Genetic Interactions of TGA Transcription Factors in the Regulation of Pathogenesis-Related Genes and Disease Resistance in Arabidopsis[1][W]

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TGA transcription factors are implicated as regulators of pathogenesis-related (*PR*) genes because of their physical interaction with the known positive regulator, nonexpresser of *PR* gene1 (NPR1). A triple-knockout mutant *tga2-1 tga5-1 tga6-1* was shown previously to be defective in the induction of *PR* genes and systemic acquired resistance, confirming their role in disease resistance. However, the contributions of individual TGA factors have been difficult to discern because of functional redundancy among these factors, as well as possible dual functions for some single factors. In this study, we characterized six TGA factors by reverse genetics. We show that TGA3 is required for both basal and 2,6-dichloroisonicotinic acid-induced transcription of *PR* genes. The *tga3-1* mutants were found to be defective in basal pathogen resistance, whereas induced resistance was unaffected. TGA1 and TGA4 play partially redundant roles in regulation of basal resistance, having only moderate effects on *PR* gene expression. Additionally, an activation-tagged mutant of *TGA6* was able to increase basal as well as induced expression of *PR1*, demonstrating a positive role for TGA6 on *PR* gene expression. In contrast, TGA2 has repressor activity on *PR* gene expression even though it can act as a positive regulator in the *tga5-1 tga6-1* null mutant background. Finally, we examined the genetic interaction between *tga2-2* and suppressor of *npr1* inducible1 (*sni1-1*). TGA2's repressor activity overlaps with SNI1 because the *tga2-2 sni1-1* double mutant shows a synergistic effect on *PR* gene expression.

Induction of plant systemic acquired resistance (SAR) correlates with the expression of pathogenesis-related (*PR*) genes (Métraux et al., 1991; Ryals et al., 1996; Durrant and Dong, 2004). These genes, some of which encode proteins with antimicrobial activity (Van Loon and Van Strien, 1999), are believed to be the effectors of SAR, conferring broad-spectrum resistance to pathogens. In some plants, such as tobacco (*Nicotiana tabacum*), cucumber (*Cucumis sativus*), and Arabidopsis (*Arabidopsis thaliana*), an increase in salicylic acid (SA) production is essential for establishment of SAR (Gaffney et al., 1993). Treatment of these plants with SA or its functional analogs 2,6-dichloroisonicotinic acid (INA) and benzothiodiazole is sufficient to induce SAR (Görlach et al., 1996; Lawton et al., 1996). In Arabidopsis, SA-mediated gene expression and disease resistance have been shown to require the function of nonexpresser of *PR* gene1 (NPR1; Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). The *npr1* mutants are not only impaired in SAR, but also show reduced basal resistance to pathogen infection (Cao et al., 1997; Delaney, 1997).

The NPR1 protein contains a BTB/POZ domain and an ankyrin repeat domain, which are known to be involved in protein-protein interaction (Cao et al., 1997; Aravind and Koonin, 1999). It was discovered recently that activation of NPR1 involves reduction of the protein, releasing it from a large oligomeric complex to translocate into the nucleus (Kinkema et al., 2000; Mou et al., 2003). Given the absence of a canonical DNA-binding domain, NPR1 was proposed to regulate *PR* gene expression as a cofactor of the TGA transcription factors, which interact with NPR1 in the yeast (*Saccharomyces cerevisiae*) two-hybrid system (Zhang et al., 1999; Despres et al., 2000; Zhou et al., 2000) and in planta (Subramaniam et al., 2001; Fan and Dong, 2002).

There are 10 TGA transcription factors in Arabidopsis (Jakoby et al., 2002) of which seven (TGA1–TGA7) have been characterized with respect to their interaction with NPR1. These seven TGAs can be further divided into three subgroups based on sequence homology: TGA1 and TGA4 comprise group I; TGA2, TGA5, and TGA6 belong to group II; and TGA3 and TGA7 make up group III (Xiang et al., 1997). TGA2, TGA3, TGA5, TGA6, and TGA7 constitutively interact with NPR1 in yeast and in planta when transiently expressed. Interestingly, the two TGA factors (i.e. TGA1 and TGA4) that showed no interaction with NPR1 in yeast were found to bind NPR1 only in SA-induced leaves. Reduction of two Cys residues that are uniquely present in TGA1 and TGA4 are responsible

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for this SA-dependent interaction (Despres et al., 2003).

To add to the complexity of TGA-regulated gene expression, linker-scanning (LS) mutagenesis of the *PR1* promoter revealed both positive and negative regulatory cis-elements (Lebel et al., 1998). Mutation in the *LS7* element that contains a TGA-binding site resulted in complete loss of gene induction, whereas mutation in another TGA-binding element (*LS5*) augmented gene expression. A second negative element (*LS4*) was found to contain a WRKY transcription factor-binding site (W box). Consistent with this result, some WRKY factors have been shown to negatively regulate pathogen-induced *PR* gene expression (Journot-Catalino et al., 2006; Wang et al., 2006; Xu et al., 2006; Zheng et al., 2007).

Previous studies using gel mobility shift and chromatin immunoprecipitation (ChIP) assays have shown that TGA factors bind to the *as-1* element (Lam and Lam, 1995; Miao and Lam, 1995; Jupin and Chua, 1996; Johnson et al., 2003; Rochon et al., 2006). Increased binding to the *as-1* element was observed with SA-induced plant extracts. Additionally, binding of in vitro-synthesized TGA2 to the *as-1* element was enhanced in the presence of NPR1 (Despres et al., 2000), suggesting that NPR1 enhances or stabilizes the binding of TGA factors to the promoter. To further prove that the TGA factors are present in the DNA-bound complex, a supershift in gel mobility was shown using an antibody against TGA2 (Lam and Lam, 1995; Pontier et al., 2002). Moreover, depletion of TGA2 and TGA3 from nuclear extracts resulted in reduced protein binding to the *as-1* element (Jupin and Chua, 1996; Zhou et al., 2000; Johnson et al., 2003). ChIP assays in SA-induced plants showed that antibodies against TGA2 and TGA3 preferentially precipitated the *PR1* promoter sequences in an NPR1-dependent manner, confirming that these factors were recruited to the *PR1* promoter in vivo (Johnson et al., 2003).

Several molecular approaches used to study TGA factors in plant defense have produced conflicting results. Overexpression of a dominant-negative mutant of TGA2 that competes with the binding of all TGAs to the target promoter showed increased expression of *PR* genes (Pontier et al., 2001; Fitzgerald et al., 2005). On the other hand, overexpression of TGA5 in Arabidopsis protected the plants against *Hyaloperonospora parasitica* NOCO$_2$ infection without affecting *PR* gene expression (Kim and Delaney, 2002). More direct genetic evidence linking TGA factors to *PR* gene induction and resistance was only obtained when a triple-deletion knockout mutant, *tga2-1 tga5-1 tga6-1*, was generated in Arabidopsis and found to be defective in SAR and *PR1* induction (Zhang et al., 2003). However, unlike *npr1-1*, the *tga2-1 tga5-1 tga6-1* mutant does not show a defect in basal resistance (Zhang et al., 2003). In fact, the triple mutant has elevated basal expression of *PR* genes. This result suggests that, besides TGA2, TGA5, and TGA6, other TGA factors may be transcriptional activators of *PR* gene

expression. Furthermore, TGA2, TGA5, or TGA6 may have dual activity. This is consistent with the results of the *PR1* promoter study showing that there are at least two TGA-binding elements in this promoter, which, when mutated, have the opposite effect on transcription (Lebel et al., 1998).

