Knockout Analysis of Arabidopsis Transcription Factors TGA2, TGA5, and TGA6 Reveals Their Redundant and Essential Roles in Systemic Acquired Resistance

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Arabidopsis nonexpresser of pathogenesis-related (PR) genes (NPR1) is the sole positive regulator that has been shown to be essential for the induction of systemic acquired resistance. In npr1 mutant plants, salicylic acid (SA)–mediated PR gene expression and pathogen resistance are abolished completely. NPR1 has been shown to interact with three closely related TGA transcription factors—TGA2, TGA5, and TGA6—in yeast two-hybrid assays. To elucidate the biological functions of these three TGA transcription factors, we analyzed single and combined deletion knockout mutants of TGA2, TGA5, and TGA6 for SA-induced PR gene expression and pathogen resistance. Induction of PR gene expression and pathogen resistance by the SA analog 2,6-dichloroisonicotinic acid (INA) was blocked in tga6-1 tga2-1 tga5-1 but not in tga6-1 or tga2-1 tga5-1 plants. Loss of INA-induced resistance to Peronospora parasitica Noco2 cosegregated with the tga6-1 mutation in progeny of multiple lines that were heterozygous for tga6-1 and homozygous for tga2-1 tga5-1 and could be complemented by genomic clones of wild-type TGA2 or TGA5, indicating that TGA2, TGA5, and TGA6 encode redundant and essential functions in the positive regulation of systemic acquired resistance. In addition, tga6-1 tga2-1 tga5-1 plants had reduced tolerance to high levels of SA and accumulated higher basal levels of PR-1 under noninducing conditions, suggesting that these TGA factors also are important for SA tolerance and the negative regulation of the basal expression of PR-1.

INTRODUCTION

Systemic acquired resistance (SAR) is a general defense response that develops in the distal, uninjured parts of plants after local infection by an avirulent pathogen (Ryals et al., 1996). SAR is effective against a broad spectrum of microbial pathogens. One important signal molecule in SAR is salicylic acid (SA), which is required and sufficient for the induction of pathogenesis-related (PR) genes and pathogen resistance during SAR. When attacked by pathogens, plants synthesize and accumulate higher levels of SA in both infected and systemic tissues (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991). Application of SA or SA analogs, such as 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole, also induces both PR gene expression and pathogen resistance in plants (White, 1979; Métraux et al., 1991; Görlich et al., 1996). In addition, blocking SA accumulation by expressing the bacterial SA-degrading enzyme salicylate hydroxylase prevents the induction of PR genes and SAR in transgenic plants (Gaffney et al., 1993). The importance of SA in plant defense also is confirmed by analyzing mutants that are deficient in SA synthesis. In Arabidopsis, mutations in enhanced disease susceptibility5 (EDS5) and SA induction-deficient2 (SID2) block pathogen-induced SA synthesis and render the plants more susceptible to pathogen infection (Rogers and Ausubel, 1997; Nawrath and Métraux, 1999). EDS5 encodes a member of the MATE transporter family and is likely to be involved in transporting one of the precursors for the biosynthesis of SA (Nawrath et al., 2002). SID2 encodes an isochorismate synthase, suggesting that SA accumulated during pathogen infection is derived from chorismate (Wildermuth et al., 2001).

Several different genetic screens were conducted to identify regulatory genes downstream of SA. All 12 SA-nonresponsive mutants identified contain mutations in nonexpressor of PR genes (NPR1) (also known as NIM1 and SAI1) (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). In npr1 plants, induction of PR genes and pathogen resistance by SA are abolished. NPR1 encodes a protein with no obvious biochemical functions except the presence of two protein–protein interaction domains, a BTB/POZ domain at the N-terminal end and an ankyrin-repeat domain in the central region (Cao et al., 1997; Ryals et al., 1997; Aravind and Koonin, 1999). The presence of protein–protein interaction domains in NPR1 suggests that NPR1 may regulate SA signaling through an association with other proteins.

