Evidence for an Important Role of WRKY DNA Binding Proteins in the Regulation of NPr1 Gene Expression

Diqiu Yu, Chunhong Chen, and Zhixiang Chen

Department of Microbiology, Molecular Biology, and Biochemistry, University of Idaho, Moscow, Idaho 83844-3052

The Arabidopsis NPr1 gene is a positive regulator of inducible plant disease resistance. Expression of NPr1 is induced by pathogen infection or treatment with defense-inducing compounds such as salicylic acid (SA). Transgenic plants overexpressing NPr1 exhibit enhanced resistance to a broad spectrum of microbial pathogens, whereas plants underexpressing the gene are more susceptible to pathogen infection. These results suggest that regulation of NPr1 gene expression is important for the activation of plant defense responses. In the present study, we report the identification of W-box sequences in the promoter region of the NPr1 gene that are recognized specifically by SA-induced WRKY DNA binding proteins from Arabidopsis. Mutations in these W-box sequences abolished their recognition by WRKY DNA binding proteins, rendered the promoter unable to activate a downstream reporter gene, and compromised the ability of NPr1 to complement npr1 mutants for SA-induced defense gene expression and disease resistance. These results provide strong evidence that certain WRKY genes act upstream of NPr1 and positively regulate its expression during the activation of plant defense responses. Consistent with this model, we found that SA-induced expression of a number of WRKY genes was independent of NPr1.

INTRODUCTION

Upon infection by a microbial pathogen, a resistant plant often is able to recognize the invading pathogen through the specific interaction between pathogen-encoded molecules called elicitors and plant host receptors, many of which may be encoded by disease resistance genes (Yang et al., 1997; Van der Biezen and Jones, 1998). This timely recognition can trigger a rapid, local death of plant cells at the sites of pathogen infection (the hypersensitive response) that may contain the invading pathogens. In addition, a local hypersensitive response often is associated with the activation of plant defense responses in the surrounding, and even distal, uninfected parts of the plants, leading to the development of systemic acquired resistance (SAR) (Hunt et al., 1996). The establishment of SAR often is associated with enhanced biosynthesis of salicylic acid (SA) and activated expression of pathogenesis-related (PR) genes (Hunt et al., 1996). Recently, certain nonpathogenic, root-colonizing rhizobacteria also have been found to induce systemic resistance in plants (Pieterse and van Loon, 1999). Unlike SAR, this induced systemic resistance is independent of SA or PR gene expression but requires the action of jasmonate and ethylene response pathways (Pieterse and van Loon, 1999). Despite this critical difference, both SAR and induced systemic resistance require the function of the Arabidopsis NPr1 (also known as NIM1) gene (Cao et al., 1994; Delaney et al., 1995; Pieterse and van Loon, 1999).

NPr1 encodes a 66-kD protein with ankyrin repeats and some homology with the animal IκB protein (Cao et al., 1997; Ryals et al., 1997). NPr1 is localized to the nucleus upon SAR induction and binds and enhances the DNA binding activity of several members of the TGA/ObF transcription family (Zhang et al., 1999; Despres et al., 2000; Kinkema et al., 2000; Niggeweg et al., 2000; Zhou et al., 2000). Thus, unlike the IκB protein, which serves as a repressor of gene expression, NPr1 functions as a transcription coactivator of genes involved in the resistance signaling pathways. More recently, a suppressor (SN1) for the mutant npr1-1 gene was identified that encodes a leucine-rich nuclear protein with some homology with the mouse retinoblastoma protein, a tumor suppressor that represses the transcription regulated by transcription factors such as E2F (Li et al., 1999). SN1 may be a negative regulator of SAR that is inactivated by NPr1 after SA treatment or pathogen infection (Li et al., 1999).