In this study, we present genetic analysis of six *TGA* genes belonging to all three subgroups of this transcription factor gene family. We found that disruption of individual *TGA4* and *TGA7* did not significantly alter *PR* gene expression. Although the *tga1-1* mutant showed a moderate decrease in INA-induced *PR* gene expression, it plays a significant role in regulating basal defense with partial dependence on TGA4. The *tga3-1* single-knockout mutations significantly compromised expression of *PR* genes and displayed enhanced disease susceptibility (EDS). We also describe a novel function for TGA2 as a repressor of *PR* gene expression that appears to perform this function through genetic interaction with SNI1, another negative regulator of *PR* gene expression.

RESULTS

Isolation of T-DNA Insertion in Different *TGA* Family Members

To perform a comprehensive genetic study of TGA transcription factors, we screened for T-DNA insertion mutants in the NPR1-interacting TGA genes. To complement the work by Zhang et al. (2003) on *tga2-1 tga5-1* (a single deletion of these adjacent genes) and *tga2-1 tga5-1 tga6-1*, we characterized a new allele of *tga2* (*tga2-2*), which is wild type for *TGA5*, and an activation-tagged *TGA6$_{ACT}$* allele that overexpresses the endogenous *TGA6* gene. In addition, we found previously uncharacterized insertion mutants *tga1-1*, *tga3-1*, *tga4-1*, and *tga7-1*, which belong to the other two subgroups of the *TGA* gene family.

The *tga1-1* and *tga4-1* mutants were isolated from the SALK collection with T-DNA insertions in the first and second exons, respectively (Fig. 1A), causing complete loss of the respective transcripts (Fig. 1B). The *tga2-2* and *TGA6$_{ACT}$* homozygous lines were screened from the T-DNA-tagged population generated at the University of Wisconsin. As shown in Figure 1A, the T-DNA insertion in *tga2-2* was found to be 1,000 nucleotides downstream of the start codon. Although no full-length cDNA could be amplified from the homozygous line (Fig. 1B), the region before the insertion site could be detected by reverse transcription (RT)-PCR (data not shown). Western blotting was then performed using a polyclonal antibody against the N terminus of TGA2 (Johnson et al., 2003), which confirmed that *tga2-2* is a knockout mutant, producing no detectable full-length or truncated protein (Supplemental Fig. S1). The insertion in *TGA6$_{ACT}$* is 100 nucleotides upstream of the start codon (Fig. 1A) and RT-PCR showed a 20-fold increase in *TGA6*
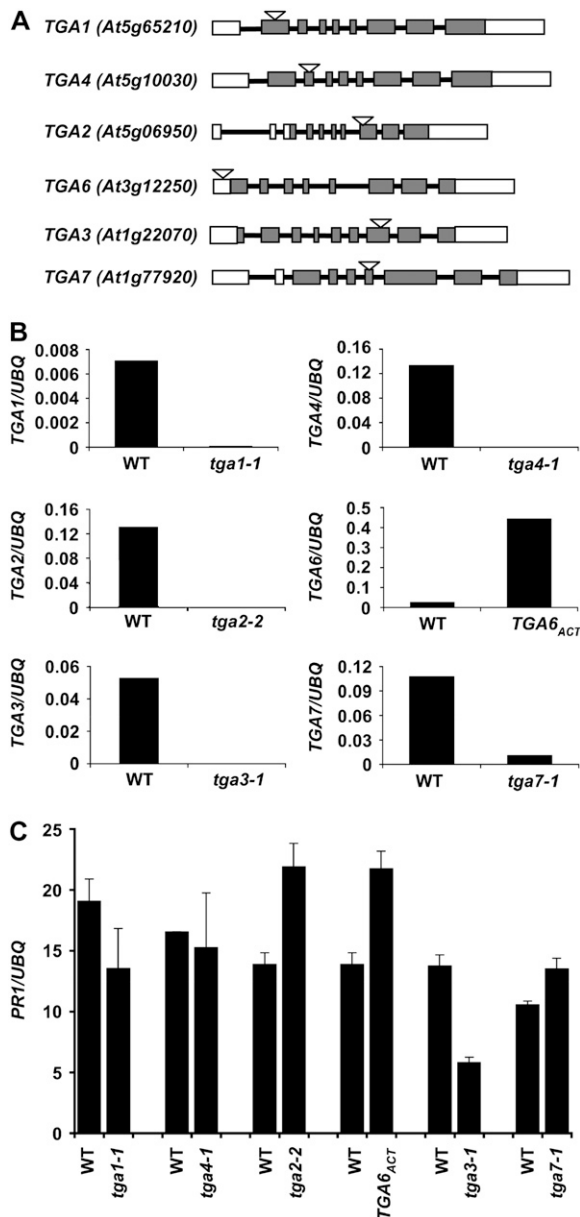
Figure 1. Characterization of *tga* T-DNA insertion mutants. A, Schematic representations of different *tga* T-DNA insertion mutations. Arabidopsis Genome Initiative numbers of the genes are shown in parentheses. Rectangular boxes represent the exons, whereas the interconnecting lines represent the introns. White rectangles show the untranslated regions in the genes. The T-DNA insertion site is represented as a triangle above each gene. B, Real-time RT-PCR analysis of *TGA* gene expression in the mutants. RNA was extracted from 2-week-old seedlings grown on a Murashige and Skoog plate. The individual bar diagrams shows the relative amount of the transcript of the corresponding *TGA* gene in the wild type and mutant normalized to the levels of a *UBQ* gene. The normalized values were multiplied by 1,000 for *TGA2, TGA3, TGA4,* and *TGA7* and by 10 for *TGA4*. C, Expression of *PR1* in each *tga* mutant was determined from 2-week-old seedlings harvested from Murashige and Skoog plates containing 20 μM INA using real-time RT-PCR. The *y* axis values are normalized to *UBQ5* expression. Error bars represent SE. The experiments were repeated at least three times with similar results. WT, Wild type; *UBQ*, ubiquitin.

expression compared to wild type (Fig. 1B). Both *tga2-2* and $TGA6_{ACT}$ mutants were backcrossed once with wild type and the resulting homozygous mutants in the F_2 generation were selected and propagated for further studies. The *tga3-1* T-DNA insertion was isolated from Thomas Jack's enhancer trap population. The insertion in TGA3 was 1,341 nucleotides downstream of the start codon (Fig. 1A) and no cDNA could be amplified from *tga3-1*, suggesting that it is a knockout mutant (Fig. 1B). The *tga7-1* mutant was obtained from the GABI-Kat population where the insertion was found to be within the fourth exon. All of these mutants bred true in multiple genetic crosses. No apparent morphological defect affecting any developmental stage was observed in any of the single mutants studied.