Several groups have performed yeast two-hybrid screens using NPR1 as bait and found multiple TGA transcription factors that can interact with NPR1 (Zhang et al., 1999; Després et al., 2000; Niggeweg et al., 2000a; Zhou et al., 2000; Chern et al., 2001; Kim and Delaney, 2002). We showed previously that TGA2 (also known as AHBP-1b), TGA5 (also known as OBF5), and TGA6 interact with NPR1, with TGA2 and TGA6 exhibiting strong affinity and TGA5 showing weaker affinity to NPR1 in the yeast two-hybrid assay (Zhang et al., 1999). The interaction between...
NPR1 and TGA2 was demonstrated in vivo (Subramaniam et al., 2001; Fan and Dong, 2002), and the involvement of TGA transcription factors in SA signaling is supported further by the presence of a TGA binding site in the PR-1 promoter that is essential for SA-induced PR-1 expression (Lebel et al., 1998). However, genetic evidence for the roles of these TGA transcription factors in SAR is lacking. Here, we report that TGA2, TGA5, and TGA6 encode redundant functions and are essential for the induction of SAR.

RESULTS

Isolation of tga6-1 and Construction of the tga6-1 tga2-1 tga5-1 Triple Mutant

Using primers flanking TGA6 (At3g12250), we screened an Arabidopsis deletion mutant population by PCR and identified a deletion mutant for TGA6 named tga6-1. Sequence analysis of the deletion mutation revealed that a fragment of ~2.7 kb between nucleotides 30,861 and 33,564 on BAC clone F28J15 was deleted (Figure 1). Sequence comparison between TGA6 cDNA and the genomic sequence revealed that the cDNA of TGA6 is transcribed from the region between nucleotides 30,249 and 33,651 of F28J15 and that the coding sequence is located between nucleotides 31,032 and 33,264. Thus, the deletion in tga6-1 occurred within TGA6 and removed the entire coding region. TGA2 (At5g06950) and TGA5 (At5g06960) are linked directly, and the distance between these two genes is ~2 kb. We reported previously the identification of a mutant with both TGA2 and TGA5 deleted (Li et al., 2001). This mutant is named tga2-1 tga5-1. To obtain a triple mutant for TGA2, TGA5, and TGA6, we crossed tga6-1 with tga2-1 tga5-1 and screened the resulting F2 population for homozygous mutants at both loci. Two independent F2 lines that are homozygous at both loci were obtained, and the progeny of these two lines were used for subsequent phenotypic analysis. F2 lines heterozygous at the tga6-1 locus and homozygous at the tga2-1 tga5-1 locus also were obtained, and they were used later for cosegregation analysis.

The TGA Triple Knockout Mutant Is More Sensitive to the Toxicity of SA

Previously, npr1-1 plants were shown to be more sensitive to high concentrations of SA (Cao et al., 1997). To determine whether the tga mutants also have altered responses to SA, the mutant seeds were plated on MS medium (Murashige and Skoog, 1962) containing 0.2 mM SA. Similar to npr1-1, tga6-1 tga2-1 tga5-1 plants were highly sensitive to SA. As shown in Figure 2, the growth of tga6-1 tga2-1 tga5-1 plants was arrested at the cotyledon stage and the seedlings were bleached, whereas tga6-1 and tga2-1 tga5-1 grew like the wild type under the same conditions. Thus, these TGA transcription factors play roles similar to that of NPR1 in the regulation of tolerance to SA.

Induction of PR-1 by INA Is Blocked in the TGA Triple Knockout Mutant

In Arabidopsis, PR-1 is highly induced during SAR (Uknes et al., 1992). The expression of PR-1 also can be induced by exogenous application of SA or the SA analog INA. Mutations in NHR1 completely block the induction of PR-1 by SA or INA. To determine whether TGA2, TGA5, and TGA6 encode functions similar to those of NHR1, we analyzed the expression levels of PR-1 in the TGA knockout mutants under inducing and noninducing conditions. Because SA is highly toxic to the TGA triple mutant, we used INA as the inducing agent.