NPr1 is expressed at low levels in healthy uninfected plants (Ryals et al., 1997). Upon pathogen infection or treatment with SA or its functional analogs, the expression of NPr1 is induced by twofold to threefold (Cao et al., 1997; Ryals et al., 1997). Recently, it was reported that transgenic Arabidopsis plants overexpressing NPr1 exhibit high levels
of resistance to both the bacterial pathogen *Pseudomonas syringae pv maculicola* ES4326 and the oomycete pathogen *Peronospora parasitica* Noco, whereas plants underexpressing NPR1 are more susceptible to these pathogens (Cao et al., 1998). These results suggest that the increased expression of NPR1 induced by pathogens and SA may be one of the critical steps in the activation of the plant defense response. Interestingly, although the expression of many defense-related genes is dependent on NPR1, accumulation of the NPR1 gene transcript is normal in npr1 mutants (Cao et al., 1997; Ryals et al., 1997). Thus, induced NPR1 gene expression is mediated by signaling mechanisms different from those responsible for the expression of many other defense-related genes; therefore, studying the expression of NPR1 may lead to the identification of novel components and mechanisms important for the activation of the plant defense response.

We are studying a group of pathogen- and SA-induced DNA binding proteins containing the novel WRKY domains (Wang et al., 1998; Yang et al., 1999; Chen and Chen, 2000). Plant WRKY proteins recognize various W-box elements with a TGAC core sequence that are present in promoters of many defense-related genes (Eulgem et al., 2000). As one approach toward identifying their functions, we are interested in identifying potential target genes regulated by these pathogen- and SA-induced DNA binding proteins. In the present study, we report the presence of W-box sequences in the promoter of NPR1 that are recognized specifically by pathogen- and SA-induced WRKY proteins. A series of molecular and genetic analyses have indicated that these W-box sequences are necessary for the induction of NPR1 transcription and for the NPR1-mediated activation of the plant defense response. These results strongly suggest that certain WRKY proteins act upstream of NPR1 and positively regulate its expression during the activation of the plant defense response.

**RESULTS**

**Recognition of W-Boxes in the NPR1 Gene Promoter by WRKY Proteins**

The likely transcriptional start site for NPR1 has been determined to be at position 199 upstream of the translation start site (Ryals et al., 1997). A putative TATA box is found 39 bases upstream of the transcriptional start site (Figure 1). Interestingly, there are three TTGAC W-box sequences within a 28-bp region from position 103 to position 129 upstream of the translation start site (Figure 1), suggesting a potential role of WRKY proteins in the regulation of NPR1 gene expression. These potential cis-acting regulatory elements are located within the transcribed but untranslated region of the NPR1 gene (Figure 1).

To determine the role of these W-box sequences in the function of NPR1, we first determined whether they were recognized specifically by SA-induced WRKY DNA binding proteins. A double-stranded DNA probe (PN1) containing these three W boxes was synthesized and labeled (Figure 2A). When incubated with the recombinant AtWRKY18 protein (Eulgem et al., 2000), this probe produced retarded bands in electrophoretic mobility shift assays (Figure 2B). When incubated with nuclear extracts isolated from Arabidopsis plants, the mutant probe failed to be recognized by the SA-induced binding activities detectable with the PN1 probe (Figure 2C). Thus,

![Figure 1. Scheme of The Arabidopsis NPR1 Gene Promoter.](image-url)
the W-box sequences in the *NPR1* gene promoter were recognized specifically by both the purified recombinant AtWRKY18 protein and the SA-induced W-box binding activities from SA-treated Arabidopsis plants.

To confirm whether the SA-induced W-box binding activities from the nuclear extracts detected by the PN1 probe were in fact WRKY DNA binding proteins, we prepared antibodies against the conserved WRKYGQK sequence found in WRKY proteins and examined their effects on the binding activities. For these assays, the antibodies were first affinity purified using the WRKYGQK peptides as affinity ligands. As shown in Figure 3A, preincubation of purified recombinant AtWRKY18 with the preimmune antiserum IgG antibodies had little effect on the intensities or migration rates of the shifted bands on electrophoretic mobility shift assays. On the other hand, preincubation of the WRKY proteins with the affinity-purified WRKY antibodies severely inhibited the binding activity of AtWRKY18 (Figure 3A). Likewise, when the nuclear extracts isolated from SA-treated Arabidopsis plants were first preincubated with the preimmune antiserum IgG antibodies, the SA-induced W-box binding activities detected by the PN1 probe were not affected significantly (Figure 3B). However, if the same nuclear extracts were preincubated with the affinity-purified WRKY antibodies, the W-box binding activities were inhibited substantially (Figure 3B). On the basis of these results, it appears that SA-induced WRKY proteins in the nuclear extracts were predominantly responsible for the DNA binding activities that recognized the W-box sequences in the *NPR1* gene promoter. Moreover, the ability of the affinity-purified WRKY antibodies to
inhibit the DNA binding activity of WRKY proteins indicated that the conserved WRKYGQK sequence is required by these proteins to bind their DNA targets. This is consistent with the finding that recombinant mutant AtWRKY18 proteins with substitutions for the Arg, Gly, or Gln residue in the conserved WRKYGQK region had greatly reduced W-box binding activity (Z. Chen, unpublished results).