PR Gene Expression in *tga* Mutants

To evaluate the role of individual TGA factors in *PR* gene expression, we tested a range of INA concentrations (2–50 μM) for induction of these genes. Plants with the Wassilewskija (Ws-0) genotype showed stress symptoms at INA concentrations higher than 20 μM. At 20 μM INA, significant differences in *PR1* gene expression were detected in *tga2-2* and $TGA6_{ACT}$ (both are in Ws-0) using real-time RT-PCR (Fig. 1C). We observed significant differences in *PR* gene expression in *tga3-1* mutants at all the concentrations tested (Fig. 1C; Supplemental Fig. S2A). The *tga1-1* mutant showed a small decrease in *PR1* expression. However, no clear-cut differences were seen in *tga7-1* and *tga4-1* in any of the INA concentrations tested. Contrary to previous reports (Fan and Dong, 2002; Zhang et al., 2003; Kang and Klessig, 2005), which suggest that TGA2 is a transcriptional activator of *PR* genes, the *tga2-2* knockout mutant showed higher expression of *PR1* in response to induction (Fig. 1C). This result suggests that, in the presence of other TGA factors, TGA2 is a transcriptional repressor of *PR* genes. The $TGA6_{ACT}$ plants showed an increase in *PR1* expression as compared to wild type, indicating that TGA6 is a transcriptional activator of *PR1* as previously reported (Zhang et al., 2003). Disruption of *TGA3* in the *tga3-1* mutant showed >50% decrease in the expression of *PR1* as compared to wild type. Similar results were obtained with SA induction of *tga3-1* plants (Supplemental Fig. S2B). A prominent role of TGA3 as a transcription activator of *PR* genes is consistent with the fact that *TGA3* is the strongest interactor of NPR1 (Zhou et al., 2000) and is the most abundantly expressed *TGA* gene in leaves (Zimmermann et al., 2004), where *PR* genes are expressed. INA responsiveness of other *PR* genes (*PR2* and *PR5*) was also examined in *tga2-2*, *tga3-1*, and $TGA6_{ACT}$, and they were found to have an expression pattern similar to *PR1* (Supplemental Figs. S2C and S3).

TGA3 Is Required for Basal Resistance and *PR* Gene Expression

To examine the effect of the *tga3-1* mutation on disease resistance, plants were infected with a low

inoculum (OD$_{600}$ = 0.0001) of *Pseudomonas syringae* pv *maculicola* ES4326 (*Psm*4326). At this level of inoculum, wild-type plants and mutants with enhanced (EDS) can be easily distinguished. In the EDS test shown in Figure 2A, wild-type plants did not show any disease symptoms, whereas *npr1-1* plants showed clear chlorosis. The *tga3-1* plants sporadically displayed disease symptoms. The *tga3-1* mutants were then analyzed for growth of *Psm*4326 in leaves. As shown in Figure 2B, wild-type plants showed limited growth of *Psm*4326, whereas the *npr1-1* mutant had substantial growth of this pathogen 3 d after inoculation. The *tga3-1* mutant, in comparison, had a bacterial titer significantly higher than wild type, but lower than the *npr1-1* mutant. Interestingly, the *tga3-1 npr1-1* double mutant had even higher pathogen growth compared to either *npr1-1* or *tga3-1* alone. These results clearly demonstrate that TGA3 is required for basal defense and that the function of TGA3 is not completely dependent on NPR1.

It has been shown previously that the *tga2-1 tga5-1 tga6-1* triple mutant fails to establish SAR similar to *npr1* (Zhang et al., 2003). However, unlike *npr1*, the triple mutant has elevated background expression of *PR* genes, accompanied by intact basal resistance to *Psm*4326. We hypothesize that this is due to the activity

of TGA3. To test this hypothesis, the *tga2-1 tga5-1 tga6-1* triple mutant was crossed with *tga3-1* to generate a quadruple-knockout mutant. As shown in Figure 2C, the *tga3-1* mutation in the quadruple mutant completely blocked the basal expression of *PR1* observed in the *tga2-1 tga5-1 tga6-1* triple mutant. Furthermore, the EDS test showed that the quadruple mutant developed symptoms similar to those observed in *npr1*, whereas wild type and *tga2-1 tga5-1 tga6-1* did not show any symptoms (Fig. 2A). However, when in planta bacterial growth was quantified, the quadruple mutant showed a bacterial titer higher than those in *tga3-1* and the triple mutant, but lower than the titer observed in *npr1-1* (Fig. 2D). These results further establish the importance of *TGA3* in the regulation of basal *PR* gene expression and resistance. Moreover, TGA3 also seems to be involved in development of disease symptoms.

The *tga1-1 tga4-1* Double Mutant Is Compromised in Resistance to *Pseudomonas* Infection

It has been shown previously that the reduced forms of TGA1 and TGA4 can interact with NPR1 (Despres et al., 2003), suggesting an important role for this subgroup of TGA factors in SAR. However, we only
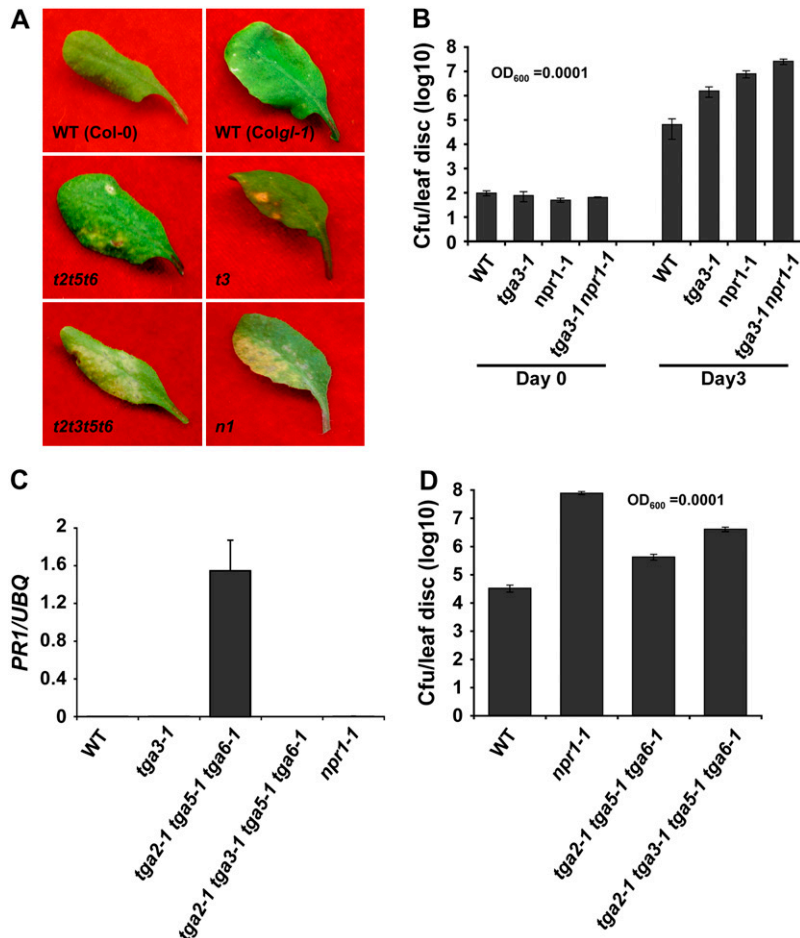