In wild-type plants, PR-1 was induced strongly by INA treatment. This induction was not affected by either tga6-1 or the tga2-1 tga5-1, because both tga6-1 and tga2-1 tga5-1 plants accumulated levels of PR-1 similar to those in wild-type plants after INA induction (Figure 3). By contrast, PR-1 was no longer induced by INA in the tga6-1 tga2-1 tga5-1 triple knockout mutant, sug-
suggesting that SAR is compromised when all three TGA factors are mutated.

Interestingly, under noninducing conditions, the basal level of PR-1 was affected in the TGA knockout mutants. In tga6-1 tga2-1 tga5-1 and tga2-1 tga5-1 plants, the expression level of PR-1 was ∼50-fold and 10-fold higher than that of wild-type plants, respectively (Figure 3). No significant change in the basal level of PR-1 was observed in tga6-1 plants.

SAR Is Abolished in TGA Triple Knockout Mutants

To determine whether INA-induced pathogen resistance was affected in the TGA knockout mutants, 2-week-old wild-type and mutant plants were treated with 0.33 mM INA and inoculated with the virulent oomycete pathogen Peronospora parasitica Noco2 after 3 days. The INA-treated wild-type plants were immune to P. parasitica Noco2 infection, because no conidiophores were observed on the plants 7 days after inoculation. This induced immunity was not affected by the tga6-1 or tga2-1 tga5-1 mutations, because both mutants were as resistant as the wild type (Figures 4A and 4B). By contrast, the INA-induced resistance was abolished completely in tga6-1 tga2-1 tga5-1, indicating that SAR is compromised in the TGA triple mutant.

We further tested whether systemic resistance can be induced by an avirulent pathogen in tga6-1 tga2-1 tga5-1 plants. As shown in Figure 5, Pseudomonas syringae pv tomato (P.s.t) DC3000 carrying avrRpt2 induced systemic resistance to Pseudomonas syringae pv maculicola (P.s.m) ES4326 in wild-type plants but not in tga6-1 tga2-1 tga5-1 plants, further suggesting that SAR is compromised in the TGA triple mutant.
time point. This experiment was repeated once with similar results. avr, and the bacterial titers were measured. Error bars represent 95% confidence limits of log-transformed data. Four samples were taken for each time point. This experiment was repeated once with similar results. avr, P.s.t. DC3000 avrRpt2; cfu, colony-forming units; f.w., fresh weight; tga triple, tga6-1 tga2-1 tga5-1.

Compromised SAR in tga6-1 tga2-1 tga5-1 Cosegregates with tga6-1

As shown in Figure 1, the deletion in tga6-1 affected only TGA6. To determine whether the loss of INA-induced resistance in the TGA triple mutant cosegregated with the tga6-1 deletion, we analyzed four independent F2 lines that were heterozygous for the tga6-1 deletion but homozygous for the tga2-1 tga5-1 deletion. The F3 plants of these lines were analyzed for susceptibility to P. parasitica Noco2 after INA treatment. As shown in Table 1, in all four lines tested, approximately one-fourth of the progeny lostINA-induced resistance to P. parasitica Noco2. To determine whether the susceptible plants were homozygous at the tga6-1 locus, DNA from each individual susceptible plant was analyzed by PCR using primers within the TGA6 homolog were used to probe the functions of TGA transcription factors in Arabidopsis and tobacco (Niggeweg et al., 2000b; Pontier et al., 2001; Fan and Dong, 2002). Phenotypes of the transgenic plants differed dramatically depending on the specific dominant-negative mutant used. In one study, tobacco plants overexpressing a dominant-negative form of TGA2 exhibited higher levels of PR gene induction by pathogen challenge and an enhanced SAR, leading to the conclusion that TGA factors are not essential for PR gene activation or SAR (Pontier et al., 2001). In another study, INA-induced PR-1 expression was reduced in plants accumulating high levels of a truncated form of TGA2, although it is unclear whether INA-induced pathogen resistance was affected in these plants (Fan and Dong, 2002). Similarly, a reduction of SA-induced PR gene expression also was observed in transgenic plants overexpressing a dominant-negative mutant of tobacco TGA2.2 (Niggeweg et al., 2000b).