**Promoter Analysis Using a Reporter Gene**

After demonstrating that the three closely linked W-box sequences in the *NPR1* gene promoter could be recognized by both a purified WRKY protein and SA-induced WRKY binding proteins in the nuclear extracts, we examined whether these W-box sequences were necessary for the inducible activity of the *NPR1* gene promoter. To investigate this possibility, we compared the activities of two constructs. The first construct consisted of the 2419-bp promoter fragment (from −2419 to +1) of the *NPR1* gene fused with the β-glucuronidase (GUS) reporter gene (*NPR1*-GUS) (Figure 4A). For the second construct, all three TTGAC sequences in the *NPR1* gene promoter were changed to TTGAA before the fragment was fused to the GUS gene (*mNPR1*-GUS) (Figure 4A). Both constructs were transformed into Arabidopsis (Columbia ecotype). More than 25 transgenic Arabidopsis plants were obtained for each construct and analyzed for the levels of both GUS gene transcripts and GUS activity. As shown in Figure 4B, in untreated Arabidopsis plants, a significant level of GUS gene transcripts was detected in the transgenic plants transformed with the *NPR1*-GUS construct. After SA treatment, the level of GUS transcript increased by twofold to threefold (Figure 4B). Thus, this promoter fragment was sufficient to confer both the basal and SA-inducible expression of the reporter GUS gene in a pattern similar to that found with the endogenous *NPR1* gene (Cao et al., 1997; Ryals et al., 1997).

Importantly, mutating the TTGAC sequences to TTGAA completely abolished the accumulation of transcripts for the GUS transgene (Figure 4B). In fact, it accumulated no more transcripts than plants containing a promoterless GUS construct (−GUS) (Figure 4B). Consistent with the RNA gel blotting, untreated transgenic plants transformed with the *NPR1*-GUS construct produced significantly more GUS activity than those transformed with the promoterless construct (Figure 4C). This level increased approximately fourfold after SA treatment (Figure 4C). On the other hand, mutations of the W-boxes reduced the GUS activity to levels close to those found in transgenic plants transformed with the promoterless GUS gene construct, regardless of whether the plants were treated with SA (Figure 4C). These results indicated that the three W-box sequences in the promoter of the *NPR1* gene are important for the basal level of expression and essential for the induction of the reporter gene.

Because the three W-box sequences in the *NPR1* gene promoter are located in its transcribed region, mutations of the sequences also could alter the sequences of the 5’ untranslated regions of the transcripts and affect their stability. This effect could account for the largely abolished accumulation of GUS transcripts and GUS activity in transgenic plants transformed with the *mNPR1*-GUS construct (Figure 4). To examine this possibility, we analyzed the transcription of the GUS reporter gene in transgenic plants using nuclear run-on assays. As shown in Figure 5, a significant background of transcription of the GUS gene was detected in transgenic plants transformed with the *NPR1*-GUS construct. After SA treatment, transcription of the reporter gene was enhanced substantially (Figure 5). In contrast, no detectable level of transcription of the GUS gene was found in the transgenic plants transformed with the *mNPR1*-GUS construct in either untreated or SA-treated plants (Figure 5). As internal controls, we detected similar levels of transcription of the cotransformed *APHII* transgene that was under the control of a nos gene promoter (Figure 5). These results indicated that the mutations in the W-box sequences in the *NPR1* gene promoter reduced the accumulation of GUS.

**Figure 3.** Antibodies against the Conserved WRKYGQK Sequence of WRKY Proteins Inhibit Binding of AtWRKY18 and SA-Induced W-Box Binding Activities to the PN1 Probe.

(A) Sequence-specific binding of PN1 by the recombinant AtWRKY18 protein without added antibodies (lane 1) or with added preimmune antiserum IgG (lane 2) or affinity-purified WRKY antibodies (lane 3).

