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Alteration of TGA factor activity in rice results in enhanced tolerance to *Xanthomonas oryzae* pv. *oryzae*

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Summary

In dicotyledonous plants broad-spectrum resistance to pathogens is established after the induction of the systemic acquired resistance (SAR) response. In Arabidopsis the NPR1 protein can regulate SAR by interacting with members of the TGA class of basic, leucine-zipper transcription factors to alter pathogenesis-related (PR) gene expression. Overexpression of (*At*)*NPR1* in Arabidopsis enhances resistance to multiple pathogens. Similarly, overexpression of (*At*)*NPR1* in rice enhances resistance to the bacterial pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). These results suggest that components of the (*At*)*NPR1*-mediated SAR defense response may be conserved between monocots and dicots. To determine whether or not rice TGA factors are involved in disease resistance responses, the effect of altering the function of rice TGA2.1 was analyzed in transgenic plants. Transgenic rice overexpressing an rTGA2.1 mutant, that can no longer bind DNA, and transgenic rice that have the endogenous rTGA2.1 silenced by dsRNA-mediated silencing were generated. Both types of transgenic rice displayed increased tolerance to *Xoo*, were dwarfed, and had altered accumulation of *PR* genes. The results presented in this study suggest that wild-type rTGA2.1 has primarily a negative role in rice basal defense responses to bacterial pathogens.

Keywords: TGA factors, SAR, rice, *Xoo*, resistance, tolerance.

Introduction

The systemic acquired resistance (SAR) response is a basal plant immune response induced after infection by pathogens that trigger localized necrosis (Ryals *et al.*, 1996). Once SAR is initiated, plants display heightened resistance to viral, bacterial and fungal pathogens. In Arabidopsis and tobacco key cellular features correlated with the onset of SAR are a rise of salicylic acid (SA) levels and the upregulation of pathogenesis-related (*PR*) gene expression (Malamy *et al.*, 1990; Maleck *et al.*, 2000; Metraux *et al.*, 1990; Vernooij *et al.*, 1994; Ward *et al.*, 1991). The Arabidopsis NPR1 (non-expressor of *PR* genes, also called NIM1, SAI1) protein is essential for the transmission of the signal associated with the rise in SA, for disease resistance and for the expression of several *PR* genes (Cao *et al.*, 1994; Delaney *et al.*, 1994, 1995; Glazebrook *et al.*, 1996; Shah *et al.*, 1997). (At)NPR1 is a novel protein containing ankryin repeats and other protein–protein interaction domains (Cao *et al.*, 1997; Ryals *et al.*, 1997). The regulation of *PR* genes by (At)NPR1 relies on the ability of (At)NPR1 to localize to the cell nucleus after induction of SAR, where it can interact with bZIP transcription factors of the TGA family (Johnson *et al.*, 2003; Kinkema *et al.*, 2000; Subramaniam *et al.*, 2001; Zhang *et al.*, 1999; Zhou *et al.*, 2000). When cellular redox alterations are triggered by rising SA levels (Mou *et al.*, 2003) (At)NPR1 undergoes a conformational change from a cytosolic oligomeric form to a monomeric form that exposes its nuclear import signal that may allow translocation into the nucleus.

(At)NPR1 is involved in multiple plant defense responses, including the jasmonic acid-mediated responses to chewing insects (Spoel *et al.*, 2003) and induced systemic resistance triggered by rhizobacteria (Pieterse *et al.*, 2001). However, one likely central function of (At)NPR1 is the regulation of defense gene expression through its interactions with the TGA factors (Zhang *et al.*, 1999). TGA factors are likely to have distinct roles in mediating gene expression. Each TGA is unique in regard to its (i) interaction with (At)NPR1, (ii) homodimerization or heterodimerization capacity, (iii) binding of consensus DNA target sites, (iv) tissue expression

pattern, and (v) post-transcriptional regulation (Despres *et al.*, 2003; Johnson *et al.*, 2001; Lam and Lam, 1995; Niggeweg *et al.*, 2000a,b; Pontier *et al.*, 2002; Zhou *et al.*, 2000). For example, (At)NPR1 does not interact equivalently with all TGA family members; TGA2 and TGA3 interact most strongly, followed by TGA5 and TGA6 (Despres *et al.*, 2000; Kim and Delaney, 2002; Niggeweg *et al.*, 2000b; Zhang *et al.*, 1999; Zhou *et al.*, 2000). The interaction of (At)NPR1 with some TGA factors can also increase the DNA-binding capacity of at least a subset of TGA factors (Despres *et al.*, 2000). Adding to this complexity, the TGA factors are likely to form heterodimers with other TGA family members, and possibly other classes of transcription factors (Niggeweg *et al.*, 2000a). For instance, TGA4 interacts with AtEBP, a protein that binds to ethylene-responsive elements in many gene promoters (Buttner and Singh, 1997). While TGA2 appears to be expressed in all plant tissues, TGA1 and TGA3 are predominantly expressed in younger tissues and plant roots (Pontier *et al.*, 2002; Schiermeyer *et al.*, 2002). Taken together, these results predict specific regulation and activity of each TGA.

TGA factors bind to the TGACGT motifs first identified in the activation sequence-1 (*as-1*) element of the cauliflower mosaic virus (CaMV) 35S promoter (Krawczyk *et al.*, 2002; Lam and Lam, 1995; Lam *et al.*, 1989; Zhang *et al.*, 1999; Zhou *et al.*, 2000). Plant genes with promoters containing *as-1*, or *as-1*-like elements, fall into at least two classes; an 'immediate early' class, that is rapidly induced by chemicals including SA and auxin, and a 'late' class associated predominantly with SA inducibility, typified by the Arabidopsis *PR-1* gene (Lebel *et al.*, 1998; Liu and Lam, 1994; Qin *et al.*, 1994). The TGA factors are proposed to differentially regulate these two gene classes through differences in affinity for the promoter consensus sites (Krawczyk *et al.*, 2002).

Precisely how TGA factors regulate *PR* gene expression in Arabidopsis and tobacco is a topic under intense investigation. While several studies indicate that interaction with (At)NPR1 is key to this regulation, transgenic Arabidopsis carrying mutations in any single TGA do not display an obvious phenotype (Fan and Dong, 2002). However, an Arabidopsis triple mutant harboring deletions of *TGA2*, *TGA5* and *TGA6* is compromised in its ability to induce *PR-1* expression and mount a SAR response, suggesting some overlapping functionalities among subsets of TGA factors (Zhang *et al.*, 2003). Several laboratories have generated transgenic Arabidopsis and tobacco that either over-expressed different TGA factors, or attempted to disrupt their function through antisense technology, dominant-negative mutants, and virus-induced gene silencing (Ekengren *et al.*, 2003; Fan and Dong, 2002; Kim and Delaney, 2002; Miao and Lam, 1995; Niggeweg *et al.*, 2000a; Pontier *et al.*, 2001). These studies indicate both positive and negative functions for two factors, TGA2 and TGA5, in regard to gene regulation and resistance against bacterial or fungal pathogens. Therefore, it does not appear that data from one plant species will be sufficient to predict the behavior of individual TGAs in all plant species. Any plant engineering for agronomical traits will require investigation of the role of TGAs in individual crops.

TGA factors appear to be conserved between dicots and monocots at the nucleotide level, but the extent of functional conservation is not yet known. At least 10 Arabidopsis TGA or TGA-like bZIP factors have been identified and four TGA factors have been isolated from tobacco (Jakoby *et al.*, 2002; Schiermeyer *et al.*, 2002). Wheat has several HBP-1b/TGA factors and at least four rice (At)NPR1-interacting TGA and TGA-like factors have been identified through yeast two-hybrid screens (Chern *et al.*, 2001; Mikami *et al.*, 1994). In previous experiments we have demonstrated that the rice TGA, rTGA2.1, binds *in vitro* to the Arabidopsis NPR1 protein (Chern *et al.*, 2001), as well as to an (At)NPR1-homolog isolated from rice (Chern *et al.*, 2005). In addition, we have shown that rTGA2.1 binds to oligonucleotides containing the *as-1*-like element from the *PR-1* gene promoter and to the promoter of the rice chitinase gene, *RCH10*. Overexpression of (*At*)*NPR1* in rice enhances resistance to the rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), indicating that several of the components mediating the SAR pathway in dicots are conserved in rice and may function analogously (Chern *et al.*, 2001). Together, these data suggest that rTGA2.1 may be involved in rice defense gene regulation through interaction with an (At)NPR1 rice homolog.

