The Plant Cell, Vol. 15, 2181–2191, September 2003, www.plantcell.org © 2003 American Society of Plant Biologists

The Arabidopsis NPR1 Disease Resistance Protein Is a Novel Cofactor That Confers Redox Regulation of DNA Binding Activity to the Basic Domain/Leucine Zipper Transcription Factor TGA1

Charles Després,[a,b] Catherine Chubak,[c] Amanda Rochon,[a] Rena Clark,[b] Terry Bethune,[b] Darrell Desveaux,[d,e] and Pierre R. Fobert[b,1]

[a] Department of Biological Sciences, Brock University, St. Catharines, Ontario, Canada L2S 3A1
[b] National Research Council Canada, Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada S7N 0W9
[c] Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 5E2
[d] Department of Biochemistry, Université de Montréal, Montréal, Québec, Canada H3C 3J7
[e] Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599

The Arabidopsis NPR1 protein is essential for regulating salicylic acid–dependent gene expression during systemic acquired resistance. NPR1 interacts differentially with members of the TGA class of basic domain/Leu zipper transcription factors and regulates their DNA binding activity. Here, we report that although TGA1 does not interact with NPR1 in yeast two-hybrid assays, treatment with salicylic acid induces the interaction between these proteins in Arabidopsis leaves. This phenomenon is correlated with a reduction of TGA1 Cys residues. Furthermore, site-directed mutagenesis of TGA1 Cys-260 and Cys-266 enables the interaction with NPR1 in yeast and Arabidopsis. Together, these results indicate that TGA1 relies on the oxidation state of Cys residues to mediate the interaction with NPR1. An intramolecular disulfide bridge in TGA1 precludes interaction with NPR1, and NPR1 can only stimulate the DNA binding activity of the reduced form of TGA1. Unlike its animal and yeast counterparts, the DNA binding activity of TGA1 is not redox regulated; however, this property is conferred by interaction with the NPR1 cofactor.

INTRODUCTION

Systemic acquired resistance (SAR) is a systemic and broad-range disease resistance in plants triggered by a localized exposure to avirulent pathogens that cause a hypersensitive response (Ryals et al., 1996). SAR is characterized by the induction of a battery of pathogenesis-related (*PR*) genes and the accumulation of PR proteins (Ward et al., 1991). Genetic and biochemical studies have identified salicylic acid (SA) as a metabolite mandatory for SAR. Exogenous application of SA to plants leads to the establishment of SAR and the induction of *PR* genes (Ward et al., 1991), whereas Arabidopsis SA induction–deficient mutants (*sid*; Nawrath and Métraux, 1999) or transgenic plants expressing the bacterial *nahG* gene encoding a bacterial salicylate hydroxylase that degrades SA to catechol are compromised in their ability to mount SAR and express *PR* genes (Delaney et al., 1994). Downstream of SA in the SAR signal transduction pathway is NPR1 (NONEXPRESSOR OF PR GENES), also known as NIM1 and SAI1 (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah et al., 1997). Arabidopsis *npr1* mutants are nonresponsive to SA, are compromised in their ability to express *PR* genes, and do not mount an effective SAR (Cao et al., 1994; Delaney et al., 1995). By contrast, overexpression of NPR1 in rice and Arabidopsis leads to enhanced resistance against various pathogens (Cao et al., 1998; Chern et al., 2001; Friedrich et al., 2001).

Cloning of *NPR1* (Cao et al., 1997; Ryals et al., 1997) revealed that the protein contains two identifiable protein–protein interaction motifs: a BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-brac/Pox virus and Zinc finger) (Aravind and Koonin, 1999) and ankyrin repeats (Sedgwick and Smerdon, 1999). NPR1 localizes to both the cytoplasm and the nucleus (Després et al., 2000). Cytoplasmic NPR1 appears to modulate crosstalk between SA- and jasmonate-dependent defense signaling through a mechanism that is not understood at present (Spoel et al., 2003). Nuclear localization, which is controlled by a bipartite nuclear localization sequence located at the C-terminal end, is required for the induction of *PR* genes (Kinkema et al., 2000).

Yeast two-hybrid screens have revealed that NPR1 interacts differentially with members of the TGA family of basic domain/Leu zipper (bZIP) transcription factors (Zhang et al., 1999; Després et al., 2000; Niggeweg et al., 2000b; Zhou et al., 2000; Chern et al., 2001), so called because their cognate DNA binding elements contain the core sequence TGACG. Upon SAR induction, NPR1 translocates to the nucleus, where it interacts with TGA factors (Kinkema et al., 2000; Subramaniam et al., 2001; Fan and Dong, 2002). NPR1 stimulates the DNA binding activity of interacting TGA factors to SA response elements, and the binding of TGA factors to cognate elements in response to SA requires functional NPR1 (Lebel et al., 1998; Després et al., 2000; Niggeweg et al., 2000a; Fan and Dong, 2002).

[1] To whom correspondence should be addressed. E-mail pierre.fobert@nrc-cnrc.gc.ca; fax 306-975-4839.
Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.012849.

A role for TGA factors in mediating SAR and *PR* gene expression was confirmed recently using transgenic plants containing *trans*-dominant negative versions of *TGA2* (Pontier et al., 2001; Fan and Dong, 2002) or one of its tobacco homologs, *TGA2.2* (Niggeweg et al., 2000a). However, contradictory results were obtained in these studies. Fan and Dong (2002) reported a suppression of SAR and *PR-1* expression, whereas Pontier et al. (2001) reported an enhancement of SAR and *PR* gene expression. Niggeweg et al. (2000a) did not test for SAR, but they reported a suppression of *PR-1* expression. Using a different approach, Kim and Delaney (2002) reported that sense and antisense overexpression of *TGA2* did not affect resistance to a virulent strain of the oomycete *Peronospora parasitica*. However, transgenic plants overexpressing *TGA5* displayed increased resistance to this parasite. This phenotype was not dependent on SA or NPR1 and was correlated with a reduction in *PR* gene expression, suggesting that it is distinct from SAR (Kim and Delaney, 2002).

Given that the interaction of NPR1 with TGA factors is not constitutive, but instead is regulated during the establishment of SAR (Subramaniam et al., 2001; Fan and Dong, 2002), our goal was to investigate the mechanism by which the NPR1–TGA interaction is regulated. Here, we report that TGA1, a member of the Arabidopsis TGA family that does not interact with NPR1 in the yeast two-hybrid system (Després et al., 2000; Niggeweg et al., 2000b; Zhou et al., 2000), interacts with NPR1 in plant cells after SA treatment. Therefore, TGA1 possesses all of the structural determinants for interaction with NPR1. A domain was identified within TGA1 that contains critical inhibitory Cys residues, because their mutation brings about an interaction with NPR1 in yeast cells. We also show that SA modulates the redox status of TGA1 Cys residues in Arabidopsis leaves, with the reduced state being required for a strong TGA1–NPR1 interaction. By contrast, the oxidized form of TGA1 does not interact with NPR1 and contains an intramolecular disulfide bridge. Consequently, NPR1 only stimulates the DNA binding activity of the reduced form of TGA1. The redox regulation of TGA1 presented here reveals one level of control for the interaction between NPR1 and the TGA transcription factors during the establishment of SAR.

RESULTS

NPR1 Interacts with TGA1 in Arabidopsis Leaves upon SA Treatment

Of the seven Arabidopsis TGA factors tested, only TGA1 and TGA4 failed to interact with NPR1 in yeast cells (Després et al., 2000; Zhou et al., 2000). We wanted to assess whether TGA1 could interact with NPR1 in Arabidopsis cells. To this end, we developed a transient assay conceptually similar to the yeast two-hybrid assay. The coding sequence of *TGA1* was ligated into a plant expression vector, downstream of the *35S* promoter of *Cauliflower mosaic virus* (*35S*) and the DNA binding domain (*DB*) of the Gal4 transcription factor. The *NPR1* coding sequence was ligated into a similar plasmid that contained the transactivation domain (*TA*) of the adenovirus viral particle 16 (VP16) instead of the *GAL4 DB*. These effector constructs both are capable of expressing N-terminal fusion proteins and are shown schematically in Figure 1A. They were transfected into Arabidopsis leaves using biolistics, and the NPR1–TGA1 interaction was monitored through the expression of a *5X UAS^{GAL4}*:*luciferase* reporter gene. After bombardment, leaves were placed on MS medium (Murashige and Skoog, 1962) with or without 1 mM SA for 24 h. This level of SA is effective at inducing *PR* gene expression and SAR in Arabidopsis (data not shown) (Cao et al., 1994).