(B) Sequence-specific binding of PN1 by SA-induced W-box binding activities in the nuclear extracts isolated from SA-treated Arabidopsis plants without added antibodies (lane 1) or with added preimmune antiserum IgG (lane 2) or affinity-purified WRKY antibodies (lane 3).
Figure 4. Importance of the W-Box Elements for NPR1 Gene Promoter Activity.

(A) Constructs of NPR1-GUS (the +1 to −2419 promoter sequence of the NPR1 gene fused to the GUS reporter gene), mNPR1-GUS (the +1 to −2419 promoter sequence with mutated TTGAA sequences fused to the GUS reporter gene), and −GUS (the GUS reporter gene with no upstream promoter). Underlining signifies W-box sequences. Asterisks represent the mutated bases in the W-box elements.

(B) RNA gel blotting of GUS transcripts in transgenic Arabidopsis plants harboring the three promoter constructs shown in (A) before SA treatment (−SA) or 24 hr after SA treatment (+SA). Ethidium bromide staining of rRNA is shown to demonstrate equal loading of RNA in each lane.

(C) GUS activities in transgenic Arabidopsis plants harboring the three promoter constructs shown in (A) before SA treatment (−SA) or 24 hr after SA treatment (+SA). MU, 4-methylumbelliferone. Error bars indicate ±SE.
transcripts and GUS activity in transgenic plants by affecting transcription rather than the RNA stability of the transgene.

Complementation Analysis

To determine if the W-box sequences are essential for the level of functions of 

\[
\begin{array}{c|c|c|c}
\text{NPR1-GUS} & \text{mNPR1-GUS} \\
\hline
\text{SA} & - & + & - & + \\
\text{GUS} & & & & \\
\text{AphII} & & & & \\
\end{array}
\]

Figure 5. Transcription of the GUS Transgene under the Control of the Wild-Type or Mutant NPR1 Gene Promoter.

\[^{32}\text{P}-\text{CTP}-\text{labeled transcripts were prepared from nuclear run-on assays of nuclei isolated from transgenic plants transformed with the NPR1-GUS or mNPR1-GUS construct before (−) and after (+) SA spraying (2 mM for 12 hr). The filters contained immobilized, linearized plasmids containing sequences for GUS and AphII genes. The AphII gene under the control of the nos promoter conferred kanamycin resistance in the transgenic plants, and its transcription was used as an internal control.}\]

Expression of SA-Induced WRKY Genes

If certain WRKY DNA binding proteins act upstream of NPR1 and positively regulate its expression during the activation of plant defense responses, their own expression would be expected to be independent of NPR1. To examine this notion, we attempted to identify SA-induced WRKY genes in Arabidopsis and to analyze the effects of NPR1 on their expression. To isolate SA-induced WRKY genes, we first used domain-specific differential display by taking advantage of the conserved WRKY domain amino acid sequence present in all isolated WRKY proteins. Total RNA was isolated from SA-treated Arabidopsis plants and converted to cDNA using reverse transcriptase with oligo(dT) molecules as primers. Two degenerate primers corresponding to the conserved WRKY domain sequence were synthesized and used in conjunction with the oligo(dT) primer for polymerase
Figure 6. Complementation for PR1 Gene Expression.

(A) Structures of the NPR1, mNPR1, and mNPR1a genes used for complementation. mNPR1 contains a mutant NPR1 gene promoter with the three TTGAC sequences mutated into TTGAA. mNPR1a contains a mutant NPR1 gene promoter with the two canonical W-box sequences mutated from TTGAC to TTGAA. Underlining signifies W-box sequences. Asterisks represent the mutated bases in the W-box elements.

(B) RNA gel blotting of PR1 gene expression in the wild type (WT), npr1-3, and npr1-3 transformants with NPR1 or mNPR1. RNA samples were prepared from 4-week-old plants 24 hr after treatment with 2 mM SA. Ethidium bromide staining of rRNA is shown to demonstrate equal loading of RNA in each lane.