To examine the role of rTGA2.1 in disease resistance, we have generated transgenic rice ectopically expressing a putative dominant-negative (DN) rTGA2.1 mutant that can no longer bind DNA and rice that have the endogenous rTGA2.1 transcripts silenced via dsRNA-mediated silencing (SI). Our data indicate that the loss of rTGA2.1 activity in both the DN and SI lines leads to reduced disease symptom development in rice infected with *Xoo* and to reduced plant stature, phenotypes that have not yet been observed in Arabidopsis or tobacco. Since a mutant form of rTGA2.1 resulted in enhanced tolerance to pathogens, the data suggest that the wild-type protein may function to both positively and negatively regulate a subset of rice defense genes.

Results

Construction and in vitro *characterization of a mutant rTGA2.1 protein that can no longer bind DNA*

Our previous work demonstrated that at least four (At)NPR1-interacting TGA-like factors exist in rice and that one of these, rTGA2.1, is competent to bind to defense gene promoters (Chern *et al.*, 2001). As the role of each TGA may be context dependent, and several key aspects of the SAR

signaling pathway are altered in rice plants (such as SA levels), we wanted to determine whether or not rTGA2.1 could be employed as a tool for generating disease-resistant rice plants. For one approach to generating plants with reduced or eliminated rTGA2.1 activity, we transformed rice with a mutant rTGA2.1, (DN), in which the DNA-binding activity was eliminated.

The rTGA2.1 (DN) mutant was constructed based on the design of the dominant-negative, tobacco TGA2.2 mutant (Niggeweg *et al.*, 2000a). Using a PCR-based strategy, full-length cDNA encoding rTGA2.1 was amplified with alterations in nucleotides that changed two alanine residues in the DNA-binding domain to prolines (Figure 1a; drawing not to scale). The introduction of prolines at these positions was predicted to disrupt the alpha helices that participate in stabilizing bZIP/DNA interactions (Miao and Lam, 1995; Pu and Struhl, 1991). Additionally, as only two amino acid residues were altered, the activities of other important protein domains in rTGA2.1 were more likely to be maintained. The DN mutant protein was also fused in-frame to a c-myc epitope tag to allow for protein detection *in planta*. Previous studies with rTGA2.1 fused to c-myc have demonstrated that the recombinant protein retains the capacity to interact with (At)NPR1 and bind to the promoters of rice defense genes (Chern *et al.*, 2001). To ensure that the DNA-binding capacity of rTGA2.1 (DN) was reduced by these mutations, the mutant protein was purified from *Escherichia coli* as a histidine-tagged protein and used in electrophoretic gel mobility assays (EMSA). For the EMSA, a double-stranded oligonucleotide containing canonical TGA-binding sites and modeled after the SA-responsive, Arabidopsis *PR-1* gene promoter (Lebel *et al.*, 1998) was labeled with ^{32}P and incubated with recombinant DN and wild-type rTGA2.1. The wild-type rTGA2.1-c-myc protein has been previously shown to bind well to this oligonucleotide sequence (Chern *et al.*, 2001). In the EMSA (Figure 1b), no protein/DNA complex is observed with control GFP::HIS protein (lane 2). Two bands corresponding to primary and secondary complexes formed between the DNA and wild-type rTGA2.1-myc are visible in lanes 3 and 4. However, when rTGA2.1 (DN) is added to the reaction no bands corresponding to DNA/protein complexes are observed even when high protein levels are added (lanes 6–8). As Western blots carried out concurrently with this EMSA show that substantial protein is present and has not been degraded (data not shown), these results indicate that the ability of rTGA2.1 (DN) to bind DNA is eliminated entirely. Therefore over-expression of this protein, *in vivo*, is predicted, although not formally shown, to interrupt the DNA-binding ability of the endogenous rTGA2.1.

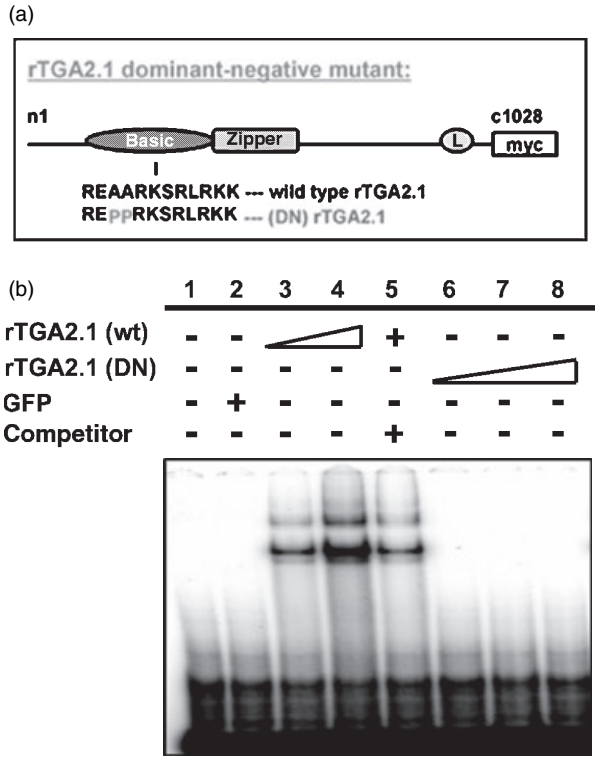


Figure 1. Construction and purification of a mutant rTGA2.1 protein.
(a) Two alanine-to-proline mutations were introduced by PCR into the basic DNA-binding domain of rTGA2.1 to create a putative dominant-negative (DN) mutant. Basic: a DNA binding domain; zipper: a leucine zipper; L: a leucine-rich domain; myc: an epitope tag fused to the C-terminus for protein detection in plants. Drawing is not to scale.
(b) Electrophoretic gel shift analysis (EMSA) using an SA-responsive element (SARE). WT and rTGA2.1 (DN) proteins were tested for the ability to bind to an oligonucleotide probe containing the TGA factor consensus binding site. Lane 1, free probe; lane 2, (6X) histidine-tagged GFP; lanes 3 and 4, increasing concentrations of WT protein (1 and 5X); lane 5, wild-type protein (5X) with wild-type non-radioactively labeled competitor oligonucleotides; lanes 6–8, increasing levels of DN protein (1, 5 and 10X). Blot is visualized using radiography.