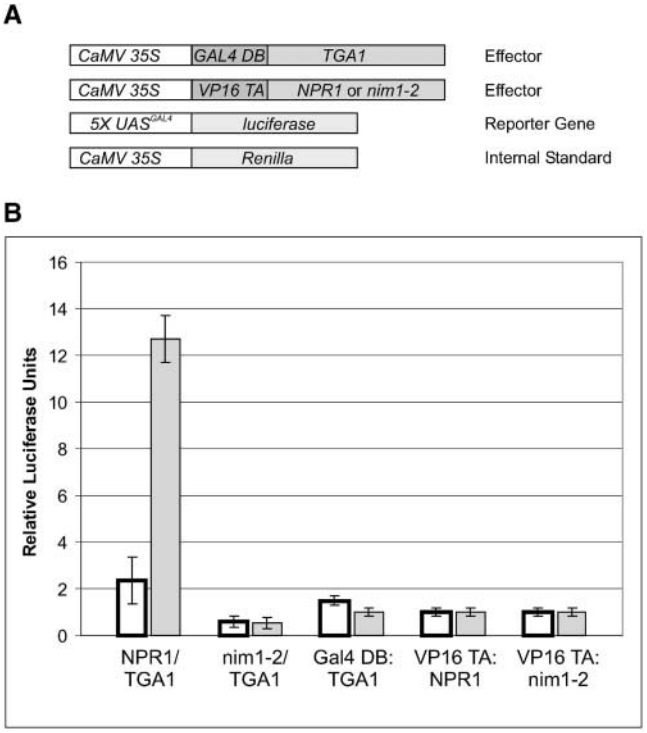

Figure 1. NPR1 Interacts with TGA1 in Arabidopsis Leaves.

(A) Schemes of the constructs used for the plant two-hybrid assays. Promoters are shown in white boxes. *CaMV 35S* indicates the double *Cauliflower mosaic virus 35S*:*Alfalfa mosaic virus* promoter. *5X UAS^{GAL4}* indicates a promoter composed of a multimerized (five elements) Gal4 upstream activating sequence fused to a minimal TATA box and the Ω translational enhancer from the *Tobacco mosaic virus*. Coding sequences are shown in dark and light gray boxes. *GAL4 DB* indicates the *GAL4* DNA binding domain. *VP16 TA* indicates the constitutive transactivation domain of viral particle 16. All constructs possess the polyadenylation signal from the *nopaline synthase* gene (not shown). The 35S:Renilla construct is an internal reference to normalize transfection efficiency. The construct containing the *NPR1*:*VP16 TA* fusion was transfected into untreated leaves along with the reporter and internal standard constructs and was given an arbitrary value of 1 relative luciferase unit ± 1 SD.

(B) Histogram illustrating the level of interaction between TGA1 and NPR1 or nim1-2 in Arabidopsis leaves treated with water (white bars) or in leaves treated for 24 h with 1 mM SA (gray bars). As controls, all Gal4 DB and VP16 TA constructs also were transfected separately with the reporter and internal standard constructs. Values represent averages ± 1 SD.

Leaves were transfected with the reporter genes along with the effector constructs TGA1:DB and NPR1:TA separately to provide a baseline activity of the reporter promoter (Figure 1B). The value obtained with NPR1:TA alone in the absence of SA was set arbitrarily to 1 relative luciferase unit. Values obtained with NPR1:TA after 24 h of SA and with TGA1:DB with and without SA were very similar and close to the baseline.

In the absence of SA treatment, leaves transfected with both TGA1:DB and NPR1:TA displayed a modest increase (2×) in luciferase activity (Figure 1B). Treatment with SA for 24 h resulted in a substantial increase of reporter gene activity. These results indicate that TGA1 interacts poorly with NPR1 in the absence of SA but that this interaction is stimulated greatly by a 24-h treatment with SA.

Although TGA factors such as TGA2 and TGA3 interact with wild-type NPR1 in yeast two-hybrid assays, they do not interact with several NPR1 mutant proteins that fail to mount SAR in plants (Zhang et al., 1999; Després et al., 2000). These observations prompted us to determine whether TGA1 was capable of interacting with a mutant form of NPR1 in Arabidopsis leaves. The coding sequence of *nim1-2*, which encodes a protein with a His-to-Tyr replacement in an ankyrin repeat at position 300 (Ryals et al., 1997), was ligated into the VP16 TA plant expression vector and transfected into Arabidopsis leaves with or without TGA1:DB. When transfected either by itself or with TGA1:DB, nim1-2:TA failed to activate the reporter gene (Figure 1B), indicating that the interaction between TGA1 and NPR1 depends on the presence of functional ankyrin repeats in NPR1.

Together, these results indicate that TGA1 contains all of the structural determinants required to interact with NPR1. However, treatment of Arabidopsis leaves with SA is required to stimulate this interaction.

A Chimeric Protein Containing Only 30 Amino Acids from TGA2 Is Capable of Interacting with NPR1 in Yeast

Unlike TGA1, TGA2 interacts with NPR1 in yeast cells (Després et al., 2000; Zhou et al., 2000) and does not require the presence of SA for interaction with NPR1 in plant cells (Subramaniam et al., 2001; Fan and Dong, 2002). Because TGA1 possesses all of the structural determinants to interact with NPR1 (Figure 1), we hypothesized that the yeast system may mimic resting Arabidopsis leaf cells (without SA treatment), which displayed poor TGA1–NPR1 interaction. To characterize TGA1 regions important for NPR1 interaction, TGA1 and TGA2 were divided arbitrarily into four regions of similar length, and chimeras containing portions of the two factors were created. The DNA constructs tested are depicted in Figure 2. *TGA2* sequences were replaced sequentially by *TGA1* sequences. The chimeric genes were ligated into the yeast two-hybrid vector pBI880 (Kohalmi et al., 1997) and introduced into yeast, in which the ability of the resulting N-terminal Gal4 TA fusion proteins to interact with NPR1:DB was examined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-GAL) filter tests. Because we were exploiting yeast as a rapid system for characterizing TGA1 domains, protein–protein interactions were not quantified in this system. Instead, we opted to quantify selected interactions in the more relevant system, Arabidopsis leaves (see below).

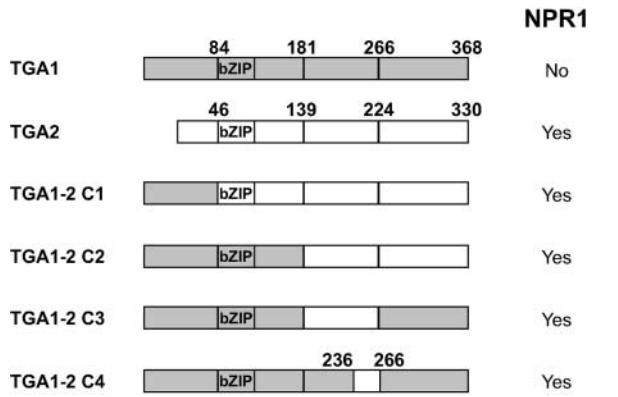

Figure 2. Identification of a 30–Amino Acid Region in TGA2 That Determines the Potential for NPR1 Interaction.