Figure 2. TGA3 is a positive regulator of *PR1* expression and basal resistance. A, Disease symptoms from representative leaves of 4-week-old plants. Infections were carried out using *P. syringae* pv *maculicola* ES4326 OD$_{600}$ = 0.0001. Photographs were taken 3 d postinfection. The experiment was repeated three times with similar results. WT, Wild type; *t3*, *tga3-1*; *n1*, *npr1-1*; *t2t5t6*, *tga2-1 tga5-1 tga6-1*; *t2t3t5t6*, *tga2-1 tga3-1 tga5-1 tga6-1*. B, Bacterial titers in the leaves of 4-week-old plants infiltrated with *Pseudomonas* as described in A. Samples were collected at 0 and 3 d postinfection. Values were averaged from eight samples per genotype and error bars represent SE. The experiment was repeated at least three times with similar results. WT, Wild type. C, Basal level expression of *PR1* in wild type, *tga3-1*, *tga2-1 tga5-1 tga-6-1*, *tga2-1 tga3-1 tga5-1 tga6-1*, and *npr1-1* in the absence of INA. RNA was extracted from 2-week-old seedlings grown on Murashige and Skoog plates. Transcript levels were quantified by real-time RT-PCR and *y* axis values were normalized to *UBQ5* expression. Error bars represent SE from three PCR reactions. The experiment was repeated at least three times with similar results. *UBQ*, Ubiquitin. D, Bacterial titers in the leaves of 4-week-old plants infiltrated with *P. syringae* pv *maculicola* ES4326 OD$_{600}$ = 0.0001. Samples were collected 3 d postinfection. Values were averaged from 16 samples per genotype and the error bars represent SE. The experiment was repeated at least three times with similar results.
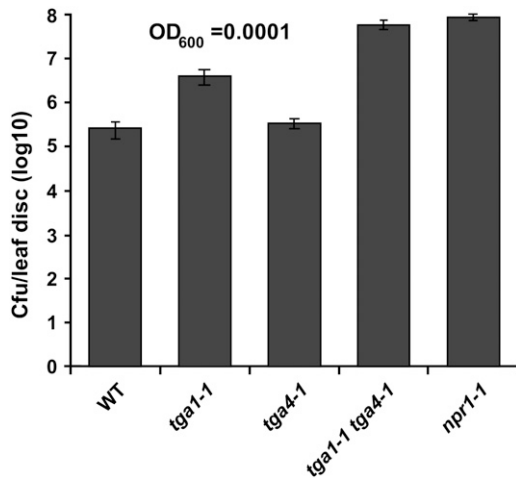
Figure 3. *tga1-1 tga4-1* mutants are compromised in basal resistance. Bacterial titers in the leaves of 4-week-old plants infiltrated with *P. syringae* pv *maculicola* ES4326 OD_{600} = 0.0001. Samples were collected 3 d postinfection. Values were averaged from 16 samples per genotype and error bars represent SE. The experiment was repeated at least three times with similar results. WT, Wild type.

observed a small decrease in *PR1* expression in the *tga1-1* mutant in response to INA induction (Fig. 1C). Expecting that this was due to functional redundancy, we generated the *tga1-1 tga4-1* double mutant through a genetic cross. Still, *PR1* expression in response to INA in the double mutant was comparable to the *tga1-1* single mutant. Similarly, the double mutant showed only a marginal effect on induced resistance (data not shown). To further assess the susceptibility of the *tga1-1 tga4-1* mutant, we carried out an EDS test using *Psm*4326 as described in the previous section. As shown in Figure 3, the *tga1-1* mutant supported significantly higher pathogen growth than the wild type, whereas the *tga4-1* mutant seemed to be similar to the wild type. Even greater susceptibility was seen in the *tga1-1 tga4-1* double mutant, which is similar to *npr1-1* (Fig. 3). These results demonstrate that TGA1 and TGA4 positively regulate basal resistance. Additionally, TGA1 and TGA4 are partially redundant, with TGA1 having a greater effect than TGA4.

TGA6 Can Activate *PR* Gene Expression in *tga2-2*

In the $TGA6_{ACT}$ mutant, the T-DNA insertion upstream of the regulatory region of *TGA6* causes overexpression of this gene (Fig. 1B). Although this does not lead to constitutive expression of *PR1*, INA-induced *PR1* transcription is significantly enhanced (Fig. 1C). This suggests that TGA6 activity may not be constitutive and may require an additional activation step. Alternatively, removal of a negative regulator may be required for TGA6 to perform its function as a constitutive transcriptional activator. To test this hypothesis, we crossed $TGA6_{ACT}$ into the *tga2-2* background where the TGA2 function is knocked out. Indeed, the *tga2-2* $TGA6_{ACT}$ double mutant showed

dramatically increased basal *PR1* expression compared to the $TGA6_{ACT}$ and the *tga2-2* single mutants (Fig. 4). This result shows that TGA6 is a constitutive transcriptional activator of *PR1* and its activity is repressed in the presence of TGA2. Because we found TGA3 to be the major transcriptional activator of *PR* genes, we tested whether the activator function of TGA6 requires TGA3. We generated the *tga2-2 tga3-1* $TGA6_{ACT}$ triple mutant as described in the experimental procedures. Because *tga2-2*, $TGA6_{ACT}$, and *tga3-1* are in two different ecotypes, we analyzed multiple progeny from each of the crosses to ensure that the observed phenotypes were from these mutations, not from mixing the genetic backgrounds. As expected, in the absence of TGA3, basal *PR1* expression observed in the *tga2-2* $TGA6_{ACT}$ double mutant was completely abolished (Fig. 4). In the *tga2-2 tga3-1* $TGA6_{ACT}$ triple mutant, INA-induced *PR* gene expression was also partially compromised compared to the double mutant, likely due to the absence of functional TGA3 (Supplemental Fig. S4).