Protein expression in rTGA2.1 (DN) transgenic rice

Much of the interest in understanding plant defense signaling pathways lies in the potential use of pathway components to engineer crop plants that are more resistant to disease. To test whether rTGA2.1 could affect rice disease resistance, the DN construct was placed under control of the strong, constitutive, maize ubiquitin promoter (Christensen and Quail, 1996) and transformed into callus tissue of the Liao Geng (LG) rice cultivar. Nine independently transformed lines were generated. The presence of the recombinant DN protein in rice leaf tissue was monitored in the (T_1) offspring plants of DN lines 1AD, 1CD, 2BD and 4AD. In the T_1 generation, plants segregate for the presence of the transgene. Protein was detected using either commercial monoclonal α-c-myc antibodies, or polyclonal α-rTGA2.1 antibodies (Figure 2a) raised against purified, full-length rTGA2.1 recombinant protein (see Experimental procedures). The α-c-myc antibody detects one predominant band corresponding to the full-length DN rTGA2.1-myc protein in lines containing the DN transgene but does not detect any

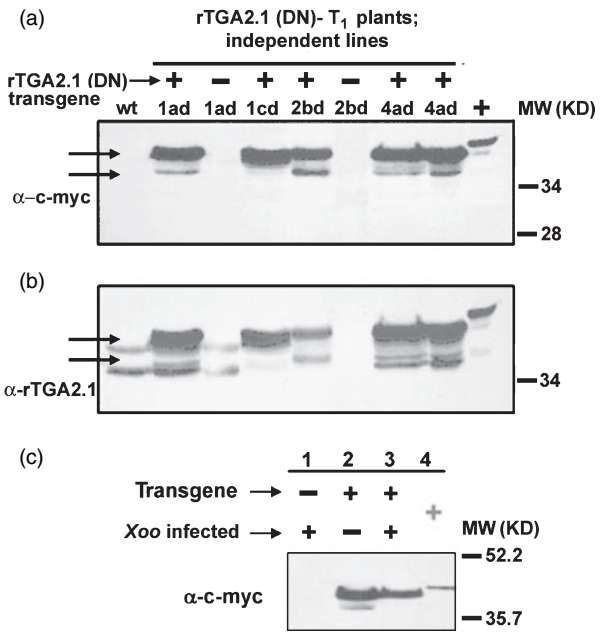


Figure 2. Mutant rTGA2.1 protein expression in transgenic rice.
(a, b) T_1 plants from four independently transformed lines (1AD, 1CD, 2BD and 4AD) express rTGA2.1(DN) protein. Mutant rTGA2.1(DN) protein was detected using antibodies to either a c-myc epitope tag (α-c-myc) or against rTGA2.1. rTGA2.1 (DN) protein and its possible degradation products are labeled with arrows. Note: the 2BD, transgene (–) sample has very low levels of endogenous protein that are observable only with longer exposure times.
(c) The rTGA2.1 (DN) protein is detected in leaf extracts from T_1 plants of rice line 4A, with (+) or without (–) infection with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Lanes with a (+) contain only *Escherichia coli*-expressed rTGA2.1 (DN) protein.

bands in the wild-type (LG) controls or transgene (–) plants (Figure 2a). This predominant band migrates more slowly than the predicted molecular mass of the protein. A smaller band that may correspond to a degradation product of the DN protein is also observed with this antibody. The α-rTGA2.1 antibody detects multiple bands on the Western blot (Figure 2b); a predominant high molecular weight band corresponding to recombinant rTGA2.1 (top arrow panels a and b), and a presumed degraded, or truncated product (top arrow panels a and b). In addition, the α-rTGA2.1 antibody detects at least two additional bands that migrate faster than the recombinant protein. These additional bands are predicted to correspond to either the endogenous or additional isoforms of rTGA2.1, or cross-detection of other rice TGA factors by this antibody. Without genetic null mutants for the rice TGA factors, these two classes cannot be distinguished easily.

The Arabidopsis TGA proteins, TGA1 and TGA3, are post-transcriptionally regulated and in some cases subject to proteolysis in mature plant tissues (Despres *et al.*, 2003; Pontier *et al.*, 2002). The presence of recombinant rTGA2.1 (DN) protein was monitored in leaf 4 of plants ranging in age from young (4 weeks) to mature (10 weeks) to evaluate mutant protein stability throughout plant development. Protein abundance in leaves of different developmental stages within mature (10 weeks old) rice, leaves 4 and 10 from individual plants were also evaluated. The DN mutant protein was detected in all samples (data not shown). The presence of the DN protein was also analyzed in rice leaves after infection with *Xoo* (Figure 2c). Again, the DN protein was present at high levels after infection. These results suggest that the DN protein is present under most conditions, and degradation, if it occurs, does not dramatically reduce the steady-state concentration of the mutant protein.

Reduction in rTGA2.1 activity by RNA-mediated silencing in transgenic rice

The DN mutant protein was designed to disrupt the ability of the endogenous rTGA2.1 to bind to gene promoters. However, if rTGA2.1 heterodimerizes with other TGAs the mutant protein may not act specifically, and may interfere with the binding of multiple TGA factors. As a means of more specifically disrupting rTGA2.1 activity, a construct predicted to silence *rTGA2.1* through double-strand (ds)-mediated RNA silencing was generated. This construct was designed to place rTGA2.1 on either side of a non-coding β-glucuronidase (GUS) fragment in an inverted repeat, thus forming an RNA with a hairpin-loop structure (Figure 3a). Similar structures have been demonstrated to generate silencing of a transgenic GUS gene in rice (Helliwell and Waterhouse, 2003; Wang and Waterhouse, 2000). One region within this fragment of rTGA2.1 shares significant nucleotide similarity with rTGA2.2, thus there is a chance that the levels of rTGA2.2 mRNA may also be affected. The silencing (SI) construct was placed under the control of the constitutive, strong maize ubiquitin promoter and also transformed into the rice cultivar LG. Seven independently transformed lines were obtained using this approach.

As the SI construct was predicted to silence *rTGA2.1*, RT-PCR was performed on leaf tissue from segregating, T_1 offspring of silencing (SI) lines 1CC and 6DD. Lines 1CC and 6DD show a 50–90% reduction, respectively, of *rTGA2.1* transcript (Figure 3b). The RT reaction was performed using primers that specifically amplified the endogenous *rTGA2.1* transcript, and that did not detect either other rTGA factors, or the *rTGA2.1* fragment contained on the SI construct. Additionally, RT-PCR results show that transcripts from a closely related TGA gene, *rTGA2.2 (OSNIF2)*, are also reduced in these lines (Figure 3b). To determine whether the loss of mRNA was associated with a loss of protein, the presence of protein in the T_1 offspring of transgenic SI lines 1CCand 6DD (Figure 3c) was analyzed. In lines 1CC and 6DD, one specific band (indicated with an arrow), corresponding to the predicted molecular weight for the endogenous rTGA2.1, was either no longer visible or was reduced. In

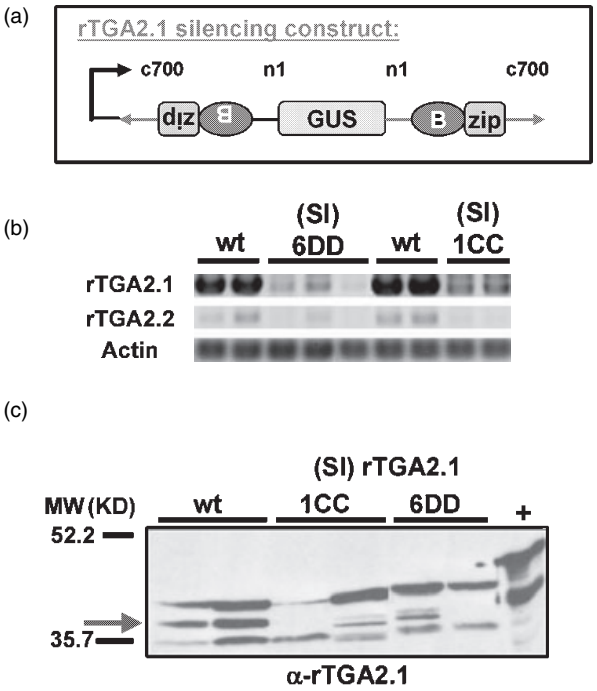


Figure 3. Characterization of rTGA2.1 silencing (SI) rice lines.
(a) Construction of a vector designed to silence endogenous rTGA2.1 through dsRNA-mediated silencing. The first two-thirds of rTGA2.1 are arranged to create RNA in a hairpin-loop structure with a fragment of the GUS gene. Drawing not to scale.
(b) RT-PCR analysis of two independently derived rTGA2.1(SI) lines, 1CC and 6DD. cDNA was amplified from three T_1 plants for each line and from two wild-type (WT) plants.
(c) Western blot of endogenous rTGA proteins as detected by an α-rTGA2.1 antibody for two T_1 individuals [all with the rTGA2.1 (SI) transgene] from the three rTGA2.1 SI lines 1CC, 5AA and 6DD. Wild-type rice have three predominant bands, whereas the SI lines show variation in intensity of the upper two bands. The bands reduced in lines 1CC and 6DD are indicated by arrows. The (+) lane contains *Escherichia coli*-produced (6X)HIS::rTGA2.1-myc protein.

the SI line, 1CC, an upper band, also appeared to have reduced levels. This result suggests that at least one of these bands represents the endogenous rTGA2.1 protein, or that multiple TGA factors are silenced in this line. Thus, the SI transgenic plants have reduced TGA protein levels as predicted.

