGA5* expression levels, and phenotypes with respect to *PR-1* inducibility and resistance to *P. parasitica*. We selected these TGA factors because all groups had found TGA2 to be a strong interactor with NIM1/NPR1, and TGA5 was also detected in our screen as a strong interactor.

Most *TGA2*-sense transgenic plant lines showed over 10-fold more *TGA2* mRNA than the low amount observed in untransformed plants. *TGA2*-antisense lines showed little *TGA2* mRNA accumulation, although because untransformed plants also accumulate little transcript, we were not able to determine using RT-PCR whether the antisense plants were suppressed for *TGA2* mRNA accumulation. We obtained surprising results with the *TGA5*-antisense plants, with the seven lines tested all showing at least 10-fold more *TGA5* mRNA than in untransformed plants, while plants containing the *TGA5*-sense construct showed no detectable increase in *TGA5* mRNA compared with controls. One explanation for the increased level of *TGA5* transcripts in the antisense lines could be non-target effects of the

antisense approach. Because *TGA5* has extensive regions of similarity to *TGA2* and *TGA6*, as well as homology to all of the estimated seven members of its gene family, it is possible that the *TGA5*-antisense plants may suppress expression of another TGA factor. If such a factor acted to negatively regulate *TGA5*, then suppression of that factor could lead to the accumulation of *TGA5* mRNA as we observed. To examine this possibility, we conducted RT-PCR assays using gene-specific primers to measure expression of several other members of the TGA gene family in the *TGA5*-antisense lines. We were unable to detect significant levels of *TGA1*, *TGA3* or *TGA4* in untransformed control plants so it was not possible to assess whether the *TGA5*-antisense plants suppressed expression of those factors (data not shown). Alternatively, increased *TGA5* accumulation in the antisense lines could be explained by negative autoregulation of the *TGA5* gene, such as has been observed for the IE2 bZIP transcription factor from human cytomegalovirus, which acts as both a general activator as well as specific inhibitor of gene expression (Lang and Stamminger, 1993). Another report described a similar finding, in which antisense plants accumulated more of the targeted mRNA, in Arabidopsis plants containing an antisense version of the *WAK4* gene (Lally *et al.*, 2001).

We then examined the *TGA2* and *TGA5* transgenic lines to determine whether they had any phenotype consistent with a role of these factors in induced resistance. We first measured induction of the SAR marker gene *PR-1* in plants 4 days after exposure to a virulent *P. parasitica* isolate or after application of INA, treatments that strongly induce *PR-1* expression in Arabidopsis. We obtained perplexing results, with some of the lines showing normal induction of *PR-1*, while others showed substantial suppression of *PR-1* inducibility after pathogen challenge or INA elicitation. Furthermore, suppression of *PR-1* induction was not correlated with expression levels of *TGA2* (e.g. lines S3 and S4), although reduced *PR-1* accumulation did correlate with the highest expressing *TGA5* lines AS15 and AS16, suggesting that high levels of *TGA5* may negatively affect *PR-1* induction. The reduced *PR-1* inducibility in the highest *TGA2* accumulator (line S3), may be a consequence of an increased *TGA2* level leading to excessive binding of the factor to the LS5 negative regulatory element in the *PR-1* promoter (Després *et al.*, 2000; Lebel *et al.*, 1998). Curiously, the lines that showed reduced *PR-1* induction by INA exhibited normal induction by pathogen, suggesting that the INA response may involve TGA2, while induction by the parasite does not.

The variable *PR-1* induction phenotype observed in *TGA2* and *TGA5* transgenic plants may reflect the complex roles played by TGA factors in regulating SAR-associated genes like *PR-1*, and other response pathways. bZIP transcription factors are versatile regulators of gene expression, in part due to their ability to dimerize with a range of similar or different transcription factors, and a connection with *PR-1* regulation is indicated by the interaction of these factors with the promoter of this SAR gene, as described above. In recent tests on transgenic tobacco plants that expressed a dominant negative *TGA2* mutant, Pontier *et al.* (2001) found enhanced expression of *PR-1* and SAR in plants with suppressed TGA binding activity, suggesting a negative role for TGA factors in SAR. These plants also showed reduced expression of other genes containing *as-1* element promoters, suggesting also positive roles of TGA factors in the expression of those genes. Thus the mechanisms by which members of the TGA family effect multiple responses in gene expression after pathogen exposure are likely to be complex, and may be sensitive to perturbation by manipulation of the levels of TGA factors, such as described in the experiments here.