TGA2 Acts Synergistically with SNI1 as a Transcriptional Repressor of *PR* Genes

The enhanced *PR* gene expression observed in the *tga2-2* knockout mutant suggests that TGA2 is a transcriptional repressor. In addition to TGA2, SNI1 is another negative regulator of *PR* genes (Li et al., 1999; Mosher et al., 2006). To determine whether there is any genetic interaction between these two negative regulators, we crossed *tga2-2* with *sni1-1*. Interestingly, the *tga2-2 sni1-1* double-homozygous plants were underrepresented in the F_2 population (observed 1:100 rather than the expected 1:16 ratio; χ^2 = 4.41; $P >$ 0.5), suggesting that plant viability is compromised by
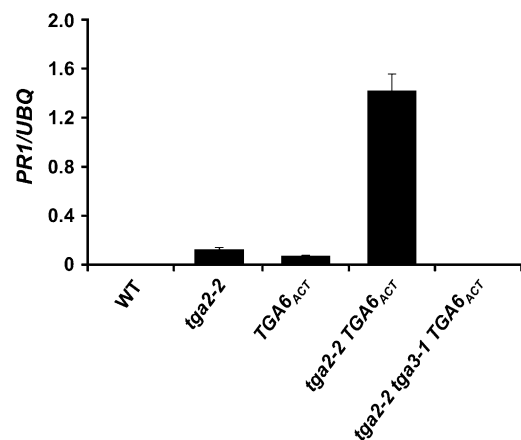

Figure 4. Overexpression of *TGA6* leads to activation of *PR1* expression in *tga2-2*. RNA was extracted from 2-week-old seedlings grown on Murashige and Skoog plates. Transcript levels were quantified by real-time RT-PCR and y axis values were normalized to *UBQ5* expression. Error bars represent SE from three PCR reactions. The experiment was repeated at least three times with similar results. WT, Wild type; *UBQ*, ubiquitin.

the presence of both mutations. We analyzed the expression of *PR1* and *PR2* in the surviving *tga2-2 sni1-1* double-homozygous plants by northern-blot analysis (Fig. 5). As reported previously, the *sni1-1* single mutant had elevated background expression of *PR1*. Interestingly, the level of *PR1* expression in the *tga2-2 sni1-1* double mutant was dramatically elevated compared to the *sni1-1* single mutant, resulting in expression levels that were similar to INA-induced wild-type plants. Expression of *PR2* was similarly induced in the double mutant. To exclude the possibility that high *PR* gene expression is due to mixing of Ws and Columbia (Col) ecotypes, we analyzed multiple *tga2-2 sni1-1* plants from independent crosses. All of the *tga2-2 sni1-1* double-mutant progeny tested had dramatically increased *PR* gene expression (data not shown). The synergistic effect of these two mutations suggests that TGA2 and SNI1 have overlapping activities as negative regulators of *PR* genes. The *PR1* transcript level in *tga2-2 sni1-1* was induced further after INA treatment, suggesting that, although TGA2 and SNI1 are the predominant repressors for this gene, other factors may also be involved. In the absence of TGA2 and SNI1, transcription of *PR1* seems to be partially dependent on NPR1 because in the *tga2-2 sni1-1 npr1-1* triple mutant, the level of *PR1* expression is significantly reduced in the presence or absence of INA (Fig. 5).

Surprisingly, the high levels of *PR* gene expression in the *tga2-2 sni1-1* are not sufficient to confer resistance against *Psm*4326 (data not shown), indicating that activation of additional genes is required. Recently, we discovered that for the *PR* proteins to be effectively folded and secreted outside the cell, genes involved in protein folding, modification, and secretion have to be coordinately up-regulated during SAR (Wang et al., 2005). A mutation in one of these genes, *BiP2*, not only compromised SAR, but also caused massive cell death upon SAR induction due to accumulation of unfolded PR proteins. This study also showed that induction of the protein secretory pathway genes requires NPR1, but not TGA factors. We tested expression of two of the genes (*BiP2* and *GRP94*)

that are required for secretion of PR proteins in the *tga2-2 sni1-1* double mutant. Real-time RT-PCR showed that the expression of *BiP2* and *GRP94* were not significantly changed in the *tga2-2 sni1-1* mutant even though the *PR* gene levels were extremely high (Supplemental Fig. S5). This may explain the lack of enhanced resistance and low viability of the double mutant. In support of this hypothesis, viability was restored in the *tga2-2 sni1-1 npr1-1* triple mutant because the triple mutant was obtained at the expected frequency (observed 1/72; expected 1/64).

DISCUSSION

PR1 expression is a paradigm for the coregulation of *PR* genes during SAR (Eulgem, 2005); therefore, understanding the regulation of this gene can help us understand SAR. The *PR1* promoter contains both negative and positive regulatory elements, the importance of which has been shown through LS mutagenesis (Lebel et al., 1998). Molecular studies of TGA factors also strongly suggest differential effects of TGAs in regulating *PR* gene expression. However, activator or repressor functions are difficult to assign to individual TGA factors due to functional redundancy and lack of knockout mutants in all of the *TGA* genes. Through our comprehensive characterization of single, double, triple, and quadruple mutants of *TGA* genes, we clearly revealed the functions of *TGA1*, *TGA2*, *TGA3*, *TGA4*, and *TGA6*.

Characterization of the *tga3-1* knockout mutant showed that this transcription factor is an important activator for both basal and induced expression of *PR* genes (Figs. 1C, 2C, and 4). This is consistent with the fact that *TGA3* is one of the most highly expressed *TGA* genes in rosette leaves (Zimmermann et al., 2004) and is the strongest interactor of NPR1 (Zhou et al., 2000). Although the *tga1-1* mutation only had a moderate effect on INA-induced *PR1* expression (Fig. 1C), it significantly impaired the plant's basal resistance to *Pseudomonas* infection (Fig. 3). TGA1 and TGA4 may have functional redundancy because the double
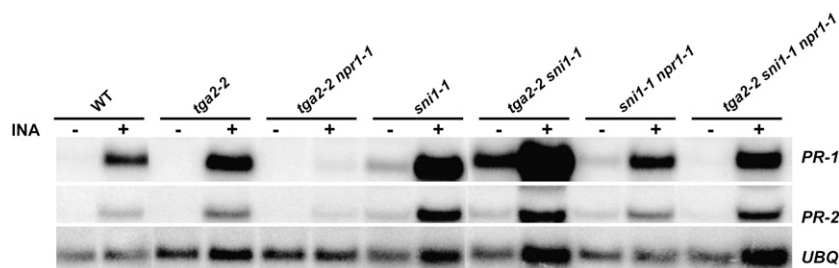

Figure 5. TGA2 is a negative regulator of *PR* gene expression. Northern blot showing expression of *PR1* and *PR2* in *tga2-2 sni1-1* and *tga2-2 sni1-1 npr1-1* mutants. Total RNA was extracted from 2-week-old seedling grown on Murashige and Skoog agar plates with (+) or without (−) INA (20 μM). *PR1* and *PR2* expression levels were analyzed by hybridizing an RNA blot with respective gene-specific probes. A UBQ5-specific probe was used to hybridize the same blot as a loading control. WT, Wild type; UBQ, ubiquitin.

mutant showed an additive effect on bacterial growth. These data indicate that the TGA1 and TGA4 subgroup of TGA factors is critical to basal resistance, with TGA1 playing a more dominant role. We were unable to find a consistent phenotype for the *tga7-1* mutant under the conditions tested. It is possible that TGA7 plays a redundant role with TGA3 because it belongs to the same subgroup.