At left are schemes of TGA1, TGA2, and the proteins encoded by various *TGA1*:*TGA2* chimeric genes used to assess interaction with NPR1. All proteins were expressed as fusions to the Gal4 TA. Numbers indicate amino acid positions within the wild-type TGA1 and TGA2 proteins. At right are results of the interaction with NPR1:DB (NPR1) in yeast using the X-GAL filter test, which monitors the activation of the *lacZ* reporter gene. "Yes" indicates that blue color was detected after 1 h, and "No" indicates that there was no blue color after 16 h.

Constructs TGA1-2 C1, TGA1-2 C2, and TGA1-2 C3, which contained ~25, 50, and 75% of *TGA1* sequences, respectively, encoded proteins that interacted with NPR1 (Figure 2). Therefore, a fourth chimeric construct (TGA1-2 C4) was created, which encodes a protein in which only 30 amino acids of TGA1, located between residues 236 and 266, are replaced by those of TGA2. This substitution was sufficient to confer the ability to interact with NPR1 upon TGA1 (Figure 2). These results indicate that these 30 amino acids of TGA2, when swapped between residues 236 and 266 of TGA1, contain critical structural information for interaction with NPR1.

Mutation of TGA1 Cys-260 and Cys-266 Brings about Interaction with NPR1 in Yeast and in Resting Arabidopsis Leaves

To identify the residues within the 30–amino acid region that are important for the interaction with NPR1, a rational site-directed mutagenesis approach was taken based on the multiple alignment of region 236 to 266 of seven TGA factors shown in Figure 3A. The selection criteria for targeting an amino acid were that the residue needed to be conserved between TGA1 and TGA4 but had to differ from that of conserved residues in the remaining TGA factors analyzed. Cys-266 is the only residue that conforms to these criteria (Figure 3A). It is conserved between TGA1 and TGA4, and in all other TGA factors a conserved Ser residue is present at the corresponding position. Because Cys is the only amino acid that can engage in disulfide bridges, Cys-260 also was targeted for mutagenesis because it occurs only in TGA1 and TGA4.

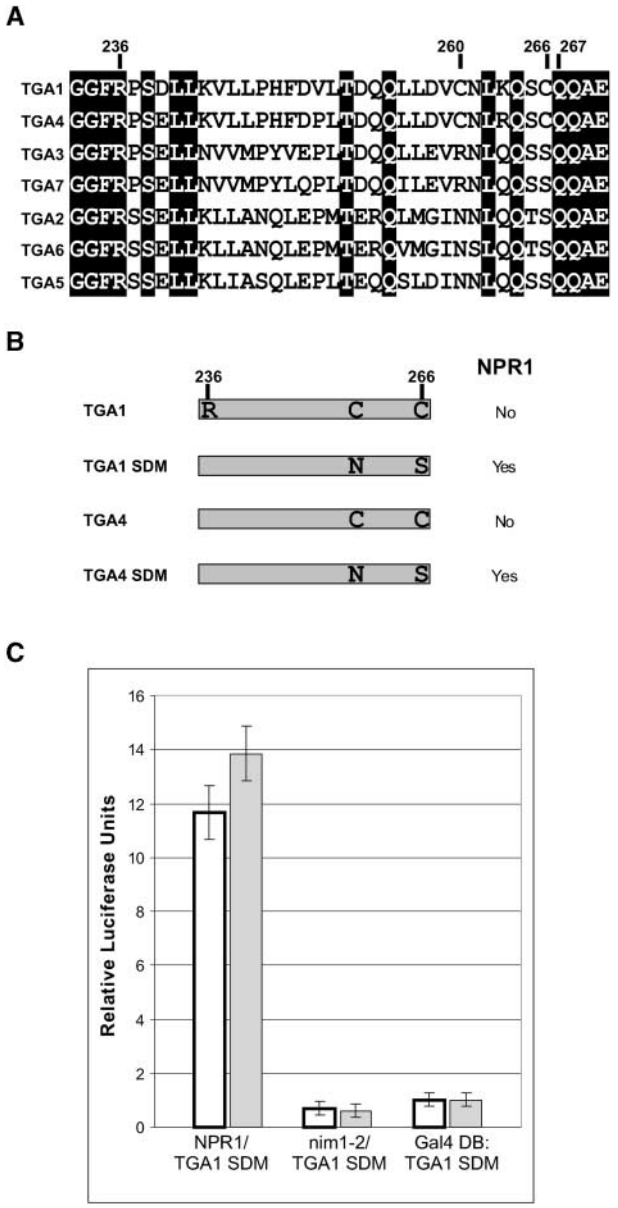

Figure 3. Cys-260 and Cys-266 in TGA1 Determine the Potential for NPR1 Interaction.

(A) Portion of a multiple alignment covering a region of TGA2 involved in interaction with NPR1. The alignment is composed of seven Arabidopsis TGA factors that have been tested in directed yeast two-hybrid assays for interaction with NPR1 (Després et al., 2000). Numbers indicate amino acid positions within wild-type TGA1.

(B) At left are schemes of the region of TGA1 and TGA4 shown in **(A)** as well as of two site-directed mutants (TGA1 SDM and TGA4 SDM) in which Cys residues were replaced by corresponding residues present in TGA2. Numbers indicate amino acid positions within the wild-type TGA1 protein. At right are results of the interaction of the full-length wild-type or site-directed mutant proteins with NPR1 in yeast using the X-GAL filter test, which monitors the activation of the *lacZ* reporter gene. "Yes" indicates that blue color was detected after 1 h, and "No" indicates that there was no blue color after 16 h.

The complete coding region of *TGA1* was used as a substrate for site-directed mutagenesis. The mutant gene encodes a full-length protein with Asn and Ser instead of Cys residues at positions 260 and 266, respectively (TGA1 SDM; Figure 3B). The wild-type and mutant genes were ligated into pBI881, and the resulting Gal4 TA fusion proteins were assessed for interaction with NPR1 in yeast using the X-GAL filter assay by coexpression with NPR1:DB. The results indicate that the mutation of Cys-260 and Cys-266 (TGA1 SDM) transformed TGA1 into a protein capable of interacting with NPR1 in yeast (Figure 3B). Similarly, mutations of the corresponding Cys residues in TGA4 (TGA4 SDM) brought about interaction with NPR1, indicating that TGA4 also possesses all of the structural determinants required to interact with NPR1.

To confirm the importance of the Cys residues in regulating the interaction with NPR1 in Arabidopsis, TGA1 SDM was tested using the biolistics assay described above. In the absence of SA, leaves transfected with constructs TGA1 SDM:DB and NPR1:TA possessed a substantial amount of luciferase activity (Figure 3C). Treatment with 1 mM SA for 24 h did not dramatically increase reporter gene activity (12 versus 14 relative luciferase units). Transfection with TGA1 SDM:DB alone or with nim1-2:TA did not activate the *luciferase* reporter gene in either the presence or the absence of SA (Figure 3C). These results indicate that unlike TGA1 (Figure 1B), TGA1 SDM interacts strongly with NPR1 in the absence of SA and that treatment with SA does not substantially enhance its interaction with NPR1. Furthermore, TGA1 SDM does not interact with the nim1-2 mutant protein in Arabidopsis leaves.

NPR1 Interacts Specifically with the Reduced Form of TGA1

Because Cys residues have the potential to engage in the formation of disulfide bridges, it is tempting to speculate that the oxidized form of Cys-260 and Cys-266 (involved in a disulfide bridge) inhibits the NPR1–TGA1 interaction in yeast. On the other hand, when these Cys residues are in a reduced form, which is mimicked by their mutation, TGA1 would become competent to interact with NPR1.