To assess whether variable levels of *TGA2* or *TGA5* mRNA accumulation influenced pathogen resistance, we inoculated plants with the virulent *P. parasitica* isolate Emwa1. Transgenic plants expressing sense or antisense *TGA2* or sense *TGA5* genes exhibited normal susceptibility to *P. parasitica* infection. By contrast, all *TGA5* over-accumulating lines showed significant resistance to the oomycete, with little sporulation and reduced hyphae evident within the leaf tissue. This was most dramatic in the AS15 and AS16 lines, which also accumulated the most *TGA5* mRNA and showed the greatest suppression of *PR-1* induction.

Together, these data indicate that while *TGA5* and *TGA2* play some role in the negative regulation of *PR-1* expression, *TGA5* may positively regulate resistance to *P. parasitica*. The resistance observed in the *TGA5*-accumulating AS15 and AS16 lines may be distinct from that expressed during SAR, because these plants show suppressed accumulation of the SAR-linked *PR-1* gene product. Examination of other defense genes in AS15 and AS16 plants 3 days after infection showed that they also do not accumulate *PR-2* or *PR-5*, or *PDF1.2*, markers of SA and JA pathways, respectively (unpublished data). Because induction of SAR requires SA accumulation and NIM1/NPR1 activity, we tested whether resistance in the most resistant AS15 line was expressed in a NahG or *nim1-1* background. Resistance was still fully expressed in AS15 NahG and AS15 *nim1-1* plants, leading us to conclude that these plants express a novel, SAR-independent form of disease resistance. This is reminiscent of induced systemic resistance (ISR), another SAR-independent form of resistance, which is induced by root-colonizing bacteria (Pieterse *et al.*, 1996). However, ISR is dependent upon NIM1/NPR1 activity and is only weakly effective against *P. parasitica* (Pieterse *et al.*, 1998; C. Pieterse, pers. comm.), indicating that AS15 plants do not express ISR.

These studies began with the identification of bZIP factors that interact with the SAR regulatory protein NIM1/

NPR1. Because accumulation of *TGA5* mRNA in AS15 and AS16 plants is not associated with *PR-1* accumulation, yet is linked to SA and NIM1/NPR1-independent resistance to *P. parasitica*, we suggest that these plants express an SAR and ISR-independent mode of resistance. The interaction between NIM1/NPR1 and different TGA factors could help explain how this protein may modulate expression of a number of distinct defense pathways, including SAR (Cao *et al.*, 1994; Delaney *et al.*, 1995), ISR (Pieterse *et al.*, 1998), and the novel form of resistance expressed in *TGA5*-accumulating AS15 and AS16 plants. The diversity of responses mediated by NIM1/NPR1 in conjunction with bZIP and possibly other proteins may be assisted by bridging proteins, such as the NIMIN proteins described that interact with NIM1/NPR1 in yeast two-hybrid screens, and simultaneously bind NIM1/NPR1 and TGA factors (Weigel *et al.*, 2001). bZIP transcription factors are versatile regulators of gene expression due to their potential for combinatorial control, which may be modified by post-translational modifications of the factors that change their protein and DNA-binding properties, as well as stability (Katagiri *et al.*, 1992; Stein *et al.*, 1993; Wolberger, 1998). Together, these properties allow bZIP proteins to regulate genes that play diverse functions, ranging from control of development to expression of multiple forms of disease resistance.