Mutant rTGA2.1 *and* rTGA2.1*-silenced transgenic rice display reduced lesion development after infection with the rice bacterial pathogen* Xoo

A serious rice disease in tropical climates is bacterial blight of rice caused by the bacterial pathogen *Xoo*. Overexpression of (*At*)*NPR1* in rice heightens the resistance to this pathogen, possibly in part through the regulation of rTGA activity (Chern *et al.*, 2001). To determine whether or not rTGA2.1 could affect bacterial disease resistance, DN and SI plants from the T_1 or T_2 generations were infected with *Xoo*, strain Philippine Race 6 (PR6), and scored for lesion development. Symptoms of *Xoo* infection were visible as chlorotic regions in leaf tissue that spread from leaf tip toward the leaf base.

For all *Xoo* inoculations, T_1 generation plants, in which the DN transgene was segregating, were used. Both transgene (+) and transgene (–) siblings were inoculated, along with the transformation recipient cultivar as controls. The presence of the transgene was confirmed by PCR amplification with primers specific to the ubiquitin promoter and the rTGA2.1 cDNA. All plants were inoculated between 5 and 6 weeks of age in a controlled growth environment and the symptoms scored 14 days post-inoculation (dpi). The spread of *Xoo*-induced lesions is more contained in the leaves of DN transgenic lines than in the wild-type control cultivar, LG. Representative pictures of lesions 21 dpi for transgene (+) and transgene (–) DN plants are depicted in Figure 4(a). When the extent of lesion spread was quantified a reduction in disease symptoms is seen in multiple, independently

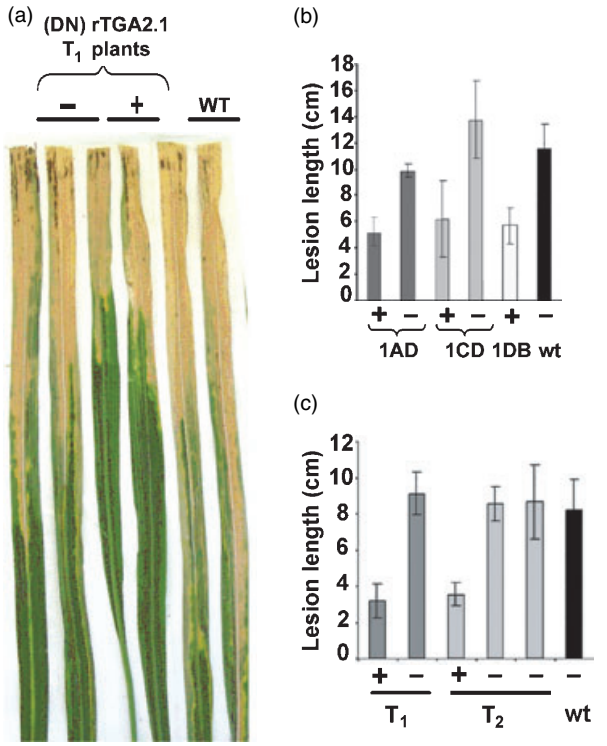


Figure 4. rTGA2.1 (DN) rice lines exhibit reduced lesion development.
(a) Representative leaves showing the extent of lesion development 21 days post-inoculation with the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in leaves of line 1CD T_1 plants with (+) and without (–) the DN transgene, and in wild-type (WT) controls.
(b) Quantitative lesion length data for three independently derived DN lines 1AD, 1CD and 1DB. The graphs depict the mean and standard deviation for T_1 plants with (+) or without (–) the DN transgene.
(c) Lesion length measurements for the T_2 generation of DN line 1AD. T_1 plants are from line 1AD, while T_2 plants are derived from the T_1 progeny 1AD-2 (transgene +, segregating) or 1AD-6 (transgene –). Lesion lengths for wild-type controls are also shown.

derived, rTGA2.1 (DN) lines, including 1AD, 1CD and 1DB (Figure 4b). The degree of symptom reduction varies among the DN lines. For the DN plants another line, 2BD, consistently developed the shortest lesions (data not shown). To confirm that the resistance phenotype was stable and heritable, *Xoo* was inoculated on T_2 offspring plants from line 1AD, a line with moderate *Xoo* resistance (Figure 4c). When infection assays were performed with the T_2 offspring of 1AD-2, *Xoo* resistance co-segregated with the presence of the transgene (Figure 4c). The extent of lesion length reduction in the T_2 plants was equivalent to that observed in the T_1 parent. As additional controls, T_2 offspring from the T_1 plant 1AD-6 (which did not carry the transgene) were also inoculated. All offspring of 1AD-6 had lesion lengths similar to that of the wild-type control. The mean lesion lengths, per line, for T_1 lines 1AD and 2BD, and the T_2 progeny of 1AD-2 and 1AD-6 were compared statistically for differences at the 0.05 significance level using an unbalanced, one-factor ANOVA analysis. As expected line 1A-6 [transgene (–) by PCR] has a *P*-value of 0.55, indicating that the mean lesion lengths for the wild type and transgene (–) are statistically equivalent. The *P*-values of all other DN T_1 and T_2 lines tested were <0.003. This value indicates that the symptom reduction in the DN lines is significantly different from wild type.

The (DN) rTGA2.1 mutant protein is likely to interfere with the ability of rTGA2.1 to bind DNA, but not its ability to homodimerize or heterodimerize or interact with other proteins. The phenotypes observed in lines overexpressing (DN) rTGA2.1, may, in part, result from dominant effects of sequestering such interacting proteins. To minimize such effects, transgenic rice which had rTGA2.1 activity altered via RNA silencing were generated. The leaves of transformed SI plants were inoculated with *Xoo* (PR6) as above. The visible reduction in lesion symptoms can be observed in representative plants leaves as shown in Figure 5(a). Multiple, independent, SI lines had reduced lesion lengths when compared with wild-type plants. The extent of lesion length reduction was similar to that observed in experiments with the DN plants (Figure 5b). Lines 1CC and 6DD had lesions that were significantly reduced ($P < 0.001$; Student's *t*-test) when compared with wild-type controls. The inoculation experiments for both SI and DN lines were repeated multiple times and the data are representative of the results in each replicate. The results of these infection assays demonstrate that *Xoo* symptom development is decreased in rice containing either the rTGA2.1 (DN) or (SI) constructs and suggest a negative role for wild-type rTGA2.1 in *Xoo* resistance.