(C) RNA gel blotting of PR1 gene expression 24 hr after SA treatment (2 mM) in the wild type (WT), npr1-3, and npr1-3 transformants with NPR1 or mNPR1a.
expression in both wild-type and WRKY genes was dependent on treated Arabidopsis plants (Figure 8). (Eulgem et al., 2000) and were found to be induced in SA-sequence tag clones correspond to characteristic of WRKY proteins. Seven of these expressed 15 expressed sequence tag clones that encode proteins with characteristic features of WRKY DNA binding proteins. Two of these three WRKY genes correspond to proteins with characteristic WRKY DNA binding proteins isolated from SA-treated Arabidopsis (Maleck et al., 2000). The third WRKY gene was not identified in this reported search and was designated \textit{AtWRKY62}. RNA gel blot analysis revealed that these WRKY genes could be induced levels of transcripts at later times after SA treatment showed normal expression at early times but greatly reduced levels of transcripts at later times after SA treatment in the \textit{npr1-3} mutant (Figure 8). \textit{AtWRKY62} was the only member of the third group whose inducible expression was abolished completely in the \textit{npr1-3} mutant (Figure 8). Thus, the SA-induced expression of a majority of these WRKY genes was independent of \textit{NPR1}.

**DISCUSSION**

DNA binding proteins containing the novel WRKY domains are a recently identified family of DNA binding proteins found only in higher plants (Eulgem et al., 2000). A number of studies have suggested that members of this gene family may play important roles in the plant defense response. A number of genes encoding WRKY proteins have been isolated from several plants, including some that are induced rapidly by pathogen infection or treatment with pathogen elicitors or SA (Eulgem et al., 1999; Chen and Chen, 2000; Dellagi et al., 2000; Hara et al., 2000; Kim et al., 2000). Plant WRKY DNA binding proteins recognize various W-box elements with a TGAC core sequence that are present in promoters of a number of defense-related genes. For example, it has been shown that the elicitor response element in the promoter of the tobacco class I chitinase gene, \textit{CHN50}, is a W-box element recognized specifically by pathogen-induced WRKY proteins (Yang et al., 1999). In addition, pathogen-induced WRKY proteins recognize the elicitor response elements of the parsley \textit{PR-1} genes (Rushton et al., 1996), suggesting that members of this group of DNA binding factors also may regulate the expression of other \textit{PR} genes. A recent microarray study further revealed that the W-box is a common promoter element in a group of defense-related genes that includes \textit{PR-1}, a reliable marker gene for SAR in Arabidopsis (Maleck et al., 2000).

In the present study, we provide strong evidence that WRKY proteins also play an important role in the regulation of transcription of \textit{NPR1}, an important regulator of the plant defense response and induced disease resistance. First, we demonstrated that the \textit{NPR1} gene promoter contains W-box sequences that are bound specifically by SA-induced WRKY DNA binding proteins isolated from SA-treated Arabidopsis plants (Figures 2 and 3). Second, we showed that these W-box sequences serve as important cis-acting elements for the transcriptional regulation of \textit{NPR1} gene expression (Figures 4 and 5), which is important for the function of the regulatory gene, as demonstrated by complementation analysis (Figure 6). Although W-box sequences have been identified previously in the promoters of many defense-related genes and in a few cases have been shown to be important...
for activating the expression of downstream genes, our studies with the NPR1 gene promoter have demonstrated that mutations in the W-box sequences can completely abolish SA-induced PR gene expression and render plants more susceptible to bacterial pathogens (Figures 6 and 7). These results provide strong evidence that WRKY proteins play important roles in the activation of plant defense responses through regulating transcription of important regulatory genes such as NPR1 as well as genes that encode defense proteins with direct or indirect antimicrobial activities (Rushton et al., 1996; Yang et al., 1999).

In untreated healthy plants, NPR1 is expressed at a low background level (Ryals et al., 1997). After pathogen infection or treatment with SA or a functional analog, NPR1 is induced severalfold (Cao et al., 1997; Ryals et al., 1997). A recent study has shown that the basal level of NPR1 gene expression is sufficient to facilitate the simultaneous expression of SAR and induced systemic resistance (van Wees et
al., 2000). However, enhanced expression of NPR1 may further increase the magnitude of disease resistance, as demonstrated in transgenic plants that overexpress the regulatory gene (Cao et al., 1998). These results indicate that both basal and induced expression of NPR1 are important for induced plant disease resistance. The expression pattern of NPR1 is correlated with the binding pattern of the W-box sequences in its promoter by SA-induced WRKY proteins (Figure 2). When incubated with nuclear extracts isolated from untreated healthy plants, significant background levels of binding activities were observed (Figure 2C). When incubated with nuclear extracts isolated from SA-treated Arabidopsis plants, these WRKY DNA binding activities increased substantially (Figure 2C). This correlated change of WRKY DNA binding activities would suggest that WRKY proteins are involved in the regulation of both basal and induced transcription of NPR1. This is consistent with the result that mutations in the W-box sequences of the NPR1 gene promoter abolished both its basal and its induced expression, as demonstrated by GUS fusion constructs (Figures 4 and 5) and the inability of the mutant NPR1 gene to complement the npr1 mutants (Figures 6 and 7).