Because dominant-negative mutants most likely affect multiple independent TGA factors to various degrees, it is difficult to determine the functions of individual TGA factors using this approach. Instead, we created knockout plants of TGA2, TGA5, and TGA6 and assayed the single and combined mutants for altered regulation of SA signaling. We found that both PR gene expression and pathogen resistance cannot be induced by INA or avirulent pathogens in the tga6-1 tga2-1 tga5-1 triple knockout mutant, suggesting that these TGA transcription factors serve as essential positive regulators of SAR. We also showed

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Figure 5. Growth of P.s.m. ES4326 in Wild-Type and tga6-1 tga2-1 tga5-1 Plants Preinoculated with P.s.t. DC3000 avrRpt2.

Two leaves from each plant were infiltrated with P.s.t. DC3000 avrRpt2 (OD600 = 0.02) in 10 mM MgCl2 solution or with the buffer alone 3 days before P.s.m. ES4326 infection (OD600 = 0.001). Leaf discs within the inoculated area were taken at 0 and 3 days after P.s.m. ES4326 infection, and the bacterial titers were measured. Error bars represent 95% confidence limits of log-transformed data. Four samples were taken for each time point. This experiment was repeated once with similar results. avr, P.s.t. DC3000 avrRpt2; cfu, colony-forming units; f.w., fresh weight; tga triple, tga6-1 tga2-1 tga5-1.

DISCUSSION

Although TGA transcription factors have been suggested to be important regulators of SA signaling, it was unclear whether they are essential for the establishment of SAR. In previous studies, transgenic plants overexpressing dominant-negative forms of TGA2 or a tobacco TGA2 homolog were used to probe the functions of TGA transcription factors in Arabidopsis and tobacco (Niggeweg et al., 2000b; Pontier et al., 2001; Fan and Dong, 2002). Phenotypes of the transgenic plants differed dramatically depending on the specific dominant-negative mutant used. In one study, tobacco plants overexpressing a dominant-negative form of TGA2 exhibited higher levels of PR gene induction by pathogen challenge and an enhanced SAR, leading to the conclusion that TGA factors are not essential for PR gene activation or SAR (Pontier et al., 2001). In another study, INA-induced PR-1 expression was reduced in plants accumulating high levels of a truncated form of TGA2, although it is unclear whether INA-induced pathogen resistance was affected in these plants (Fan and Dong, 2002). Similarly, a reduction of SA-induced PR gene expression also was observed in transgenic plants overexpressing a dominant-negative mutant of tobacco TGA2.2 (Niggeweg et al., 2000b).

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that genomic clones containing either TGA2 or TGA5 can complement the loss of the SAR phenotype in the TGA triple mutant. Thus, either TGA2 or TGA5 is sufficient for INA-induced PR gene expression and pathogen resistance. Because the loss of the SAR phenotype cosegregated with the *tga6-1* mutation and was observed only in the triple mutant but not in *tga2-1 tga5-1*, TGA6 also is sufficient for INA-induced PR gene expression and pathogen resistance. These data demonstrate that TGA2, TGA5, and TGA6 encode redundant functions in the induction of SAR.

Furthermore, we found that *tga2-1 tga5-1* and *tga6-1 tga2-1 tga5-1* accumulated increased levels of PR-1 under noninducing conditions, suggesting that these TGA transcription factors repress the basal expression of PR-1. The higher basal level of
PR-1 in the triple mutant, compared with the double mutant, suggests that TGA6 is partially responsible for the negative regulation of basal levels of PR-1. On the other hand, either TGA2 or TGA5 appeared to be sufficient to suppress the basal expression of PR-1, because transforming the genomic clone of either TGA2 or TGA5 into the triple mutant reverted PR-1 expression to the wild-type level. The increased PR-1 expression probably is the result of the loss of binding of TGA factors to a negative element on the PR-1 promoter. This hypothesis is supported by the presence of an as-1-related TGACG element (LS5) that functions as a weak silent in the PR-1 promoter (Lebel et al., 1998).