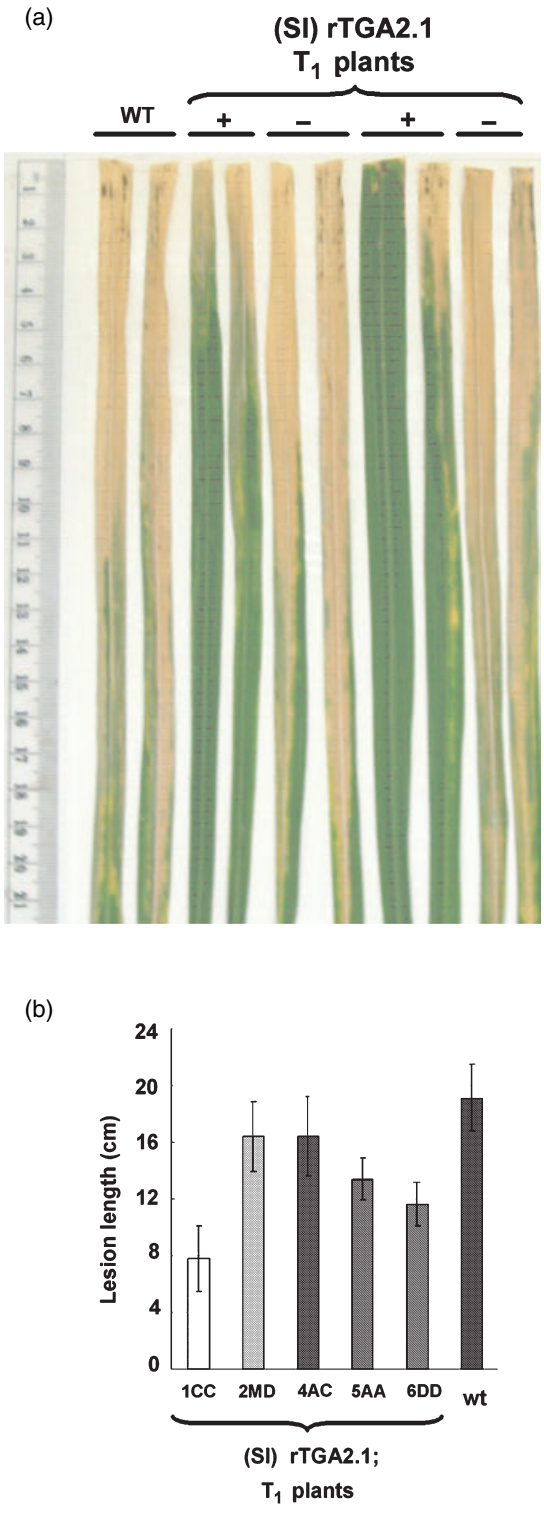


Figure 5. rTGA2.1 (SI) silenced lines exhibit reduced lesion development.
(a) Representative leaves showing the extent of lesion development 21 days post-inoculation with the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in leaves of T_1 from SI line 6DD plants with (+) and without (–) the rTGA2.1 (SI) transgene, and in wild-type (WT) controls.
(b) The graph depicts the mean and standard deviation for T_1 plants with the rTGA2.1 (SI) transgene for independent lines 1CC, 2MD, 4AC, 5AA and 6DD.

Bacterial titer in rTGA2.1 transgenic rice is not decreased concomitantly with lesion length reduction

Progression of the lesion spread is thought to correspond to multiplication of the *Xoo* bacteria. *Xoo* is a vascular pathogen whose excessive production of extracellular polysac-

charides eventually impedes nutrient movement through xylem tissues (Ou, 1972).

To determine whether the reduced symptom development correlated with reduced bacterial multiplication, growth curve analyses were performed on T_1 rice plants, from independent DN lines 1CD and 2BD, inoculated with *Xoo* (PR6). The growth curve analyses indicate that bacterial concentrations in both wild type and transgenic lines were similar at the initial inoculation (day 0) and that the populations at 10 and 14 dpi were also similar reaching a titer of up to 10^8 colony forming units (cfu) per leaf (Figure 6a). This result was surprising as there are striking lesion length differences at these time points. Growth curve analyses were also carried out on the rTGA2.1 (SI) lines. SI lines 1CC and 6DD infected with *Xoo* (PR6) demonstrate again that although these lines show a reduction in lesion development there is not a large reduction in the total number of bacteria found within plant leaves (Figure 6b) at 0, 10 or 14 days after inoculation. The reduction in disease symptoms, but lack of effects on bacterial population growth at these time points in transgenic DN and SI plants indicates that reducing rTGA wild-type activity promotes enhanced tolerance to *Xoo*. Tolerance, as defined here and in studies by Bent *et al.* (1992), refers to decreased symptom development without effects on pathogen viability.

To determine whether differences in *Xoo* populations existed at early time points after infection additional growth curves with DN line 1CD and SI line 6DD were performed (Figure 6c). At most time points the *Xoo* populations in the wild type or rTGA2.1 DN and SI lines are similar. Day 6 has the greatest variation between the lines, but as the error in the measurements is also large at this time, no statistical difference can be concluded from these data. Other transgenic rice lines with partial resistance to *Xoo* show significant differences in *Xoo* populations at this time point after infection (Andaya and Ronald, 2003; Wang *et al.*, 1998). Therefore, the plants used in this study may simply have been too heterogenous to detect small, but important differences. Alternatively, while pathogen multiplication may not be substantially decreased, pathogen aggressiveness and motility within the leaf may be altered. Together the results with both the DN and SI plants indicate that reduction in rTGA2.1 activity increased tolerance to *Xoo*, and further, suggest a negative role in basal defense responses for wild-type rTGA2.1.

Overexpression of an rTGA2.1 mutant and silencing of rTGA2.1 *reduce plant growth*

Many Arabidopsis mutants display enhanced resistance to pathogens, but this resistance is achieved at a cost to the plant. For example, the Arabidopsis *cpr* mutants display dwarfism and growth defects along with enhanced resistance (Bowling *et al.*, 1994, 1997; Clarke *et al.*, 1998). While transgenic plants with reduced or eliminated TGA factor activity have been made in tobacco and Arabidopsis, no visible effects on plant growth have been observed (Ekengren *et al.*, 2003; Fan and Dong, 2002; Kim and Delaney, 2002; Miao and Lam, 1995; Niggeweg *et al.*, 2000a; Pontier *et al.*, 2001). In the transgenic DN and SI rice rTGA2.1 plants, however, there are obvious morphological alterations. Plants that have reduced rTGA2.1 are reduced in both overall

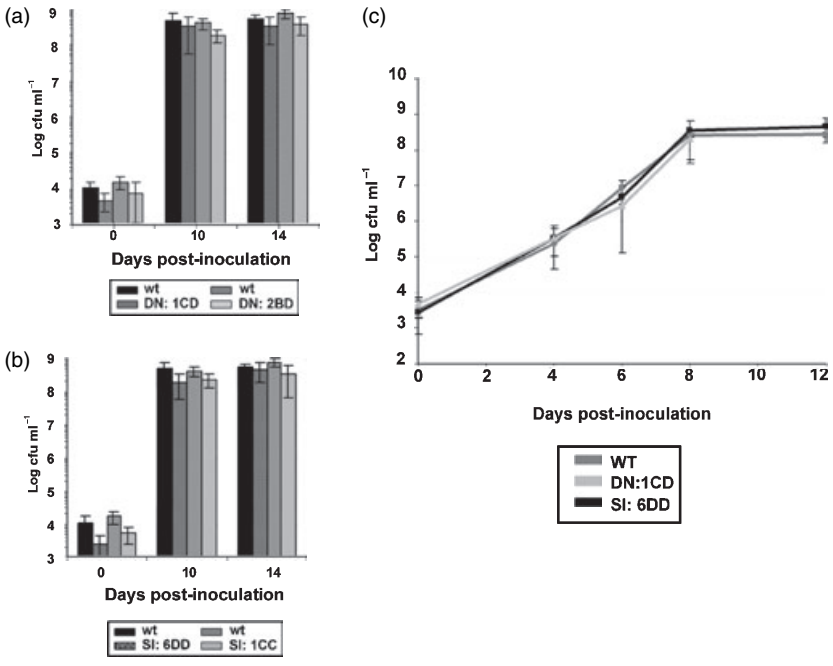
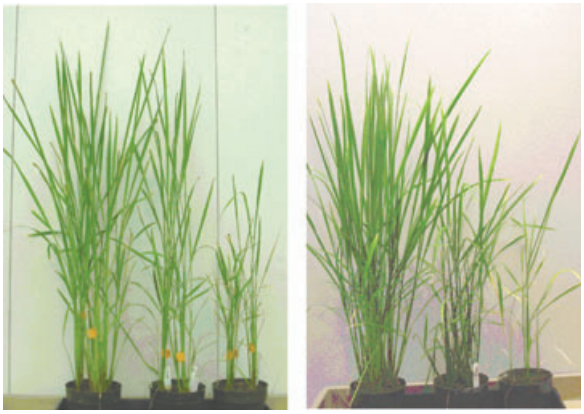