Transcriptional regulation of NPR1 gene expression is likely to play a critical role in determining the protein level of NPR1 in plant cells, a critical factor in induced disease resistance, as demonstrated in the npr1-3 mutant plants transformed with NPR1 genes containing mutations in their promoter sequences (Figures 6 and 7). Because plants overexpressing NPR1 show enhanced resistance to different types of pathogens without constitutively expressing the PR genes, it has been suggested that the NPR1 protein requires activation to be functional (Cao et al., 1998). One mechanism of activation could involve the translocation of NPR1 to nuclei. Using an NPR1-green fluorescent protein fusion protein, it has been shown that NPR1 accumulates in the nucleus in response to activators of SAR (Kinkema et al., 2000). Using a steroid-inducible system, it was further shown that the nuclear localization of NPR1 is essential for its activity in inducing PR gene expression (Kinkema et al., 2000). Together, these studies indicate that both the level and the activity of NPR1 are important for its action in plant defense response.

In the last several years, genetic strategies have been used to determine SA signal transduction pathways by isolating and characterizing SA-insensitive mutants in Arabidopsis (Glazebrook et al., 1996; Ryals et al., 1997; Shah et al., 1997; Cao et al., 1998). These screens have identified a number of mutants that fail to express PR genes and that exhibit enhanced disease resistance in response to treatment with SA or its functional analogs. Interestingly, many of these reported SA-insensitive mutants are allelic, caused by mutations in the NPR1 genes (Ryals et al., 1997; Shah et al., 1997; Cao et al., 1998). If WRKY genes act upstream of NPR1, they should have been identified in these genetic screens for mutants compromised in induced disease resistance, yet none has been reported to date. Failure to identify WRKY gene mutants might suggest that the mutant screens have not saturated the whole Arabidopsis genome, although the repeated identification of npr1 mutants by a number of independent groups would indicate otherwise. Alternately, some of the WRKY genes may be functionally redundant in the regulation of plant defense responses, including the activation of NPR1 gene expression. The substantial number of SA-induced WRKY genes in Arabidopsis is consistent with this possibility (Figure 8). Furthermore, some of the WRKY genes might be regulators of essential biological processes so that mutations causing severe reduction in their biological activities may be deleterious or even lethal for the plants. These questions can now be addressed in a systematic manner by means of reverse genetics to assess the functions of individual WRKY genes.

Transcriptional regulation of plant host genes plays a central role in the activation of plant disease resistance response. Therefore, an important step toward understanding the regulation of plant defense response is to identify regulatory components and establish pathways of transcriptional regulation of plant defense gene expression. Studies during the last several years have identified several families of sequence-specific DNA binding (SSDB) transcription factors that are likely involved in the transcriptional regulation of plant defense genes. These SSDB proteins include members of the ethylene-responsive element binding protein family of DNA binding proteins, Myb-like proteins, bZIP proteins, and W-box binding WRKY proteins (Rushton and Somssich, 1998). A number of studies have further revealed potential communication among different classes of transcription regulatory components through direct protein–protein interactions. For example, an ocs element binding protein (OBF4, a bZIP factor) has been shown to interact with an ethylene-responsive element binding protein (Buttner and Singh, 1997). More recently, NPR1 has been shown to bind and enhance DNA binding activities of several bZIP proteins (Zhang et al., 1999; Despres et al., 2000; Niggeweg et al., 2000; Zhou et al., 2000).