To examine the redox status of TGA1 in whole-cell extracts, we adapted a strategy developed by Bayer and colleagues (1987) to label and distinguish between protein sulfhydryls (reduced Cys residues) and disulfides (oxidized Cys residues). Figure 4A shows a flow chart of this approach. Protein extracted from yeast or Arabidopsis leaves was divided into two aliquots and immediately treated for sulfhydryl (SH) or disulfide

(C) Histogram illustrating the level of interaction between TGA1 SDM and NPR1 or nim1-2 in Arabidopsis leaves treated with water (white bars) or in leaves treated for 24 h with 1 mM SA (gray bars). As a control, *TGA1 SDM* in the Gal4 DB vector was transfected separately with the reporter and internal standard constructs. Values for the VP16 TA constructs transfected separately with the reporter and internal standard constructs are presented in Figure 1B. Values represent averages ± 1 SD.

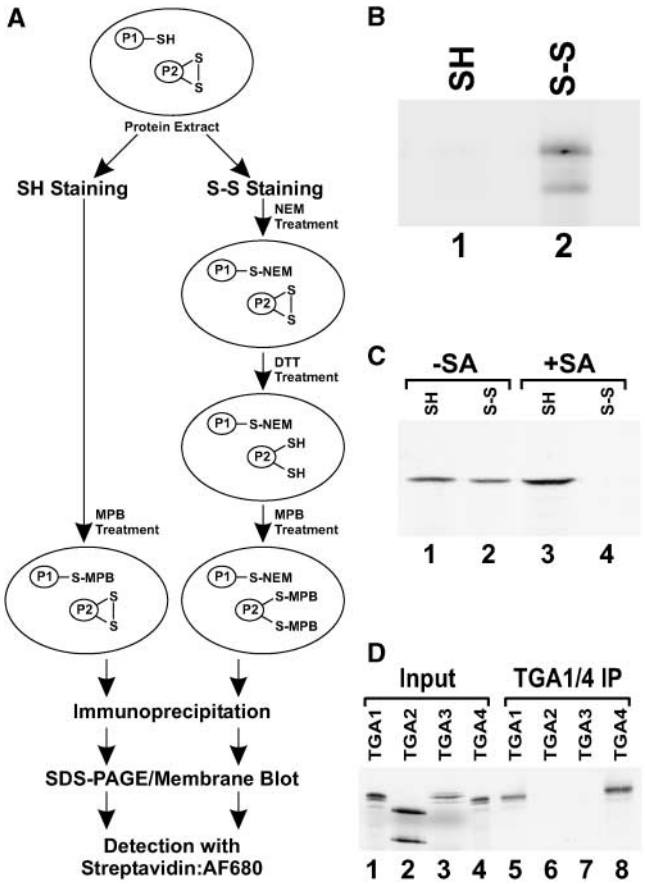

Figure 4. Determination of the in Vivo Redox Status of Cys Residues Present in TGA1.

(A) Flow chart of the method used to determine the in vivo redox status of Cys residues in TGA1. P1 and P2 represent proteins 1 and 2, respectively. SH represents a reduced Cys, and S-S represents a disulfide bridge between two Cys residues. MPB is an oxidizing alkylating agent that is biotinylated; it is used to modify and biotinylate free SH groups. NEM (*N*-ethylmaleimide) is an oxidizing alkylating agent used to block free SH groups. DTT is a reducing agent. After Cys residues have been modified by MPB, TGA1 is purified by immunoprecipitation, and the immunoprecipitated protein is blotted and reacted with a streptavidin: Alexa Fluor 680 (AF680) conjugate that will interact with biotin moieties and fluoresce at 702 nm after excitation at 679 nm.

(B) Blot analysis of TGA1 immunoprecipitate used to assess the in vivo redox status of Cys residues of TGA1 expressed in yeast cells. TGA1 was expressed as a Gal4 TA fusion, and the immunoprecipitation was performed using an anti-FLAG antibody linked to agarose beads. Lane 1, immunoprecipitate from proteins labeled for reduced Cys residues (SH); lane 2, immunoprecipitate from proteins labeled for oxidized Cys residues (S-S).

(C) Blot analysis of TGA1 immunoprecipitate used to assess the in vivo redox status of Cys residues of endogenous TGA1 present in cells of Arabidopsis leaves. The immunoprecipitation was performed using an anti-TGA1/TGA4 antibody raised against a peptide common to TGA1 and TGA4. Lanes 1 and 3, immunoprecipitates from proteins labeled for reduced Cys residues (SH); lanes 2 and 4, immunoprecipitate from proteins labeled for oxidized Cys residues (S-S). Lanes 1 and 2 contain proteins from untreated Arabidopsis, whereas lanes 3 and 4 contain proteins from Arabidopsis treated for 24 h with SA.

(S-S) staining. Although the Cys labeling is not done in vivo, the extract is assumed to represent the in vivo redox status of Cys residues. Protein sulfhydryls (SH) were stained by treatment with 3-(*N*-maleimido-propionyl) biocytin (MPB). Protein disulfides (S-S) were stained by first blocking the sulfhydryls with *N*-ethylmaleimide before reducing the disulfides with DTT. The reduced disulfides then were stained with MPB. TGA1 was purified from the bulk of proteins by immunoprecipitation, separated by SDS-PAGE, blotted onto a membrane, and revealed using a streptavidin:fluorophore conjugate capable of interacting with the biotin moiety of MPB.

In yeast cells, no signal or a very weak signal was detected for the reduced form of Cys residues, whereas the signal corresponding to oxidized Cys residues was detected readily (Figure 4B, lanes 1 and 2, respectively). Given that TGA1 fails to interact with NPR1 in yeast cells (Després et al., 2000; Zhou et al., 2000) and that mutations of Cys-260 and Cys-266 in TGA1 bring about interaction with NPR1, these results demonstrate that the oxidization of TGA1 Cys residues inhibits the interaction with NPR1 in this system.

SA Reduces Cys Residues of TGA1 in Arabidopsis Leaves

Results from Figure 4B prompted us to test the redox status of Cys residues in Arabidopsis cells under a condition that favors interaction with NPR1 (24 h of SA) and one that does not (no SA) (Figure 1).

The technique depicted in Figure 4A was used to detect the redox status of specific proteins (TGA1 and/or TGA4) present in a whole-cell extract of wild-type Arabidopsis plants before and after treatment with SA. Immunoprecipitation was performed with an antibody raised against a peptide common to TGA1 and TGA4 and that specifically recognized these two TGA factors (Figure 4D). In resting Arabidopsis cells (i.e., in the absence of SA treatment), a signal was detected for both the reduced and oxidized forms of Cys residues (Figure 4C, lanes 1 and 2), indicating that both forms coexisted in TGA1 and/or TGA4. However, after treating Arabidopsis leaves for 24 h with SA, a signal was detected only for the reduced form of Cys residues (Figure 4C, lanes 3 and 4), indicating that the Cys residues of TGA1 and/or TGA4 became fully reduced. These results indicate that the redox status of the Cys residues in TGA1 and/or TGA4 is controlled by SA. In addition, they demonstrate that, in Arabidopsis leaves, Cys residues of these TGA factors are reduced predominantly in conditions of high interaction with NPR1 (Figure 1B). In untreated Arabidopsis leaves, weak interaction with NPR1 is associated with lower signals for reduced Cys residues as well as the presence of a substantial signal for oxidized Cys residues (Figure 1B).

(D) Specificity of the anti-TGA1/TGA4 antibody used in the immunoprecipitations. TGA1, TGA2, TGA3, and TGA4 were translated and radiolabeled in vitro and were immunoprecipitated using the anti-TGA1/TGA4 antibody. Lanes 1 to 4, 10% of the input radiolabeled TGA factors; lanes 5 to 8, immunoprecipitates (IP).

TGA1 Can Form an Intramolecular Disulfide Bridge

Yap1 is a yeast bZIP transcription factor that regulates oxidative stress. Upon activation by hydrogen peroxide, Yap1 is oxidized and forms an intramolecular disulfide bridge, resulting in a conformational change that is observable by a mobility shift in nonreducing SDS-PAGE (Delaunay et al., 2002). This phenomenon is well documented and occurs in proteins with diverse functions (Mahoney et al., 1996; Delaunay et al., 2002). In a similar manner, TGA1 is a bZIP transcription factor that regulates the expression of glutathione *S*-transferase genes (Johnson et al., 2001), which encode proteins involved in the control of oxidative stress (Chen and Singh, 1999). These parallels between Yap1 and TGA1 prompted us to investigate whether TGA1 also forms an intramolecular disulfide bridge.