Our study of the $TGA6_{ACT}$ mutant is consistent with that of Zhang et al. (2003), where TGA6 is a transcription activator of *PR* genes. Interestingly, we found that overexpression of *TGA6* in $TGA6_{ACT}$ is not sufficient to cause a significant increase in *PR* gene expression in the absence of an SAR inducer (Fig. 4). Rather, TGA6 activity requires removal of TGA2 because only in the *tga2-2* knockout background did overexpression of TGA6 result in a significant increase in *PR* gene expression. Additionally, in the absence of TGA2, TGA6 may also be able to replace the activator function of TGA2. Moreover, this activity is dependent on TGA3 because in the *tga2-2 tga3-1 $TGA6_{ACT}$* mutant, basal *PR* gene expression is abolished.

Studies carried out by Fan and Dong (2002) using a chimeric TGA2-GAL4 fusion protein showed that TGA2 can act as an activator of a GAL4-mediated reporter gene in an NPR1-dependent manner. The role of TGA2 as a transcription activator was also shown by the loss of *PR1* induction in *tga2-1 tga5-1 tga6-1*. When the wild-type TGA2 gene was transformed back into this triple mutant, the inducibility of *PR* genes was restored (Zhang et al., 2003). This indicated that, in the absence of TGA5 and TGA6, TGA2 can be a transcription activator. In contrast, studies with dominant-negative mutants of the TGA2 homologs in tobacco and rice (*Oryza sativa*) showed a negative role for TGA2 in *PR* gene expression and disease resistance (Pontier et al., 2001; Fitzgerald et al., 2005; Thurow et al., 2005).

However, the role of Arabidopsis TGA2 alone was previously unknown because the *tga2* single-knockout mutant was not available. Interestingly, we observed that, in the *tga2-2* single mutant, both the basal and the induced levels of *PR1* expression were moderately increased (Figs. 1C and 4). The phenotype of *tga2-2* was synergistically enhanced in the *sni1-1* mutant background (Fig. 5). To reconcile the negative role of TGA2, we hypothesize that TGA2 may function both as a transcription activator and as a repressor. The dual function of TGA2 could be achieved by any of several mechanisms. First, it may function as an activator or a repressor depending on which of the two *as-1*-type elements it binds in the *PR1* promoter. TGA2 has been shown to bind to both the negative (*LS5*) as well as positive (*LS7*) TGA-binding element in the *PR1* promoter (Despres et al., 2000). Contrary to the previous report, a recent publication (Rochon et al., 2006) showed constitutive recruitment of TGA2 to the *PR1* promoter by ChIP. This result supports our hypothesis that TGA2 may be present at the promoter to repress transcription before induction. However, the low resolution of the ChIP experiments did not distinguish TGA2 binding between the two TGA-binding elements in the *PR1* promoter. Some transcription factors have been known to have dual activities. One such example is the tumor suppressor protein p53 whose activation and repression activities largely depend on the specific sequence it binds (Marks et al., 2003). The second possible mechanism to explain the dual functions of TGA2 is through association with other trans-acting factors or the basal transcription machinery. A third mechanism for the dual functions is through posttranslational modification. A good example is the SP3 transcription factor, which acts as a repressor of the transforming growth factor-β receptor type II gene when unmodified whereas acetylated SP3 acts as a

Figure 6. Proposed model for TGA transcription factors in the regulation of *PR1*. A, Flow chart showing the genetic interactions between key regulatory components in the regulation of *PR* gene expression. Arrows represent activation and blocked arrows indicate repression. B, Mechanistic model showing recruitment of different factors under uninduced (−SA) and induced (+SA) conditions on the *PR1* promoter. T, TGA transcription factors (TGA1–TGA6); T2, TGA2; N, NPR1; N*, activated NPR1; W, WRKY transcription factor; BTM, basal transcription machinery. The negative cis-regulatory elements, *LS5* and *LS4*, are shown as hatched boxes and the positive element, *LS7*, is shown by a dotted rectangle. The dotted trans-acting factors represent an inactive form. Thickness of the arrow represents the level of expression and the blocked arrow represents repression.
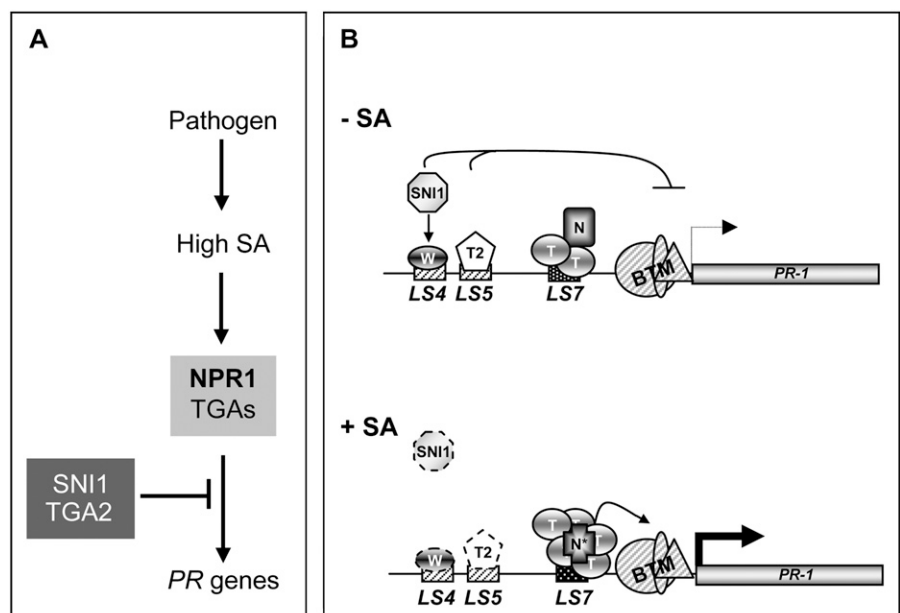
Table I. *Summary of primer pairs used for genotyping and RT-PCR*

Primer pairs listed in the first column were used to amplify either the genomic DNA or the cDNA from wild-type and mutant plants. The gene names are given before the kind of template used for amplification.

Primer Pair	Amplification
TGAKOF + TGAKOR	*TGA1* wild type genomic
LBb1 + TGA1KOF	*TGA1* T-DNA insertion
KOTGA2 + TGA2RKO	*TGA2* wild type genomic
KOTGA2 + JL202	*TGA2* T-DNA insertion
TGA4KOF + TGA4KOR	*TGA4* wild type genomic
LBb1 + TGA4KOF	*TGA4* T-DNA insertion
KOT6 + TGA6RKO	*TGA6* wild type genomic
JL202 + TGA6RKO	*TGA6* T-DNA insertion
TGA7F + TGA7LCR	*TGA7* wild type genomic
TGA1LCF + TGA1LCR	*TGA1* cDNA
TGA2LCF + TGA2LCR	*TGA2* cDNA
TGA3LCF + TGA3LCR	*TGA3* cDNA
TGA4LCF + TGA4LCR	*TGA4* cDNA
TGA6LCF + TGA6LCR	*TGA6* cDNA
TGA7LCF + TGA7LCR	*TGA7* cDNA

transcriptional activator (Ammanamanchi et al., 2003). Phosphorylation has also been shown as a repressor-to-activator switch for c-jun, a bZIP transcription factor (Lamph et al., 1990). The unphosphorylated c-jun is part of the histone deacetylase inhibitory complex and, upon phosphorylation, c-jun dissociates from the his-

tone deacetylase repressor complex and acts as an activator (Weiss et al., 2003). The role of phosphorylation was also proposed in inhibiting the binding of TAG1a to the *as-1* element in tobacco (Jupin and Chua, 1996). A recent study showed that TGA2 is phosphorylated upon SA activation (Kang and Klessig, 2005). Even though mutating the putative phosphorylation site in TGA2 did not affect its binding activity to the *LS7* element, it remains to be tested whether it affects binding to the negative (*LS5*) element.