In addition to the loss of SAR phenotypes, npr1 plants also exhibited reduced tolerance to high concentrations of SA and enhanced susceptibility to the bacterial pathogen P.s.m. (Lebel et al., 1998). The primers used to amplify TGA genes. The distance between the primers is 51,840 lines was screened by PCR using primers flanking the gene. The primers used to amplify

**METHODS**

**Generation of Triple Knockout Mutants for TGA2, TGA5, and TGA6**

TGA2 and TGA5 are located next to each other, and the identification of tga2-1 tga5-1 was described previously (Li et al., 2001). To identify a deletion mutant for TGA6, an Arabidopsis thaliana deletion mutant population of 51,840 lines was screened by PCR using primers flanking the gene. The distance between the primers is ~9 kb. The PCR extension time was set at 1.5 min to avoid amplification of the wild-type DNA fragment. A single deletion mutant, tga6-1, was detected initially in one of the megapools containing 2592 lines. Individual mutant plants were isolated subsequently by deconvolution as described previously (Li et al., 2001). tga6-1 tga2-1 tga5-1 was generated using pollen from tga6-1 plants to fertilize tga2-1 tga5-1 plants. The F1 plants were selfed, and tga6-1 tga2-1 tga5-1 identified in the F2 generation by PCR using primers within the deletions to confirm homozygosity at both loci.

**Analysis of PR Gene Expression in the Mutant Plants**

To analyze gene expression levels by real-time reverse transcription-PCR, total RNA samples were prepared from 20-day-old plants grown on MS medium (Murashige and Skoog, 1962), with or without INA, using the Totally RNA kit from Ambion (Austin, TX). Reverse transcription of the cDNA was performed using the RT-for-PCR kit from Clontech (Palo Alto, CA). Real-time PCR was performed using the QuantiTect SYBR Green PCR kit from Qiagen (Valencia, CA). The primers used to amplify PR-1 were 5’-GTAGGTTGCTTCTTGGTCCC-3’ and 5’-CACATATAATTCCCAAGCATC-3’. The primers used to amplify ACTIN1 were 5’-CGATGAGGCTOAATCTCAAACGA-3’ and 5’-CAGAGTCAGACAGAATACCCG-3’.

**Pathogen Infections**

Both tga6-1 and tga2-1 tga5-1 are in the Columbia ecotype background, and Pseudomonas syringae pv maculicola (P.s.m.) is virulent on this ecotype. Infection of wild-type and tga plants with P. parasitica Noco2 was performed by spraying a suspension of conidia (~5  × 10^5 spores/mL water) on 2-week-old soil-grown plants. Inoculated plants were maintained subsequently in a Conviron TC16 growth chamber (Winnipeg, Canada) at 18°C with a 12-h photoperiod and ~80% RH. A disease rating was determined for each plant according to Cao et al. (1998) at 7 days after inoculation. For each genotype and treatment, 25 plants were scored.

Infection with the virulent bacterial pathogen Pseudomonas syringae pv maculicola (P.s.m.) was performed by infiltrating leaves of 4-week-old soil-grown wild-type and mutant plants with a bacterial suspension at OD600 = 0.001, which is the dose that normally causes disease in wild-type plants. Symptoms were examined 3 days after inoculation. The bacterial titer in the leaves was measured according to a previously described procedure (Cao et al., 1994).

**Complementation of tga6-1 tga2-1 tga5-1 Plants by Wild-Type Genes**

A 5.9-kb KpnI-BamHI fragment (MOJ9, nucleotides 28,249 to 34,162) containing TGA2 was subcloned from P1 clone MOJ9 to pGreen229 (Hellens et al., 2000) to create pG229-TGA2. A 4.6-kb EcoRI-SacI fragment (MOJ9, nucleotides 33,110 to 37,773) containing TGA5 was subcloned from MOJ9 to pGreen229 to create pG229-TGA5. pG229-TGA2 and pG229-TGA5 were transformed into tga6-1 tga2-1 tga5-1 using the floral-dip method (Clough and Bent, 1998), and transformants were selected on soil by spraying the T1 plants with the herbicide glucosinolate. At least 10 transformants were obtained for each construct. All assays on the complementing lines were performed on glucosinolate-resistant T2 plants.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Yuelin Zhang, yuelin@interchange.ubc.ca

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