In vitro–translated TGA1 was incubated with the oxidizing agent diamide to allow for the potential formation of intramolecular or intermolecular disulfide bridges. Oxidized TGA1 then was reduced by excess 2-mercaptoethanol or left in its oxidized form before electrophoresing on an SDS-polyacrylamide gel. As shown in Figure 5A, the oxidized TGA1 (lane 2) had a slightly slower mobility than the reduced form (lane 1). No bands were observed in the region corresponding to twice the apparent molecular mass of TGA1 (~95 kD), as would be expected with the formation of an intermolecular bond. Rather, the slight change of mobility observed in oxidized TGA1 is diagnostic for the presence of an intramolecular disulfide bridge (Benezra, 1994; Mahoney et al., 1996; Delaunay et al., 2002). To further substantiate this result, we repeated the experiment with a site-directed mutant of *TGA1* encoding a protein with Cys-260 altered to Asn, the amino acid found in the corresponding position of TGA2. Unlike wild-type TGA1, the mutant protein migrated at the same mobility under both reducing and oxidizing conditions (Figure 5B).

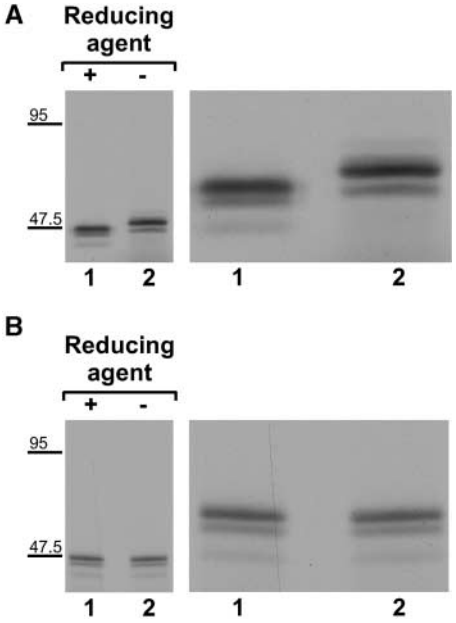

Figure 5. An Intramolecular Disulfide Bridge in TGA1 Affects Its Electrophoretic Mobility.

(A) Oxidized TGA1 was either reduced with 2-mercaptoethanol (lane 1) or left in its oxidized form (lane 2) before loading for SDS-PAGE. Reduced TGA1 migrates with an apparent molecular mass of 47.5 kD. An intermolecular disulfide bridge between two TGA1 monomers would have an apparent molecular mass of ~95 kD. At right is a close-up of the gel at left emphasizing the different electrophoretic mobilities of reduced (lane 1) and oxidized (lane 2) TGA1.

(B) Oxidized TGA1, containing a Cys-to-Asn mutation at position 260, was either reduced with 2-mercaptoethanol (lane 1) or left in its oxidized form (lane 2) before loading for SDS-PAGE. The protein migrates with an apparent molecular mass of 47.5 kD. At right is a close-up of the gel at left showing that the protein has the same electrophoretic mobility under both reduced (lane 1) and oxidized (lane 2) conditions.

Redox Changes Do Not Directly Regulate the DNA Binding Activity of TGA1

The redox regulation of the DNA binding activity of transcription factors has been demonstrated for members of several families, including the Rel-related NF-κB and the bZIP factors c-Jun and c-Fos (Abate et al., 1990; Toledano and Leonard, 1991). This stimulated us to investigate whether redox conditions would affect the DNA binding activity of TGA1, and more specifically, whether the formation of an intramolecular disulfide bridge would interfere with DNA binding. Figure 6 shows the results of electrophoresis mobility shift assays performed in the absence (lane 1) or presence of the oxidizing agent diamide (lane 2) or of the reducing agent DTT (lane 3). The data indicate that the DNA binding activity of TGA1 is unaltered by a vast molar excess of redox-regulating compounds. In addition, because bZIP transcription factors bind their cognate DNA elements as dimers (Lamb and McKnight, 1991), we also conclude from these results that the dimerization of TGA1 is not affected by redox conditions.

NPR1 Enhances the DNA Binding Activity of the Interacting Reduced Form of TGA1

NPR1 has been demonstrated to stimulate the DNA binding activity of TGA factors (Després et al., 2000; Fan and Dong, 2002). In electrophoretic mobility shift assays, NPR1 enhances the DNA binding activity of interacting TGA factors, such as TGA2, but fails to do so with TGA factors that do not show interaction in yeast, such as TGA1 and TGA4 (Després et al., 2000). Because NPR1 interacts with the reduced form of TGA1 but not with the oxidized form, we investigated the effect of redox agents on NPR1's ability to stimulate the DNA binding activity of TGA1. The addition of NPR1 to the oxidized form of TGA1 had no effect on its DNA binding activity (Figure 6, lane 4). By contrast, NPR1 stimulated the DNA binding activity of the reduced form of TGA1 (lane 5). These results confirm that the DNA binding activity of only reduced TGA1 is amenable to NPR1-mediated enhancement.

As observed with TGA2 (Després et al., 2000), NPR1 did not alter the mobility of the reduced TGA1/*as-1* (*activating se-*

quence-1) complex. We suggested previously that NPR1 enhances the DNA binding activity of the TGA factor without binding stably to the TGA/DNA complex (Després et al., 2000). This notion is consistent with a recent report (Fan and Dong, 2002) showing that the mobility of a TGA2:Gal4 DB/*UAS*^{GAL4} oligonucleotide probe complex was the same in wild-type and *npr1-2* mutant plants and that the mobility of the complex was not supershifted by the addition of two antibodies against different parts of NPR1. It is possible that the NPR1/TGA complexes are insufficiently robust to withstand the PAGE or that, upon binding DNA, the TGA factors release NPR1 (Fan and Dong, 2002). Enhancements of DNA binding activity without changes in complex mobility also have been reported for the human viral protein TAX, which stimulates the DNA binding of bZIP proteins (Wagner and Green, 1993), and the Arabidopsis OBP1 protein, which stimulates the DNA binding of TGA4 and TGA5 to the *as-1* element (Zhang et al., 1995). Furthermore, it was reported recently that the tobacco ankyrin repeat protein ANK1 inhibits the binding of the bZIP factor BZI-1 to its cognate promoter element in electrophoretic mobility shift assays without altering the complex mobility (Kuhlmann et al., 2003).

DISCUSSION

Previous studies have indicated that NPR1 does not interact with Arabidopsis TGA1 and TGA4 in yeast, whereas it does with TGA2, TGA3, TGA5, TGA6, and TGA7 (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000). Here, we show that TGA1 possesses all of the required structural elements to interact with NPR1. Indeed, our data demonstrate that this interaction occurs in Arabidopsis leaf cells in an SA-dependent manner. The generation of chimeric proteins of TGA1 and TGA2 using a domain-swapping approach followed by rational site-directed mutagenesis revealed that the interaction with NPR1 is regulated by the oxidation status of critical Cys residues within TGA1. In their oxidized state, Cys residues in TGA1 form an intramolecular disulfide bridge that inhibits interaction with NPR1. The reduced state is permissive for interaction with NPR1; consequently, NPR1 only stimulates the DNA binding activity of the reduced form of TGA1. In vivo, an accumulation of SA reduces the Cys residues in TGA1 and, accordingly, stimulates the NPR1–TGA1 interaction.