The synergistic effect of *tga2-2* and *sni1-1* mutations and the involvement of WRKY factors on *PR* gene expression suggest the presence of multiple negative regulators with overlapping functions. This effect of the *tga2-2 sni1-1* double mutant on *PR* genes could not be explained by replacement of TGA2 function by TGA6 because the *TGA6$_{ACT}$ sni1-1* mutant did not show the similar effect as *tga2-2 sni1-1* on the expression of *PR* genes (data not shown). Although we have not detected any direct physical interaction between TGA2 and SNI1 (data not shown), the genetic interaction of these two factors could be at the level of chromatin modification. The SNI1 has been shown to be a nuclear protein with significant structural similarities to armadillo repeats that are present in many regulatory proteins, such as armadillo and β-catenin. Moreover, loss of SNI1 function leads to chromatin modifications at the *PR1* promoter that mimic SAR

Table II. *Sequences of primers used in this study*

Gene	Primer Name	Primer Sequence (5′-3′)
TGA1	TGA1LCF	GGATTTCGACCCTCCG
TGA1	TGA1LCR	GGCTCTAAGCCGTTGA
TGA1	TGA1TF	TCTTCGAAGAATTTGGCGAAGA
TGA1	TGA1TR	TTCCTGCTGTTCCATGGGAAGTAT
TGA2	TGA2LCF	GACGACACAGATCATCCT
TGA2	TGA2LCR	TCAAGCGGCTGTTCTC
TGA2	TGA2RKO	CGGATGAACGAAATCCACCGA
TGA2	KOTGA2	ATCAAGCCCTTTACTTGTGCACCTTCAAG
TGA3	TGA3LCF	GGGAATGTGGCGAACT
TGA3	TGA3LCR	CTTTCGACCAAACCCTG
TGA4	TGA4KOF	GTTCCACCGAGAAGGTTTG
TGA4	TGA4KOR2	TATCACTAACTTGTCCATGT
TGA4	TGA4LCF	ATGCGTTATCCCAAGG-
TGA4	TGA4LCR	TGTCGCGTGGTTAAGAT
TGA6	TGA6LCF	AGTTGGATCAAAAGACCCT
TGA6	TGA6LCR	CCATTGCCCACCAGTAG
TGA6	TGA6RKO	GACTATTCTCCAGCTGCTGAACA
TGA6	KOT6	GGACTGATGTCTCAACTGATGGTGACACAG
TGA7	TGA7F	GTCCCAATACTGCTACTTCCTC
TGA7	TGA7LCF	CGTTTAGCGCAGAAC
TGA7	TGA7LCR	CTTGTAGCCAGTGTGAAT
TGA7	TGA7LCR	CTTGTAGCCAGTGTGAAT
NPR1	NPR1F	GATCATAAGGCACTTGACTCG
NPR1	NPR1R	ATGAGTGCGGTTCTACCTTC
PR1	PR-1LCF	CTCATACACTCTGGTGGG
PR1	PR-1LCR	TTGGCACATCCGAGTC
SNI1	sni1F	TGGTTTTGTTTTGCAGGCTTGGTCACCATT
SNI1	sni1R	TCTTGCTCATGTTCTTAAGCTAGATTTCAC
UBQ5	UBQLCF	GACGCTTCATCTCGTCC
UBQ5	UBQLCR	GTAAACGTAGGTGAGTCCA

induction (Mosher et al., 2006). Because it is known that NPR1 resides predominantly in the cytosol before induction, it is intriguing and interesting that NPR1 is required for the high level of *PR1* expression seen in *tga2-2 sni1-1.* To understand this phenomenon, we first measured the SA levels in the *tga2-2 sni1-1* plants and found them to be similar to wild type. We then measured reduced glutathione levels in the *sni1-1* and *tga2 sni1-1* double mutant because reducing conditions cause monomerization and nuclear localization of NPR1. We detected high levels of reduced glutathione and constitutive nuclear localization of NPR1 in the *sni1-1* mutant (Z. Mou and X. Dong, unpublished data). The levels of reduced glutathione remained high in the *tga2-2 sni1-1* double mutant, which can explain the requirement of NPR1 for the high levels of *PR* gene expression observed in this mutant. The nuclear NPR1 may recruit positive TGA factors, such as TGA3 and TGA1, to the *PR1* promoter in the absence of the negative regulators. Interestingly, the *sni-1 tga2-2* double mutant is further inducible by INA, which is probably because nuclear-localized NPR1 needs further activation to be fully functional. This is consistent with the recent study by Rochon et al. (2006).

This study has defined the transcriptional activities of five TGA transcription factors in *PR* gene expression and disease resistance. It also revealed genetic interactions between these factors and other known regulators of *PR* genes. TGA4 and TGA7 may still be important in the regulation of *PR* gene expression and disease resistance under conditions not tested here. Based on the information generated in this study and previous work, we propose the following model (Fig. 6A): NPR1, in association with TGA factors, is required for basal and induced *PR* gene expression. In the absence of an inducer, TGA2 and SNI1 are responsible for repression of *PR* genes. As illustrated in Figure 6B, the *PR1* promoter contains both positive (*LS7*) and negative (*LS4* and *LS5*) cis-elements. Before induction, TGA2 and SNI1 repress basal expression through direct and indirect interactions, respectively, with the negative elements. We do not rule out constitutive binding of transcription activators such as TGA1, TGA3, and TGA6 to the positive element in the uninduced state. But this does not lead to gene expression in the presence of TGA2 and SNI1. Upon induction, TGA2 and SNI1 repression is removed through an unknown mechanism and transcription is activated by NPR1 in association with the positive TGA transcription factors, including TGA1, TGA2, TGA3, TGA5, and TGA6. Further biochemical studies are required to understand the mechanism by which TGA2 represses transcription.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown either on soil (Metro-Mix 200; Grace Sierra) or on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2% Suc and 0.8% agar under conditions described in Clarke et al. (1998).