Figure 6. rTGA2.1 DN and rTGA2. SI lines exhibit increased tolerance of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*).
(a) Growth of *Xoo* in rTGA2.1 (DN) rice lines or (b) rTGA2.1 (SI) rice lines at 0, 10 and 14 days post-inoculation. *Xanthomonas oryzae* pv. *oryzae* was inoculated onto plants from two DN lines, 1CD and 2BD, in two independent experiments. The wild-type (WT) control is shown for each experiment. For each time point, the mean of the bacterial colony count from eight plants per line is shown. Error bars indicate the standard deviation.
(c) Time course analysis of *Xoo* bacterial growth 0–8 days after inoculation of rTGA2.1 (DN) line 1CD and 0–12 days after inoculation of rTGA2.1 (SI) line 6DD and WT lines. The WT control is shown in dark gray. The graph depicts the mean and standard deviation for measurements from five leaves (for WT and SI), or three leaves (DN) per cultivar, per time point. cfu, colony forming units of bacteria per ml of ground leaf tissue.

height, and in total plant biomass when compared with the wild-type parent, or siblings that do not contain the transgene (Figure 7a). In the DN lines, the height differences range from minor (line 1AD) to strong (line 2BD). However, even for plants with seemingly slight height reductions such as line 1AD, both the height and tiller number reductions are statistically significant ($P << 0.001$, Student's *t*-test) (Figure 7c). The silencing lines, in general, show greater reductions than the DN plants in both plant height and in tiller abundance (Figure 7b). Interestingly, mature leaves in the SI lines also senesce prematurely. This leaf death is exacerbated by growth under low light, high humidity, conditions (as in growth chambers) as well as by infection with *Xoo*. Indeed, if the SI plants are kept in low light conditions for over 4 weeks, the leaf death will eventually overtake the whole plant. A similar leaf death is observed in rice plants that overexpress the rTGA2.1-interacting factor, (At)NPR1, when they are grown under the same conditions (Fitzgerald *et al.*, 2004). Therefore, while the rTGA2.1 factors can be manipulated to help increase plant defenses, a substantial loss of rTGA2.1 activity may result in unwanted reductions in plant biomass, and likely a subsequent unwanted reduction of seed yield.

(a) (b)



(c)

	Height (cm)	Tiller #
WT	83.1 +/- 7.1	4.7 +/- 1.3
(DN) 1AD	64.6 +/- 4.3	2.6 +/- 0.6

(d)

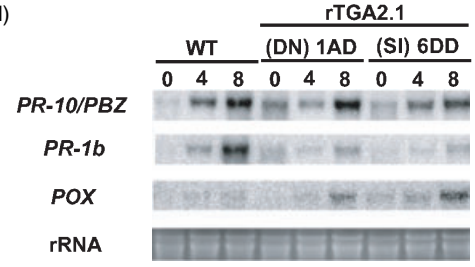


Figure 7. Reduced plant size of rTGA2.1 (DN) and rTGA2.1 (SI) transgenic rice.
(a) Representative, wild-type (WT) and DN lines displaying reductions in plant height and girth. Plants are 6 weeks old. Three plants per pot are shown.
(b) Some lines have height variation within the transgene (+) plants of the T_1 generation as is the case with the rTGA2.1 (SI) line 6DD.
(c) Quantitation of reduced plant growth in DN line 1AD compared with WT controls. Plant measurements were taken from greenhouse-grown plants at 6 weeks of age.
(d) Northern blot analysis of *PR-10/PBZ*, *PR-1b* and *POX* gene expression in leaf tissue of WT, (DN) line 1CD and (SI) line 6DD pre (0 dpi) and post-inoculation (4 and 8 dpi) with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*).

Alteration of defense gene expression in rTGA2.1 (DN) transgenic plants

Many mutant Arabidopsis that display heightened defense responses are not only dwarfed, but also have constitutive expression of *PR* genes (Bowling *et al.*, 1994; Shah *et al.*, 1999). Genes with similar sequences to the Arabidopsis *PR* genes have been identified in rice. The rice genes *PR-1* and *PR-10/PBZ* have elevated expression after treatment of wild-type rice with SAR-inducing chemicals and are constitutively expressed in several rice lesion-mimic mutants (Agrawal *et al.*, 2000; McGee *et al.*, 2001; Midoh and Iwata, 1996; Takahashi *et al.*, 1999). To determine the effect of rTGA2.1 (DN) on defense gene expression, accumulation of the rice *PR*-like defense genes, *PR-1b*, *PR-10/PBZ* and *POX*, was monitored using RNA blot analyses (Figure 7d). The levels of *PR-10/PBZ* mRNA were elevated in DN and SI lines compared with wild-type prior to inoculation with the *Xoo* pathogen (day 0). *PBZ* accumulation was lower than, or equal to, WT levels at both 4 and 8 dpi. Slight elevation of *PR-1b* expression was also observed in DN and SI lines prior to inoculation. Notably, *PR-1b* expression was much lower at 4 and 8 dpi than in WT for these lines. The *POX* genes demonstrated higher accumulation at 8 dpi in both the DN and SI rice. As *POX* is a sensitive marker for redox change, its accumulation likely reflects increases in microscopic cell death resulting from the beginning of leaf lesion formation. Studies monitoring *PR* gene expression in an Arabidopsis *tga2 tga5* double knockout also demonstrate an elevated basal expression of *PR* genes, followed by subsequent lack of induction after pathogen challenge (Zhang *et al.*, 2003). These reports support our observation that wild-type rTGA2.1 interferes with expression of a subset of rice defense genes under non-inducing conditions. Taken together our data are consistent with a negative role for rTGA2.1 in regulating defense gene expression in rice.

Discussion

Members of the TGA class of bZIP transcription factors in Arabidopsis and tobacco are predicted to transduce the SAR-inducing signal into a cellular response by regulating

transcription of defense genes. The defense genes code for a large variety of proteins including some which act enzymatically to directly contribute to plant defense against pathogen attack (Uknes *et al.*, 1992; Ward *et al.*, 1991). Studies in dicots indicate that each TGA factor has either positive or negative effects on disease susceptibility and defense gene expression (Fan and Dong, 2002; Niggeweg *et al.*, 2000a; Pontier *et al.*, 2001; Zhang *et al.*, 2003). Therefore, the relative contribution to defense gene regulation is likely to differ for each TGA factor and each plant species.

We previously isolated four rice (At)NPR1-interacting TGA factors through yeast two-hybrid screens (Chern *et al.*, 2001). One factor, rTGA2.1, has 78% amino acid similarity to Arabidopsis TGA2. In dicots, TGA2 has been most strongly implicated in SAR signaling. It interacts with NPR1 more strongly than other Arabidopsis TGA factors, and appears to be the main component of the anti-microbial, activating sequence factor-1 (ASF-1) and SA response protein activities, in both tobacco and Arabidopsis (Niggeweg *et al.*, 2000a; Zhang *et al.*, 1999; Zhou *et al.*, 2000). Therefore, as a first step in determining the involvement of the TGA factors in rice defense responses, transgenic rice expressing a putative dominant-negative mutant form of rTGA2.1, and rice engineered with a vector to silence endogenous rTGA2.1 by dsRNA-mediated silencing, were generated. The resultant transgenic plants were characterized for disease resistance, protein accumulation, morphological variations and defense gene expression.