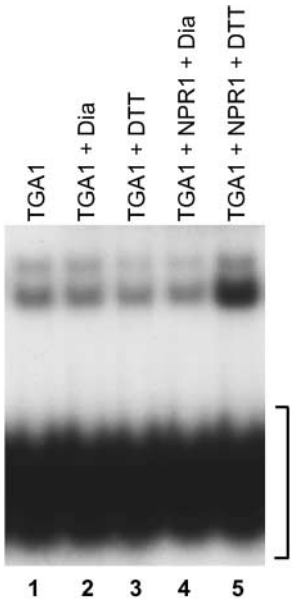

Figure 6. Binding of Reduced TGA1 to the *as-1* Element Is Enhanced by NPR1.

Electrophoretic mobility shift assay using untreated (lane 1), diamide-treated (Dia; lanes 2 and 4), or DTT-treated (lanes 3 and 5) in vitro–translated TGA1 and the *as-1* DNA element as a probe (lanes 1 to 5). NPR1 was added to the TGA1–*as-1* reaction (lanes 4 and 5). The bracket indicates the position of the free probe. An equal amount of reticulocyte lysate extract was used in all lanes.

SAR is associated with the accumulation of SA and the generation of reactive oxygen intermediates in a phenomenon described as the oxidative burst (Lamb and Dixon, 1997). It has been proposed that the role of SA is to inhibit catalase and ascorbate peroxidase and to potentiate the formation of reactive oxygen intermediates (Chen et al., 1993; Durner and Klessig, 1995). These compounds, particularly hydrogen peroxide, act as second messengers to activate disease resistance and SAR (Alvarez et al., 1998). However, a later study argued against SA being an inhibitor of ascorbate peroxidase; indeed, it was shown that SA is a substrate of this enzyme (Kvaratskhelia et al., 1997). In addition, the irreversible catalase inhibitor 3-amino-1,2,4-triazole was found to be a weak inducer of the tobacco *PR-1* gene, although this induction still was dependent on the production of SA (Bi et al., 1995; Neuenschwander et al., 1995). Therefore, the mechanism by which SA acts as a mediator of redox changes during disease resistance remains obscure.

Our results clearly indicate that SA is a modulator of the redox status of Cys residues and that its effect is exerted, at least in part, through TGA factors. Redox-sensitive transcription factors whose activities are modulated by the post-translational modification of reactive Cys residues have been identified in other organisms. Examples include prokaryotic and eukaryotic hydrogen peroxide sensors such as OxyR and Yap1 that are activated through oxidation and the formation of disulfide bridges between critical Cys residues (Zheng et al., 1998; Delaunay et al., 2000). OxyR senses hydrogen peroxide directly, whereas Yap1 does so indirectly, through a glutathione peroxidase–like enzyme (Delaunay et al., 2002). Because SA is a generator of prooxidants such as hydrogen peroxide, the observed SA-mediated reduction of TGA1 Cys residues cannot proceed through a direct sensing of hydrogen peroxide. One possibility is that under oxidizing conditions, TGA1 Cys residues may be reduced by a mechanism exemplified by the mammalian Activator Protein-1 (AP-1) complex, as detailed below.

AP-1, which includes such proteins as c-Jun and c-Fos, is part of the same family of bZIP transcription factors that includes yeast Yap1 (Moye-Rowley et al., 1989). Like Yap1 and OxyR, the DNA binding activity of AP-1 is regulated through a redox mechanism, and in certain cell types, AP-1 also is acti-

vated by prooxidants such as hydrogen peroxide. Paradoxically, the reduction of a conserved Cys residue located in the DNA binding domain is required for DNA binding activity, whereas the oxidation of the Cys has an inhibitory effect (Abate et al., 1990). The reduction of this critical Cys in oxidizing conditions is performed by the apurinic/apyrimidinic endonuclease Ref-1, which is translocated to the nucleus upon generation of sublethal doses of reactive oxygen intermediates (Ramana et al., 1998). This reduction also requires thioredoxin, which interacts physically with Ref-1 (Hirota et al., 1997). Ref-1 homologs and thioredoxins are present in the Arabidopsis genome (data not shown), suggesting that the reduction of TGA1 in prooxidizing conditions could occur through a similar mechanism.

Redox regulation of eukaryotic transcription factors generally affects Cys residues in their DNA binding domain, and the reduced form is required for full binding activity (Dalton et al., 1999). Cys residues in TGA1 are located outside of the DNA binding domain, and binding to DNA is independent of their redox status, because the addition of excess DTT or diamide does not affect band intensity in electrophoretic mobility shift assays. Therefore, although TGA1 is redox regulated by SA, at first glance one might be tempted to conclude that its DNA binding activity is not redox regulated. However, the results presented here demonstrate that the DNA binding activity of TGA1 is redox regulated but that this level of control is indirect and requires the redox-regulated recruitment of NPR1, which acts as a cofactor to stimulate TGA1 DNA binding activity.

NPR1 has been proposed to be a homolog of the mammalian protein IκB (Ryals et al., 1997), which regulates the subcellular localization and DNA binding activity of transcription factor NF-κB, whereas TGA1 is a bZIP transcription factor related to AP-1. Interestingly, searches of Arabidopsis genomic databases failed to reveal NF-κB–related sequences (Arabidopsis Genome Initiative, 2000). Consequently, it is tempting to speculate that plants have evolved an IκB-like (NPR1) function independent of NF-κB but that acts on AP-1–like (TGA1) factors.

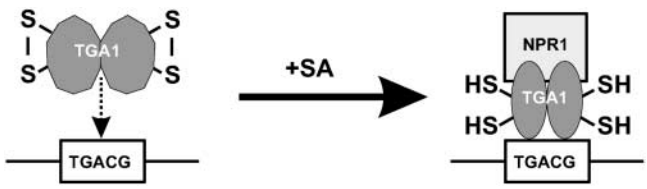

Figure 7. A Working Model Depicting the Indirect Redox Control of NPR1 on TGA1 DNA Binding Activity.

In resting Arabidopsis cells, oxidized TGA1 forms an intramolecular disulfide bridge, which alters its conformation and prevents interaction with NPR1. In the absence of NPR1 interaction, the binding of TGA1 to the cognate *cis* element is low (dotted arrow). When SA accumulates inside Arabidopsis cells, TGA1 becomes reduced and adopts a conformation that allows interaction with NPR1. The binding of NPR1 to TGA1 stimulates its DNA binding activity to the cognate *cis* element. TGA1 binds DNA as a dimer; therefore, it is represented as a homodimer.

As detailed in Figure 7, our data provide a mechanistic understanding of how the NPR1–TGA1 and/or the NPR1–TGA4 interaction might be regulated in vivo and of its effect on the stimulation of DNA binding activity by these transcription factors in situations of increased SA concentration, such as during SAR. Although we have not tested the remaining Arabidopsis TGA factors, we anticipate that they are not regulated in the same manner because they interact with NPR1 in yeast and lack Cys residues at positions corresponding to Cys-260 and Cys-266 of TGA1 (Figure 3A). Therefore, our model is intended to add to, rather than replace, other recently proposed models for the regulation of NPR1–TGA interactions (see Introduction).

Previous functional studies of TGA factors have focused on members of the family that interact with NPR1 (Niggeweg et al., 2000a; Pontier et al., 2001; Fan and Dong, 2002; Kim and Delaney, 2002). The conditional, SA-regulated interaction between TGA1 with NPR1 reported here clearly implicates this transcription factor as well as TGA4 in SAR signaling and hopefully will stimulate similar research on these transcription factors.

METHODS

Bacterial Strains and General Cloning Vectors

The *Escherichia coli* strain DH12S (Gibco BRL, Grand Island, NY) was used for plasmid maintenance and production. Cloning plasmids used were pBC SK(+) (Stratagene, La Jolla, CA) and pCR2.1 (Invitrogen, Carlsbad, CA). Large-scale plasmid purification was performed using Endo-Free GigaPrep Kits (Qiagen, Mississauga, Ontario, Canada). All constructs used were verified by sequencing.