Additional studies are required to determine the associations between TGA5 and other transcription factors or cofactors that positively regulate the SAR-independent defense pathway described for *TGA5*-accumulating plants. The targeted disruption of these factors followed by pathogen susceptibility assays will validate their significance. Further experiments using DNA microarray analysis will profile the gene expression characteristics of AS15 plants. Such studies will help assess the novelty of the SAR-independent resistance phenotype in these plants, and will point to potential effector genes responsible for preventing growth of *P. parasitica*.

Experimental procedures

Yeast strain and plasmids

Yeast two-hybrid screens were conducted using the *Saccharomyces cereviseae* strain YRG-2 (Stratagene, La Jolla, CA, USA), which carries auxotrophic markers for leucine, tryptophan and histidine. This strain also contains the *His3* and *lacZ* genes under control of Gal4 transcription factor responsive promoters. An Arabidopsis cDNA prey library and two-hybrid plasmids pBI880 and pBI771 (Kohalmi *et al.*, 1995) were provided by Dr W. Crosby (Plant Biotechnology Institute, NRC, Canada). The prey plasmid library contains Arabidopsis cDNAs cloned into vector pBI771, which produces fusion proteins between the cDNA product and Gal4 transcription activation domain. The full length *NIM1/NPR1* gene was cloned by recombinant PCR of its four exons (White, 1993), and introduced into the bait vector pBI880, which creates a fusion protein between the gene of interest, the Gal4 DNA binding domain, and an N-terminal FLAG-epitope (Kohalmi *et al.*, 1995). The entire *NIM1/NPR1* gene and flanking sequences from the recombinant pBI880 were confirmed by sequence analysis. After transformation of YRG-2 with the recombinant pBI880, expression of the fusion protein was confirmed by western analysis using an anti-FLAG monoclonal antibody (Eastman Kodak Co., Rochester, NY, USA). Two-hybrid screens were performed by PEG-mediated transformation of the bait strain with the prey library, and filter assays to detect β-galactosidase activity in yeast colonies performed as described (Kohalmi *et al.*, 1995).

Quantification of β-galactosidase activity

To quantify β-galactosidase activity in yeast strains carrying the NIM1/NPR1 bait plasmid alone, or in combination with either *TGA2* and *TGA5* prey plasmids, 5 ml cultures were grown to OD600 = 1.0, cells were permeabilized, resuspended in Z buffer (Kohalmi *et al.*, 1995), and β-galactosidase units calculated using an o-Nitrophenyl β-D-galactopyranoside substrate as described by Ausubel *et al.* (1997).

Production of NIM1/NPR1, TGA2, and TGA5 protein

NIM1/NPR1 was expressed in *Spodoptera frugiperda* Sf9 cells provided by Dr G. Blissard (Boyce Thompson Institute, Ithaca, NY, USA) using the Bac-to-Bac system (Gibco-BRL, Carlsbad, CA, USA). A full-length *NIM1/NPR1* gene was cloned behind the polyhedrin promoter in the baculovirus donor vector, pFASTBAC1, and transformed into *E. coli* strain, DH10BAC, carrying the bacmid bMON14272 and the helper plasmid pMON7124. *E. coli* transformants were selected on plates containing gentamicin, kanamycin, tetracycline, IPTG (isopropylthio-β-D-galactoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), according to the manufacturer's recommendations, and recombinant clones containing bacmids harboring the *NIM1/NPR1* gene identified by their white color. Recombinant bacmid DNA was prepared from the positive transformants and used to transfect Sf9 insect cells to produce recombinant baculovirus particles expressing NIM1/NPR1 protein. These initially tranfected cells were tested for the expression of NIM1/NPR1 protein by loading a crude extract of the transfected insect cells onto an SDS-PAGE gel and immunoblot analysis using an anti-NIM1 antibody. Upon confirmation of the expression of the full length NIM1/NPR1 protein from the recombinant baculovirus particles, fresh insect cells were transfected with the virus stock to produce additional NIM1/NPR protein for further analysis. TGA2 and TGA5 proteins were made in *E. coli* host strain BL21(DE3) by cloning full length *TGA2* and *TGA5* genes into the pET21a + expression vector (Novagen, Madison, WI, USA). Cloning into pET21a + created translational fusions between *TGA2* or *TGA5* to a T7 epitope and 6xHis affinity tag to their amino and carboxyl terminal ends, respectively. BL21(DE3) carrying either pET21 + *TGA2* or pET21 + *TGA5* was grown overnight in LB medium containing 100 μg ml^{-1} ampicillin. Fresh LB-Amp was inoculated with 1/100 volume of the overnight culture and allowed to grow to an OD600 = 0.6–1.0, and then induced for 3 hours with 1 mM IPTG (Isopropyl-Thio-β-D-Galactopyranoside). Cells were collected by centrifugation and resuspended in sonication buffer (50 mM Na-phosphate pH = 7.8, 300 mM NaCl). After sonication (2 × 15 sec, 300 watts) to lyse the cells, *TGA2* and *TGA5* fusion proteins were purified under native conditions using a Ni-NTA resin following the manufacturer's protocol (Qiagen, Valencia, CA, USA).