Isolation of TGA T-DNA Insertion Mutants

The *tga2-2* and *TGA6*$_{ACT}$ T-DNA insertion alleles were isolated from the Ws-0 ecotype of Arabidopsis. The insertion was identified by PCR screening of pooled genomic DNA using the protocol provided by the Arabidopsis gene knockout research facility at the University of Wisconsin (Krysan et al., 1999). The *tga2-2* and *TGA6*$_{ACT}$ homozygous plants were obtained by PCR screening the progeny using two gene-specific primers flanking the T-DNA insertion and a primer complementary to the T-DNA left-border sequence. Sequencing of the PCR product from the left border revealed the exact insertion site of the T-DNA. The primer pairs used for PCR are shown in Table I. The sequences of the primers are described in Table II. Plants in which a PCR product was detected using one gene-specific primer and the T-DNA primer were identified and homozygosity for the insertion mutation was confirmed by the lack of segregation in the next generation. The *tga3-1* mutant was identified in Thomas Jack's T-DNA-tagged population (http://www.arabidopsis.org/abrc/jack.jsp) made in the Col-*gl1* ecotype by performing three rounds of PCR with pooled genomic DNA. The exact insertion site was determined by sequencing the region flanking the T-DNA. Homozygous *tga3-1* plants were obtained by genomic Southern blotting. The T-DNA insertion was detected by the presence of an extra *Hin*dIII site in the T-DNA. Southern blotting was carried out using a standard protocol and the full-length *TGA3* coding region was used to make the probe. The *tga7-1* T-DNA insertion mutant (no. 434F04) was obtained from German plant genomic research plan Kolner Arabidopsis (GABI-Kat; Rosso et al., 2003). The homozygosity of the insert was confirmed by the absence of the wild-type PCR product using gene-specific primers (Table I). The *tga1-1* and *tga4-1* mutants were identified in the T-DNA insertion collection from the SALK Institute Genomic Laboratory (http://signal.salk.edu). T$_3$ progeny (SALK_082821 and SALK_127923 for *tga1-1* and *tga4-1*, respectively) were analyzed individually by PCR. Insertion was identified as described above for *tga2-2* using the T-DNA left-border primer and gene-specific primers (Table I).

Generation of Double, Triple, and Quadruple Mutants

Because *tga3-1* was generated in the Col ecotype containing the recessive glabrous mutation (*gl1*), it was used as female in all of the crosses, from which the F$_1$ progeny would be distinguishable from the *tga3-1* parent by the presence of trichomes. To obtain *tga3-1 npr1-1* and *tga2-2 tga3-1*, *tga3-1* was crossed with *npr1-1* and *tga2-2*, respectively. F$_1$ plants (with trichomes) were allowed to self-fertilize and double mutants were screened from the F$_2$ population by identifying *tga2-2* homozygous T-DNA insertion using PCR as described above. The *npr1-1* allele was also confirmed by PCR using the cleaved-amplified polymorphic sequence marker primers described in Table II, followed by digestion with *Nla*III. The *tga3-1* mutation was screened using Southern hybridization as described in the previous section. To create the *tga1-1 tga4-1* double mutant, *tga4-1* plants were used as pollen donors and F$_1$ plants were analyzed for the presence of the TGA4 T-DNA allele; the successful F$_1$ plants were self-fertilized to produce F$_2$ seeds. These seeds were planted and screened for the homozygous *tga1-1* and the *tga4-1* allele by PCR using the T-DNA left-border primer and gene-specific primers to *TGA1* and *TGA4*. Multiple homozygous plants were recovered and used for further analysis. To make the *tga2-2 TGA6*$_{ACT}$ double mutant, *tga2-2* was used as the pollen donor and the F$_1$ plants were identified by the presence of a band using the *TGA2* gene-specific primer and the T-DNA left-border primer. The F$_2$ progeny were then generated through self-fertilization and screened for T-DNA insertion at both *TGA2* and *TGA6* loci as described above. To obtain *tga2-2 tga3-1 TGA6*$_{ACT}$, *TGA6*$_{ACT}$ was crossed with the *tga2-2 tga3-1* double mutant and the F$_2$ progeny containing homozygous mutation at *TGA2*, *TGA3*, and *TGA6* loci were screened as described above. To generate *tga2-1 tga3-1 tga5-1 tga6-1* quadruple mutant, *tga3-1* was crossed with the *tga2-1 tga5-1 tga6-1* triple mutant (Zhang et al., 2003). The *tga2-1 tga5-1* deletion mutation in the progeny was obtained by lack of the PCR product using gene-specific primers for *TGA2*. Similarly, the *tga6-1* deletion was confirmed by lack of the PCR product using the *TGA6* gene-specific primer. To obtain *tga2-2 sni1-1* and *tga2-2 sni1-1 npr1-1*, *tga2-2* pollen was used to fertilize *sni1-1* and *sni1-1 npr1-1* plants, respectively. The recessive *sni1-1* mutation has a pleiotropic effect on plant morphology; therefore, wild-type-looking F$_1$ plants were progeny of successful crosses and were allowed to self-fertilize to produce F$_2$ plants.

These plants were first screened for the *sni1-1* locus using cleaved-amplified polymorphic sequence markers (Table II) followed by digestion with *Tsp*509I. The *npr1-1* and *tga2-2* loci were identified as described above. To make *tga2-2 npr1-1*, *tga2-2* pollens were used to fertilize *npr1-1* plants. The F$_1$ progeny that expressed the *PR* gene were chosen and selfed to produce F$_2$ plants. Genotyping of *tga2-2* and the *npr1-1* allele was carried out as described above. Homozygosity for all of the mutants identified in the F$_2$ generation was further confirmed in the F$_3$ by the lack of segregation.

RNA Extraction and Detection

Seedlings were grown on plates containing Murashige and Skoog medium or on Murashige and Skoog medium supplemented with different concentrations of INA for 2 weeks. RNA was extracted from seedlings by using the method described previously (Bowling et al., 1994). To synthesize cDNA, RNA (10 μg) was treated with 1 unit of *DNase*I (Ambion) for 30 min at 37°C prior to RT. First-strand cDNA synthesis was carried out with the oligo(dT) primer using the first-strand cDNA synthesis kit (Invitrogen) following the manufacturer's protocol. Gene-specific primers were designed using LightCycler probe design software (Roche). Real-time PCR was carried out on a 10× diluted reverse-transcribed template using a Quantitect SYBR green PCR kit (Qiagen) in LightCycler (Roche) following the manufacturer's protocol. Primers used for real-time PCR are described in Tables I and II. Northern-blot analysis was carried out as described previously in Clarke et al. (1998).

Pathogen Infection

Fully expanded leaves of 3- to 4-week-old soil-grown plants were infiltrated with *Psm*4326 suspension in 10 mM MgCl$_2$ at OD$_{600}$ = 0.0001. Disease symptoms were recorded 4 d postinfection. To determine bacterial growth, infected leaves were collected at 0 and 3 d after inoculation. Leaf discs of the same size were made using a hole puncher and bacterial titers from those leaf discs were determined.

Supplemental Data

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

Supplemental Figure S1. Western blot of TGA2 in *tga2-2* mutant.

Supplemental Figure S2. Expression of *PR* genes in *tga3-1*.

Supplemental Figure S3. Expression of *PR* genes in *tga2-2* and *TGA6$_{ACT}$*.

Supplemental Figure S4. Relative expression of *PR-1* in *tga2-2 TGA6$_{ACT}$* and *tga2-2 tga3-1 TGA6$_{ACT}$*.

Supplemental Figure S5. Expression of *BiP2* and *GRP94* in *tga2-2 sni1-1* mutant.

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