Yeast Two-Hybrid Assays and in Vitro Translation of TGA Factors

Plasmids pBI880 and pBI881 (Kohalmi et al., 1997) in conjunction with the *Saccharomyces cerevisiae* strain YPB2 were used throughout this study. All procedures for the yeast two-hybrid system and the in vitro translation of TGA factors were described previously (Després et al., 2000).

Chimeric and Site-Directed Mutants of TGA Factors

Chimeric genes between *TGA1* and *TGA2* were created by PCR stitching using appropriate primers. Single– and double–amino acid substitution mutants were created using the Quick-Change site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions.

In Vivo Determination of the Cys Redox Status of TGA1

Proteins extracted from yeast cells or *Arabidopsis thaliana* leaves were separated into two aliquots and processed immediately and in parallel. For detection of reduced Cys residues, one aliquot was treated with 0.2 mM 3-(*N*-maleimido-propionyl) biocytin (MPB) for 1 h in the dark at room temperature. Proteins then were precipitated four times with acetone. For the detection of oxidized Cys residues, the second aliquot was treated with 20 mM *N*-ethylmaleimide for 1 h, precipitated with acetone, and then treated with 20 mM DTT for 1 h. After another acetone precipitation, proteins were treated with 0.2 mM MPB for 1 h in the dark at room temperature followed by two acetone precipitations. Cys-labeled proteins then were subjected to immunoprecipitation to purify TGA1.

Immunoprecipitation of TGA1 and Detection of Modified Cys Residues

The Cys-labeled proteins were incubated for 16 h at 4°C with the anti-TGA1 antibody coupled to protein A–Sepharose beads (Bio-Rad, Mississauga, Ontario, Canada). The beads were washed subsequently three times in IP buffer (0.1% SDS, 1% Triton X-100, 50 mM Hepes, pH 7.9, 150 mM NaCl, and 1 mM EDTA), and the proteins were eluted by boiling in 2× sample buffer (0.12 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, and 0.05% bromphenol blue). The supernatant was electrophoresed on an 8% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose and reacted according to the manufacturer's instructions (Li-Cor, Lincoln, NE) with a streptavidin:Alexa Fluor 680 conjugate (Molecular Probes, Eugene, OR) and detected by infrared imaging using an Odyssey imager (Li-Cor). For the immunoprecipitation of TGA1:TA from yeast cells, we used an anti-FLAG M2 antibody conjugated to agarose (Sigma-Aldrich, Oakville, Ontario, Canada) and omitted coupling with protein A. The procedure was otherwise identical and produced similar results. The anti-TGA1 antibody was raised in rabbits against the immunizing peptide CEDTSHGTAGTPHMFDQEAST and affinity purified by Alpha Diagnostics International (San Antonio, TX).

Plasmid Constructs for Plant Two-Hybrid Assays

The *GAL4 DB*, from the yeast two-hybrid vector pBI880, the *VP16 TA* (provided by Brian Miki, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada), and the *Renilla luciferase* from plasmid pRL-null (Promega, Madison, WI) were amplified by PCR using appropriate primers and ligated into the expression vector pBI524 (Datla et al., 1993) containing a modified double *Cauliflower mosaic virus 35S* promoter with the *Alfalfa mosaic virus* translational enhancer. The *TGA1* and *NPR1* coding sequences were amplified by PCR and ligated into pBI524 containing *GAL4 DB* or *VP16 TA* in the appropriate reading frame to permit the expression of N-terminal fusion proteins. The *5X UAS^{GAL4}:luciferase* reporter construct was provided by Masaru Ohme-Takagi National Institute of Advanced Industrial Science and Technology (Tsukuba, Japan; Fujimoto et al., 2000).

Plant Two-Hybrid Assays

Arabidopsis (ecotype Columbia) leaves were harvested from 4-week-old plants grown at 21°C (day) and 18°C (night) with a 10-h photoperiod and transferred to Petri dishes containing MS salts (Murashige and Skoog, 1962) and micronutrients supplemented with B5 vitamins, 1% sucrose, and 0.8% agar at pH 5.8. When required, filter-sterilized salicylic acid was added to the medium at a final concentration of 1 mM. Bombardment and sample processing were as described by Fujimoto et al. (2000). Each experiment consisted of five samples and was repeated three times (15 values per point).

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were performed as described previously using *as-1* as a probe (Després et al., 2000). To test their effects on the DNA binding activity of TGA1, diamide and DTT were used at a final concentration of 10 mM. To investigate the NPR1 stimulatory effect, they were used at a final concentration of 1 mM.

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 P. Fobert, pierre.fobert@nrc-cnrc.gc.ca.

ACKNOWLEDGMENTS

We are grateful to Normand Brisson for helpful comments on the manuscript. We thank Brian Miki and Masaru Ohme-Takagi for providing the *VP16 TA* and *5X UAS^{GAL4}:luciferase* constructs, respectively, and the DNA Technology Unit of the Plant Biotechnology Institute (PBI) for oligonucleotide synthesis and DNA sequencing. This research was supported by the Saskatchewan Agri-Food Innovation Fund (P.R.F.), by National Research Council Canada PBI core funding (P.R.F.), by the Natural Sciences and Engineering Research Council (NSERC) discovery grant program (C.D. and P.R.F.), and by NSERC postgraduate scholarship (D.D.) and undergraduate science research award (A.R.) programs. This is National Research Council Canada publication 45269.

Received April 14, 2003; accepted June 26, 2003.

REFERENCES

Abate, C., Patel, L., Rauscher, F.J., III, and Curran, T. (1990). Redox regulation of fos and jun DNA-binding activity in vitro. Science **249,** 1157–1161.

Alvarez, M.E., Pennell, R.I., Meijer, P.J., Ishikawa, A., Dixon, R.A., and Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell **92,** 773–784.

Arabidopsis Genome Initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408,** 796–815.

Aravind, L., and Koonin, E.V. (1999). Fold prediction and evolutionary analysis of the POZ domain: Structural and evolutionary relationship with the potassium channel tetramerization domain. J. Mol. Biol. **285,** 1353–1361.

Bayer, E.A., Safars, M., and Wilchek, M. (1987). Selective labeling of sulfhydryls and disulfides on blot transfers using avidin-biotin technology: Studies on purified proteins and erythrocyte membranes. Anal. Biochem. **161,** 262–271.

Benezra, R. (1994). An intermolecular disulfide bond stabilizes E2A homodimers and is required for DNA binding at physiological temperatures. Cell **79,** 1057–1067.

Bi, Y.M., Kenton, P., Mur, L., Darby, R., and Draper, J. (1995). Hydrogen peroxide does not function downstream of salicylic acid in the induction of PR protein expression. Plant J. **8,** 235–245.

Cao, H., Bowling, S.A., Gordon, S., and Dong, X. (1994). Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6,** 1583–1592.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X. (1997). The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88,** 57–63.

Cao, H., Li, X., and Dong, X. (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc. Natl. Acad. Sci. USA **95,** 6531–6536.

Chen, W., and Singh, K.B. (1999). The auxin, hydrogen peroxide and salicylic acid induced expression of the Arabidopsis *GST6* promoter is mediated in part by an ocs element. Plant J. **19,** 667–677.

Chen, Z., Silva, H., and Klessig, D.F. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science **262,** 1883–1886.

Chern, M.S., Fitzgerald, H.A., Yadav, R.C., Canlas, P.E., Dong, X., and Ronald, P.C. (2001). Evidence for a disease-resistance pathway in rice similar to the *NPR1*-mediated signaling pathway in Arabidopsis. Plant J. **27,** 101–113.

Dalton, T.P., Shertzer, H.G., and Puga, A. (1999). Regulation of gene expression by reactive oxygen. Annu. Rev. Pharmacol. Toxicol. **39,** 67–101.

Datla, R.S.S., Bekkaoui, F., Hammerlindl, J., Pilat, G., Dunstan, D.I., and Crosby, W.L. (1993). Improved high-level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci. **94,** 139–149.