Several strategies have been employed to make TGA 'dominant-negative' mutants in dicots and each strategy has yielded different interpretations of the overall roles of the TGA factors (Niggeweg *et al.*, 2000a; Pontier *et al.*, 2002). Tobacco plants overexpressing TGA2.2 (the tobacco homolog of TGA2) displayed increased expression of the 'immediate early' genes *PR-1a* and *ParA*, and the expression of the dominant-negative mutant eliminated gene induction in response to SA, suggesting a positive role for TGA2.2 (Niggeweg *et al.*, 2000a). Conversely, Pontier *et al.* (2001) over-expressed a dominant-negative, Arabidopsis TGA2 in tobacco and observed increased expression of the canonical 'late' *PR* genes *PR-1*, *PR-2* and *PR-3* along with increased systemic defenses to the bacterial pathogen *Pseudomonas syrigae* pv. *tabaci*. In that study, three amino acids within the basic DNA-binding region were altered and a flexible linker was inserted before the TGA leucine zipper domain (Pontier *et al.*, 2001). The results from this latter study suggest a negative role for TGA2 in gene expression and defense responses. Thus, these two studies do not allow a precise interpretation of the role TGA2 or its tobacco homolog. More recent data obtained by analyzing triple knockout mutants of Arabidopsis *TGA2*, *TGA5* and *TGA6* by Zhang *et al.* (2003) demonstrate that although collectively the Arabidopsis TGA factors are essential, positive regulators for SAR and can serve as negative regulators of *PR* gene expression, specific roles in defense responses have not yet been assigned to individual TGA factors.

We predicted that transgenic rice with altered rTGA2.1 activity would indicate a positive role in defense responses for the rice TGA factors, particularly as the (DN) rTGA2.1 mutant is over-expressed in the same, not a heterologous, plant species. Surprisingly, all transgenic rTGA2.1 rice with decreased rTGA2.1 DNA-binding activity, or silenced rTGA2.1, displayed increased tolerance to the rice pathogen *Xoo* and demonstrated elevation of basal rice PR-like gene expression suggesting a negative basal role in defense responses for these factors. The extent of tolerance to *Xoo* is similar for both the DN and SI lines, plants that have rTGA2.1 activity reduced through two very different techniques, suggesting that the phenotypes observed in the DN lines result from a lack of normal TGA2.1 activity. It appears likely that the interplay between TGAs is complex and varies due to the roster of individual factors present in each plant species. It is also possible that in our study and that of Pontier *et al.* (2001) that the mutant proteins are expressed to a high level and are either more effective at inhibiting TGA2 function, or have additional effects due to sequestration that Niggeweg *et al.* (2000a) did not observe. We observed high levels of (DN) rTGA2.1 protein in plant leaves regardless of plant age, leaf stage or infection status. However, as each of the studies, including the present study, have monitored different classes of defense genes, similarities may be more apparent when identical genes and pathogens are assayed.

Pontier *et al.* (2001) observed enhanced systemic resistance to bacteria as measured by population colony counts in secondary leaf infections. Our data demonstrate that the rTGA2.1 (DN) and (SI) transgenic rice have attenuated disease symptom development after infection with *Xoo*. However, the bacterial population growth in infected leaves does not differ when compared with wild-type plants at time points late after infection (0, 10 or 14 dpi). It may be that we simply were not able to detect a subtle difference in bacterial growth at these time points using our assays, or these results could indicate a specific defense mechanism, for example cell death, that involves the function of the TGA factors. Enhanced pathogen tolerance (fewer symptoms, similar pathogen populations) has also been observed in Arabidopsis overexpressing the *ACD2* gene and in the *ein2* Arabidopsis mutants (Bent *et al.*, 1992; Lund *et al.*, 1998; Mach *et al.*, 2001).

Other Arabidopsis mutants that show enhanced disease resistance include the *cpr* (constitutive expresser of PR genes) mutants (Bowling *et al.*, 1994, 1997; Clarke *et al.*, 1998, 2000). The *cpr* mutants display growth defects, constitutive *PR* gene expression and elevated endogenous SA levels. If the TGA factors are the main regulators of the *PR* genes, over-activation of TGA factors could be predicted to

yield plants with phenotypes similar to the *cpr* plants. None of the various tobacco or Arabidopsis TGA mutants or TGA-overexpressing plants has yet displayed any morphological variations. However, both the rTGA2.1 (DN) and (SI) transgenic rice displayed morphological defects that were positively correlated with reduction in disease symptoms. The most obvious effect was a reduction in the number of tillers and overall plant height. Transgenic DN and SI lines that displayed higher tolerance to bacteria were the most severely dwarfed, while several lines with lesser tolerance had only minor, although consistent, reductions in size. The dwarfism in these transgenic plants is consistent with costs imparted by constitutive *PR* gene expression and is similar to that observed in the *cpr* mutants.

It is not yet clear which suites of genes are regulated by individual TGA factors. Neither is it known how the TGA factors themselves are regulated. Our data suggest that lack of TGA factor activity affects not only disease symptom development, but also plant growth and basal defense gene expression patterns. These processes have not been previously connected to the TGA factors but will provide fertile ground for future investigations of the multiple functions carried out by this family of transcription factors.

Experimental procedures

Construction of the rTGA2.1 DN mutants and the SI silencing vector

To generate a mutant (DN) rTGA2.1, the N-terminal approximately 200 bp of rTGA2.1 (also called OsNIF1, Genbank accession no. AB051295) was amplified by PCR, from a plasmid containing full-length rTGA2.1 DNA (Chern *et al.*, 2001), using the primers MN1-4 (5′-AAAGGATCCCATGGCAGATGCTAGTTCAAGGACTGACACATC-GATTG-3′) and DN-1(5′-TTTTCTTGGTGGCTCACGATTTTG-3′). The C-terminal approximately 800 bp of rTGA2.1 was amplified by PCR using DN2 (5′-CGTGAGCCACCAAGAAAAAGTCGG-3′) and pr-MN1-myc (5′-AAAGGATCCACTAGTTTACTTCTCCAGCAGATCCTCCT-CAGAAATCAG-3′), which also codes for a c-MYC tag. The N and C-terminal fragments were mixed together and amplified in a second PCR round using only primers MN1-4 and pr-MN1-myc. The final PCR product was gel purified and ligated to the *E. coli* pCR-Blunt II-TOPO plasmid (Invitrogen, Carlsbad, CA, USA). To generate the rTGA2.1 silencing (SI) vector, a yeast two-hybrid vector containing 90% of rTGA2.1 (Chern *et al.*, 2001) was digested with *Eco*RI and *Pst*I restriction enzymes. This yielded an approximately 700-bp rTGA2.1 fragment that was subcloned into the pBSK II vector digested with the same enzymes to generate plasmid pHF20. The GUS gene was digested with *Eco*RI and ligated in three parts to pHF20, digested with *Eco*RI and *Bam*HI and to the plant transformation vector pCambia Ubi/NC3300 digested with *Bam*HI only (construction of modified pCambia vectors has been described previously, Chern *et al.*, 2001). The newly created vector (pHF21) contained the truncated rTGA2.1 in a non-coding orientation after the constitutive maize ubiquitin promoter. It was fused to a truncated GUS fragment and at the 3′-end of the GUS fragment was fused another rTGA2.1, *Eco*RI/*Pst*I fragment.

Protein expression and purification in E. coli

The construction, expression and purification of recombinant wild-type rTGA2.1 and wild-type GFP protein have been described in detail (Chern *et al.*, 2001). Wild-type rTGA2.1 fused to c-myc and with an N-terminal 6X-Histidine tag, purified previously (Chern *et al.*, 2001) was injected into rabbits to generate polyclonal α-rTGA2.1 antisera. To create a 6X-histidine fusion with the rTGA2.1 (DN) mutant, pHF13 was digested with *Bam*HI and subcloned into pET15B (Novagen, Madison, WI, USA) also digested with *Bam*HI to generate pHF16. DN rTAG2.1 (HIS/myc tagged) was transformed into *E. coli*, strain BL21(DE3)pLysS (Novagen). Induction of expression and purification of native protein using Ni-NTA agarose resin were carried out according to the manufacturer's protocols (Qiagen, Chatsworth, CA, USA). Briefly, the over-expressed recombinant protein was allowed to bind to the resin and was subsequently washed five times with 40 mM imidazole, 150 mM imidazole and eluted twice with 500 mM imidazole. The elution was dialyzed into protein buffer S (20 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 10% glycerol and 0.5 mM DTT) and aliquots were used for gel mobility shift assays. Protein purity was assessed by separating protein on 10% SDS-PAGE gels and visualized after staining with Coomassie blue dye (Sambrook *et al.*, 1989).