In vitro *binding assays*

His-tagged TGA2 or TGA5 protein was immobilized on a Ni-NTA resin column, and incubated with a crude extract of insect cells expressing NIM1/NPR1 protein for 60 min at 4°C. The resin was washed three times with wash buffer (50 mM Na-phosphate, 300 mM NaCl, 10% glycerol, pH = 6.0). TGA2 or TGA5 was then eluted with wash buffer containing 250 mM imidazole. A sample of the eluate was analyzed by SDS-PAGE and transferred to a PVDF membrane (Immobile-P; Millipore, Bedford, MA, USA). For western analysis, blotted proteins were incubated with a primary polyclonal antibody raised in a rabbit injected with the carboxyl-terminal region of NIM1/NPR1 containing amino acids 502–582 (G. Rairdan and T. Delaney, unpublished results; Cornell Center For Research Animal Resources), which was detected using the Western-Star immunodetection system (Tropix, Foster City, CA, USA).

Transgenic plants: Cloning into binary vector pKYLX71 and Agrobacterium tumefaciens-mediated transformation of Arabidopsis plants

Full-length *TGA2* and *TGA5* cDNAs were cloned into the pKYLX71 binary vector with a dual 35S promoter and enhancer (provided by Dr A. Hunt, University of Kentucky, USA). For cloning *TGA2* and *TGA5* sense strands into pKYLX71, PCR primers were used that introduced 5′ XhoI and 3′ XbaI sites into the amplimer. PCR products were digested with XhoI and XbaI and ligated into the pKYLX71 vector previously digested with these enzymes. For cloning *TGA2* and *TGA5* antisense strands into pKYLX71, a different set of primers was used that introduced 5′ XbaI and 3′ XhoI sites into the amplimer, which was then digested and ligated into the vector as described for the sense constructions. *E. coli* cells were transformed with the *TGA2* or *TGA5* sense or antisense constructs, and sequences were obtained to verify the orientation and integrity of the *TGA2* and *TGA5* inserts. Arabidopsis plants were transformed by dipping them into suspensions of *Agrobacterium tumefaciens* carrying pKYLX with either sense or antisense oriented *TGA2* or *TGA5* (Clough and Bent, 1998). Transgenic (T1) seedlings were identified by expression of the neomycin phosphotransferase gene contained on the transformation vector pKYLX71, which confers resistance to kanamycin. Because *TGA5*-antisense plants displayed an unexpected accumulation of *TGA5* mRNA, we verified that these lines contained the expected transgene by PCR amplification, and sequencing of the product.