Delaney, T.P., Friedrich, L., and Ryals, J.A. (1995). Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc. Natl. Acad. Sci. USA **92,** 6602–6606.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gutrella, M., Kessmann, H., Ward, E., and Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. Science **266,** 1247–1250.

Delaunay, A., Isnard, A.D., and Toledano, M.B. (2000). H_2O_2 sensing through oxidation of the Yap1 transcription factor. EMBO J. **19,** 5157–5166.

Delaunay, A., Pflieger, D., Barrault, M.B., Vinh, J., and Toledano, M.B. (2002). A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. Cell **111,** 471–481.

Després, C., DeLong, C., Glaze, S., Liu, E., and Fobert, P.R. (2000). The Arabidopsis NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors. Plant Cell **12,** 279–290.

Durner, J., and Klessig, D.F. (1995). Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense responses. Proc. Natl. Acad. Sci. USA **92,** 11312–11316.

Fan, W., and Dong, X. (2002). In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid–mediated gene activation in Arabidopsis. Plant Cell **14,** 1377–1389.

Friedrich, L., Lawton, K., Dietrich, R., Willits, M., Cade, R., and Ryals, J. (2001). *NIM1* overexpression in Arabidopsis potentiates plant disease resistance and results in enhanced effectiveness of fungicides. Mol. Plant-Microbe Interact. **14,** 1114–1124.

Fujimoto, S.Y., Ohta, M., Usui, A., Shinshi, H., and Ohme-Takagi, M. (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box–mediated gene expression. Plant Cell **12,** 393–404.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1996). Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics **143,** 973–982.

Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997). AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. Proc. Natl. Acad. Sci. USA **94,** 3633–3638.

Johnson, C., Boden, E., Desai, M., Pascuzzi, P., and Arias, J. (2001). In vivo target promoter-binding activities of a xenobiotic stress-activated TGA factor. Plant J. **28,** 237–243.

Kim, H.S., and Delaney, T.P. (2002). Over-expression of *TGA5*, which encodes a bZIP transcription factor that interacts with NIM1/NPR1, confers SAR-independent resistance in *Arabidopsis thaliana* to *Peronospora parasitica*. Plant J. **32,** 151–163.

Kinkema, M., Fan, W., and Dong, X. (2000). Nuclear localization of NPR1 is required for activation of *PR* gene expression. Plant Cell **12,** 2339–2350.

Kohalmi, S.E., Nowak, J., and Crosby, W.L. (1997). The yeast two-hybrid system. In Differentially Expressed Genes in Plants: A Bench Manual, E. Hansen and G. Harper, eds (London: Taylor and Francis), pp. 63–82.

Kuhlmann, M., Horvay, K., Strathmann, A., Heinekamp, T., Fischer, U., Böttner, S., and Dröge-Laser, W. (2003). The alpha-helical D1 domain of the tobacco bZIP transcription factor BZI-1 interacts with the ankyrin-repeat protein ANK1 and is important for BZI-1 function, both in auxin signaling and pathogen response. J. Biol. Chem. **278,** 8786–8794.

Kvaratskhelia, M., George, S.J., and Thorneley, R.N. (1997). Salicylic acid is a reducing substrate and not an effective inhibitor of ascorbate peroxidase. J. Biol. Chem. **272,** 20998–21001.

Lamb, C., and Dixon, R.A. (1997). The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 251–275.

Lamb, P., and McKnight, S.L. (1991). Diversity and specificity in transcriptional regulation: The benefits of heterotypic dimerization. Trends Biochem. Sci. **16,** 417–422.

Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J., and Ward, E. (1998). Functional analysis of regulatory sequences controlling *PR-1* gene expression in Arabidopsis. Plant J. **16,** 223–233.

Mahoney, C.W., Pak, J.H., and Huang, K.P. (1996). Nitric oxide modification of rat brain neurogranin: Identification of the cysteine residues involved in intramolecular disulfide bridge formation using site-directed mutagenesis. J. Biol. Chem. **271,** 28798–28804.

Moye-Rowley, W.S., Harshman, K.D., and Parker, C.S. (1989). Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. Genes Dev. **3,** 283–292.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15**, 473–497.

Nawrath, C., and Métraux, J.P. (1999). Salicylic acid induction-deficient mutants of Arabidopsis express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11,** 1393–1404.

Neuenschwander, U., Vernooij, B., Friedrich, L., Uknes, S., Kessmann, H., and Ryals, J. (1995). Is hydrogen peroxide a second messenger of salicylic acid in systemic acquired resistance? Plant J. **8,** 227–233.

Niggeweg, R., Thurow, C., Kegler, C., and Gatz, C. (2000a). Tobacco transcription factor TGA2.2 is the main component of *as-1*-binding factor ASF-1 and is involved in salicylic acid- and auxin-inducible expression of *as-1*-containing target promoters. J. Biol. Chem. **275,** 19897–19905.

Niggeweg, R., Thurow, C., Weigel, R., Pfitzner, U., and Gatz, C. (2000b). Tobacco TGA factors differ with respect to interaction with NPR1, activation potential and DNA-binding properties. Plant Mol. Biol. **42,** 775–788.

Pontier, D., Miao, Z.H., and Lam, E. (2001). Trans-dominant suppression of plant TGA factors reveals their negative and positive roles in plant defense responses. Plant J. **27,** 529–538.

Ramana, C.V., Boldogh, I., Izumi, T., and Mitra, S. (1998). Activation of apurinic/apyrimidinic endonuclease in human cells by reactive oxygen species and its correlation with their adaptive response to genotoxicity of free radicals. Proc. Natl. Acad. Sci. USA **95,** 5061–5066.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., and Hunt, M.D. (1996). Systemic acquired resistance. Plant Cell **8,** 1809–1819.

Ryals, J.A., Weymann, K., Lawton, K., Friedrich, L., Ellis, D., Steiner, H.Y., Johnson, J., Delaney, T.P., Jesse, T., Vos, P., and Uknes, S. (1997). The Arabidopsis NIM1 protein shows homology to the mammalian transcription factor inhibitor IκB. Plant Cell **9,** 425–439.

Sedgwick, S.G., and Smerdon, S.J. (1999). The ankyrin repeat: A diversity of interactions on a common structural framework. Trends Biochem. Sci. **24,** 311–316.

Shah, J., Tsui, F., and Klessig, D.F. (1997). Characterization of a salicylic acid–insensitive mutant (*sai*) of *Arabidopsis thaliana* identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. Mol. Plant-Microbe Interact. **10,** 69–78.

Spoel, S.H., et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15,** 760–770.

Subramaniam, R., Desveaux, D., Spickler, C., Michnick, S.W., and

Brisson, N. (2001). Direct visualization of protein interactions in plant cells. Nat. Biotechnol. **19,** 769–772.

Toledano, M.B., and Leonard, W.J. (1991). Modulation of transcription factor NF-kappa B binding activity by oxidation-reduction in vitro. Proc. Natl. Acad. Sci. USA **88,** 4328–4332.

Wagner, S., and Green, M. (1993). HTLV TAX protein stimulation of DNA binding of bZIP proteins by enhancing dimerization. Science **262,** 395–399.

Ward, E., Uknes, S., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Métraux, J.P., and Ryals, J. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3,** 1085–1094.

Zhang, B., Chen, W., Foley, R.C., Buttner, M., and Singh, K.B. (1995). Interactions between distinct types of DNA binding proteins enhance binding to *ocs* element promoter sequence. Plant Cell **7,** 2241–2252.

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. Proc. Natl. Acad. Sci. USA **96,** 6523–6528.

Zheng, M., Aslund, F., and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. Science **279,** 1718–1721.

Zhou, J.-M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., and Klessig, D.F. (2000). NPR1 differentially interacts with members of the TGA/OBF family of transcription factors that bind an element of the *PR-1* gene required for induction by salicylic acid. Mol. Plant-Microbe Interact. **13,** 191–202.