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# 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

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**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).

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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).

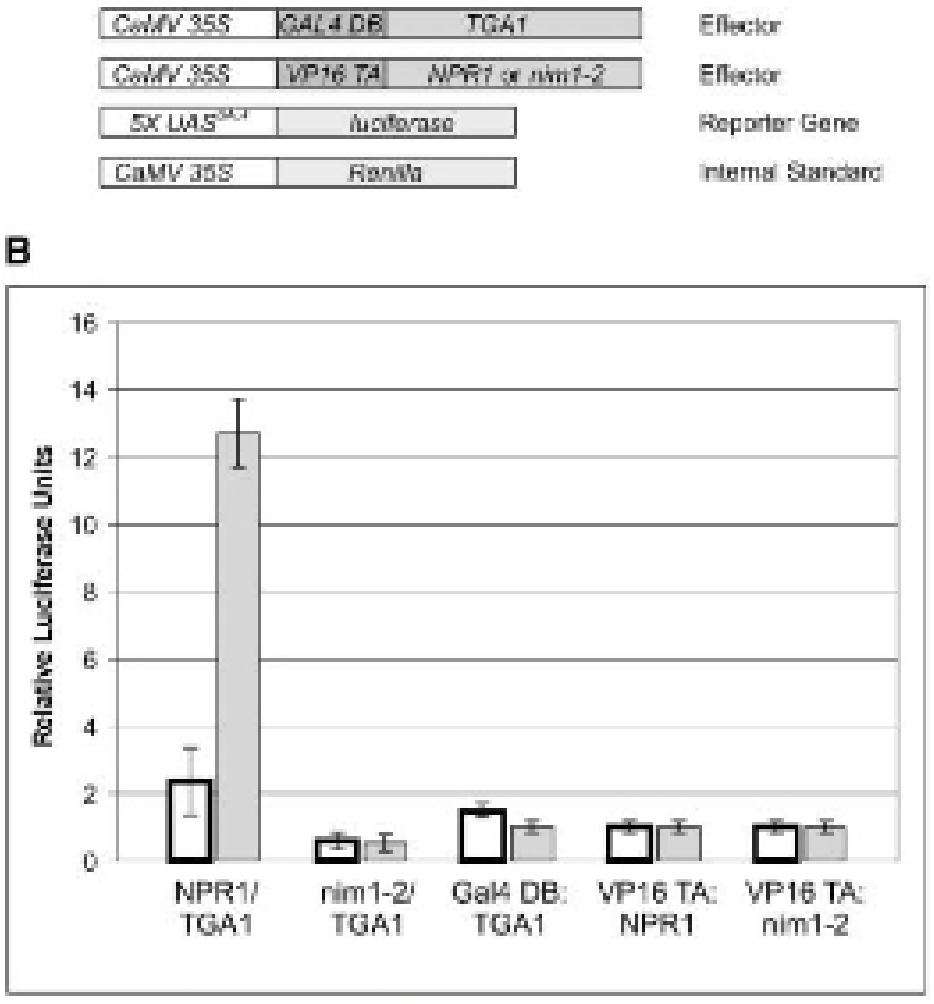


**Figure 1.** NPR1 Interacts with TGA1 in Arabidopsis Leaves.

**(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).

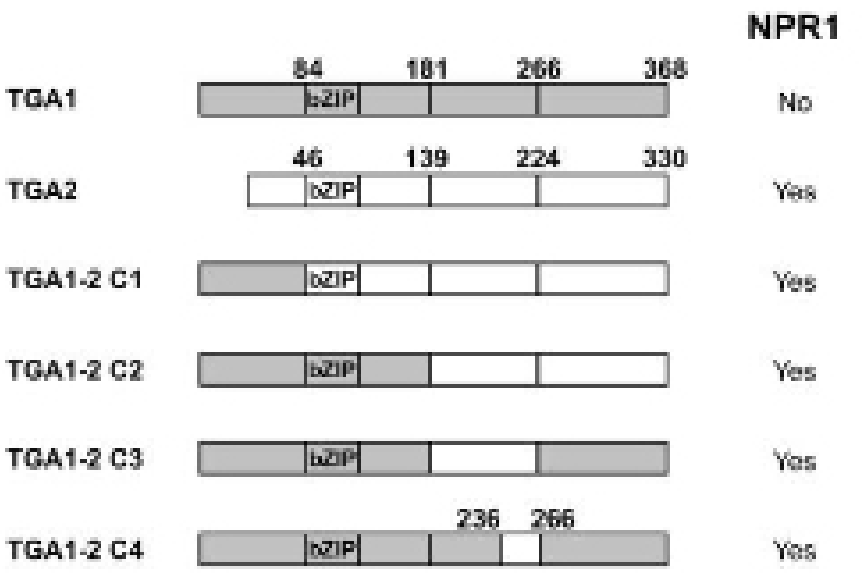


**Figure 2.** Identification of a 30–Amino Acid Region in TGA2 That Determines the Potential for NPR1 Interaction.

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.