Quantitative RT-PCR

To assess *TGA2* and *TGA5* expression levels in the sense and antisense *TGA2* and *TGA5* transformed lines, quantitative RT-PCR was performed. Total RNA from *TGA2* and *TGA5* sense and antisense lines were isolated according to Lagrimini *et al.* (1987). PolyA + RNA for cDNA synthesis was recovered using oligo-dT conjugated paramagnetic beads (Promega, Madison, WI, USA) and a magnet. After first strand cDNA synthesis primed using oligo-dT (Lambert and Williamson, 1997), PCR was performed using *TGA2* or *TGA5* gene-specific primers designed to cross a splice junction to prevent amplification of contaminating genomic DNA. PCR primers also included those to amplify a β-tubulin cDNA as an internal control and normalization standard. Samples were amplified by 17 cycles of PCR (95°, 30 sec; 50°, 60 sec; 72°, 60 sec), which allowed observing differences between the *TGA2* or *TGA5* accumulating lines, without reaching an amplification plateau. PCR products were electrophoresed on a 1.0% TBE gel and analyzed by Southern analysis using radioactive probes corresponding to β-tubulin and *TGA2* or *TGA5*.

Treatment of Arabidopsis plants with INA

Two-week-old wild-type and transgenic plants were sprayed to imminent runoff with a 0.25 mg ml^{-1} solution of 25% 2,6-dichloroisonicotinic acid (in a wettable powder; Syngenta, Research Triangle Park, NC, USA) in sterile water using a Preval spray mister (Preval Sprayer Division, Precision Valve Corp. Yonkers, NY, USA). Leaf tissue was collected from plants for RNA gel blot analysis 0, 2, 4 and 6 days after treatment.

RNA gel blot analysis

RNA was extracted from leaf tissue frozen in liquid nitrogen using a hot phenol/chloroform method followed by lithium chloride precipitation (Verwoerd *et al.*, 1989). Electrophoresis was performed with approximately 2.0 μg total RNA containing ethidium bromide (0.05 mg ml^{-1}) in formaldehyde agarose gels as described by Uknes *et al.* (1992). Gels were photographed under UV light to visualize the samples and to assess equal loading and blotted onto Hybond N + nylon membranes (Amersham Life Science, Arlington Heights, IL, USA). The Arabidopsis *PR-1* cDNA probe (Uknes *et al.*, 1992) was labeled with 32P using the random priming method and a commercial kit (Gibco Life Technologies, Carlsbad, CA, USA). Hybridization conditions and subsequent washings were carried out following Church and Gilbert (1984).

Inoculation of Arabidopsis plants with Peronospora parasitica

Wild-type and transgenic Arabidopsis plants 2 weeks of age were inoculated with a *P. parasitica* conidial suspension (approximately 10^5 spores ml^{-1}) using a Preval sprayer. Inoculated plants were incubated at 18°C under plastic domes to maintain approximately 100% relative humidity in Percival growth chambers equipped with VHO cool white fluorescent lamps (approximately 150 uE), under a 12-h light/dark cycle. Living plants were assessed for pathogen growth seven days after inoculation using a Leica MZ8 stereomicroscope. To view pathogen development within plant tissues, leaves were collected 7 days after inoculation and fixed in lactophenol trypan blue for 1 hour before being cleared with chloral hydrate (Uknes *et al.*, 1993). Leaf samples were mounted on glass slides and photographed using the stereomicroscope.

Construction of AS15 nim1-1 and AS15 NahG lines

AS15 line was crossed to *nim1-1* mutants and to *nahG* expressing transgenic lines. F_1 seedlings from the AS15 × *nim1-1* cross were then grown on MS plates containing 50 μg ml^{-1} kanamycin to select for the presence of the *TGA5*-antisense construct, which expresses the neomycin phosphotransferase gene. F_2 progeny that were homozygous for Kan resistance were then tested for homozygosity of the *nim1-1* mutation using *nim1-1* allele specific PCR primers that are able to distinguish the *nim1-1* mutation from wild-type *NIM1* sequence. F_1 seedlings from the AS15 *nahG* cross were similarly tested. F_2 progeny that were homozygous for the TGA5-antisense construct were then tested for homozygosity of the *nahG* gene. Selection for the presence of the *nahG* gene, however, involved resistance to hygromycin, as the construct carrying the *nahG* gene confers resistance to hygromycin. F_2 progeny that were

thus tested in pathogen assays were homozygous resistant to both kanamycin and hygromycin.

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