Electrophoretic gel mobility shift assay

Gel mobility shifts were performed as described previously using the SARE-1 and SARE-3 oligonucleotides as a probe (Chern *et al.*, 2001). Wild-type competitor probe was also prepared as before using oligos SARE-1 and SARE-2. All radioactive oligoucleotide probes were prepared by fill-in reactions of overhanging double-stranded ends with the Klenow enzyme and ^{32}P-dCTP. The rTGA2.1 wild type, rTGA2.1 (DN) mutant, and GFP recombinant 6XHis-tagged proteins used in the assay are described above.

Generation of transgenic rice plants

The rice rTGA2.1 (DN) mutant and rTGA2.1 (SI) constructs were used to generate transgenic rice with altered rTGA2.1 activity. pHF13, containing the rTGA2.1 (DN), was digested with *Bam*HI and *Spe*I enzymes and subcloned into pCambia Ubi/NC1301 (M.-S. Chern unpublished data, Cambia, Canberra, Australia) digested with the same enzymes, to create plasmid pHF23. pHF 23 places rTGA2.1 expression under the control of the strong, constitutive maize ubiquitin promoter, a NOS terminator and the selectable markers hygromycin and kanamycin. To place the silencing construct into a rice transformation vector, the rTGA2.1 (SI) insert was released from pHF21 using *Bam*HI and subcloned into the same site of plasmid Ubi/NC1301 creating plasmid pHF22. Both the overexpressing DN and SI vectors were transformed into *Agrobacterium tumefaciens* (strain EHA105) by electroporation according to the manufacturer's instructions, using a Cell-Porator (Life Technologies, Rockville, MD, USA). For transformation, callus tissues derived from the mature embryos of rice cultivars LG or Taipei 309 were inoculated with *A. tumefaciens* carrying either the DN or SI rTGA2.1 vectors, using a modification of the standard (Hiei *et al.*, 1994) transformation protocol (Cheng *et al.*, 1997). Transgene-positive *Agrobacteria* were selected by growth on media containing kanamycin (50 μg ml^{-1}). Transgene-positive rice were selected on plates containing 50 mg l^{-1} hygromycin and *Agrobacteria* growth was simultaneously selected against with carbenicillin at 250 mg l^{-1}.

Plant materials, growth conditions and pathogen infection

Rice plants were grown in autoclaved clay soil in greenhouse conditions (8–16 h day/night cycle; >80% RH; 20–35°C) unless otherwise specified. Plants were fertilized twice in the growing period with ammonium sulfate. For *Xoo* inoculation experiments, rice plants were grown in the greenhouse until 6 weeks of age and then transferred to growth chambers (12 h day/night: light provided by incandescent and metal halide lights; average light intensity of 100 μmol photons m^{2-} sec^{1}; 85% day/95% night RH; 28°C). Transgenic rice plants were grown in a similar manner once seedlings had regenerated to approximately 10 cm in height. All rice plants were allowed to self-pollinate and seeds collected from individual plants. Transgene-positive T_1 and T_2 transgenic rice, carrying either the DN or SI constructs, were verified by PCR with primers specific to portions of the rTGA2.1 gene and the ubiquitin promoter. Bacterial blight inoculation experiments were performed with the Philippine race 6 (Pxo99) *Xoo* strain using a scissors-dip method (Kauffman *et al.*, 1973). *Xoo* cultures were grown on PSA plates (10 g peptone, 10 g sucrose, 16 g agar/ all per liter of H_2O) at 30°C. Lesion development was scored on rice leaves 14 dpi by measuring symptom progression with a ruler. Bacterial growth curve analyses were performed by inoculating rice with PX099 (OD_{600} 0.5) as above, and then excising 16 cm of the youngest, fully expanded rice leaves at time points of 0, 10 and 14 dpi. One leaf per rice plant, for eight rice plants per cultivar per time point was harvested. The excised leaves were ground using a mortar and pestle with sea sand into 10 ml of water. Aliquots from serial dilutions of the leaf homogenate were plated onto PSA plates and the number of bacterial colonies counted after 4 days.

DNA and RNA isolation, blotting and PCR and RT-PCR analysis

For both RNA and DNA extractions, rice leaves were ground using liquid nitrogen and a mortar and pestle or with a 1-ml pipette tip attached to a drill and modified bit. Genomic DNA was extracted from rice leaves according to the protocol of Dellaporta *et al.* (1984). Detection of the rTGA2.1 (DN) transgene in rice plants was performed using the primers Ubi-1 (5′-TGATATACTTGGATGATGGCA-3′) and MN1-12 (5′-GCGGATCCACTAGTAGGCTTGCCGGATCGTC-AGAA-3′). Detection of the rTGA2.1 (SI) transgene was performed using the Ubi-1 primer and primer MN1spec1F (5′-GCGGATCCCACCAGTTAGAAAACGGACAT-3′). Total RNA was isolated from leaf homogenates using the Trizol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. RT-PCR analysis was carried out on total RNA from mature leaf extracts from T_1 plants of rTGA2.1 (SI) lines. For each sample, 1.5 μg of RNA was used in a 20-μl RT reaction. First strand cDNA synthesis was performed using an rTGA2.1 endogenous gene-specific primer MN1-12, or rTGA2.2 primer (OSNIF1), and a rice actin-specific primer (Actin AS) as internal controls (Yoshimura *et al.*, 1998). PCR was performed on 5 μl of the RT reaction using the rTGA2.1 primers MN1-4 and MN1-12 or the actin primers (AS and S) with 35 cycles of: 94°C, 3 min; 94°C, 1 min; 54°C, 1 min; 72°C, 1 min. PCR product was separated on an agarose gel and visualized with ethidium bromide.

Protein extraction and immunodetection

Rice leaves (0.2 g per sample) harvested at various developmental stages (Century *et al.*, 1999) were ground using liquid nitrogen and the homogenate resuspended in buffer R (125 mM Tris-HCL, pH 8.0, 20% glycerol, 2% SDS) with protease inhibitors (mini-Complete, protease-inhibitor tablet; Boerhinger-Ingelheim, Ridgefield, CT, USA). Approximately 0.1 ml of glass beads (425–600 μm) was added to the homogenate. The slurry was mixed at RT for 5 min, subjected to centrifugation (15 000 ***g***, 5 min), and the supernatant removed. β-Mercaptoethanol was added to the supernatant to a final concentration of 5%, and the solution frozen at −80°C until further use. Equal volumes of protein extracts were mixed with 2X Laemmli sample buffer separated on 10% SDS-PAGE gels and electrotransferred to nitrocellulose membranes (Sambrook *et al.*, 1989). Loading equivalency was determined through Coomassie staining of the SDS-PAGE gels. Non-specific sites on membranes were blocked by overnight incubation of the membrane in a solution of 5% non-fat milk in 1X TBS-T (100 mM Tris, pH 7.6, 150 mM NaCl, 0.1% Tween-20). Membranes were probed in T-TBST with primary antibodies. Either a polyclonal α-cMYC antibody (Invitrogen) at a dilution of 1:1000, or a polyclonal α-rTGA2.1, at a dilution of 1:5000 of the final rabbit bleed, was used. Proteins were detected using either α-mouse IGG (for c-myc) or α-rabbit IGG (for rTGA2.1) secondary antibodies linked to horseradish peroxidase and the ECL-plus, chemiluminesence system, according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, USA).

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