In the present study, we have shown that some members of the WRKY gene family act upstream of NPR1 and positively regulate its transcription. In addition, SA-induced expression of AtWRKY62 is completely abolished or reduced in the npr1-3 mutant, indicating that this WRKY protein acts downstream of the regulatory gene. In previous studies, W-box sequences were identified as important cis-acting elements for transcriptional regulation of a pathogen elicitor-induced WRKY gene from parsley, suggesting that the gene could be regulated by itself or by other members of the gene family (Eulgem et al., 1999). Thus, it appears that transcriptional regulators of plant defense response also interact extensively at the transcriptional level. Because many of the transcriptional regulators function by sequence-specific interactions with DNA, the completed genome of Arabidopsis will facilitate attempts to identify potential target genes of SSDB proteins through genome sequence analysis. Development of these analyses in combination with other ex-
perimental approaches such as microarray analysis may provide important insights into the pathways and network of transcriptional regulation important for the activation of plant defense response.

**METHODS**

**Materials**

\[ ^{32}P\text{-dATP} \text{ and } ^{32}P\text{-CTP (>3000 Ci/mmoll) were obtained from Du-} \]

\[ \text{Pont–New England Nuclear; other common chemicals were pur-} \]

\[ \text{chased from Sigma. Arabidopsis thaliana plants were grown in a} \]

\[ \text{growth chamber at } 22^\circ \text{C under } 180 \mu \text{E-m}^{-2}\text{-sec}^{-1} \text{ light with a short} \]

\[ \text{day photoperiod (8-hr-light/16-hr-dark). Plant inoculations were per-} \]

\[ \text{formed by infiltration with the Pseudomonas syringae pv tomato} \]

\[ \text{DC3000 strain (OD}_{600} = 0.001 \text{ in } 10 \text{ mM MgCl}_2}. \]

Salicylic acid (SA) was dissolved in water as 100 mM stock solutions and adjusted to

\[ \text{pH 6.5 with KOH. SA treatment was performed by spraying the plants with 2 mM solution.} \]

**Domain-Specific Differential Display**

Total RNA was isolated from untreated and SA-treated Arabidopsis plants using the TRIzol reagent purchased from BRL Life Technologies according to the manufacturer’s instruction. WRKY domain-specific differential display was performed as described previously for the isolation of pathogen-induced WRKY genes from tobacco (Chen and Chen, 2000). Briefly, cDNA was synthesized from total RNA with Superscript reverse transcriptase (BRL Life Technologies) with oligo(dT) as primers. Two degenerate primers (5‘-TGGCGN-

\[ \text{AARTAYGGNCARAAR-3} \text{ and } 5’-TGGAGRAARTAYGGNCARAAR-3’} \text{ that} \]

\[ \text{correspond to the WRKYQGK amino acid sequence were used} \]

\[ \text{for polymerase chain reaction (PCR) amplification in conjunction with} \]

\[ \text{an oligo(dT) primer using synthesized cDNA as a template. PCR am-} \]

\[ \text{plification was performed at } 94^\circ \text{C for 30 sec, } 55^\circ \text{C for 30 sec, and} \]

\[ 72^\circ \text{C for 60 sec for 25 cycles. The amplified samples were size frac-} \]

\[ \text{tionated on a } 1\% \text{ agarose gel, and the resolved fragments were} \]

\[ \text{eluted, cloned into pCR2.1 (Invitrogen, Carlsbad, CA), and partially} \]

\[ \text{sequenced.} \]

**Construction and Screening of cDNA Libraries**

The cDNA library was prepared from Arabidopsis plants harvested 4 hr after spraying with 2 mM SA. The library (in ZAP Express λ vector from Stratagene) of 10^6 phages was screened using ^32P-labeled PCR-amplified products from domain-specific differential display or cDNA fragments of expressed sequence tag clones. The hybridization was performed in a buffer of 5 × SSPE (1 × SSPE is 0.115 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4), 0.5% SDS, 5 × Denhardt’s solution (1 × Denhardt’s solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), and 100 μg/mL salmon sperm DNA for 16 hr at 55°C. The filters were washed three to four times for 30 min each with 0.5 × SSPE and 0.5% SDS at 55°C. The phagemid for each isolated clone was obtained through in vivo excision according to the manufacturer’s instruction. DNA sequencing was performed by the dideoxynucleotide chain termination method at the DNA Sequencing Facility of Washington State University (Pullman).

**Production of Recombinant WRKY Proteins, Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays**

For generation of recombinant AtWRKY18 proteins, its full length cDNA clone was cloned into pET32 (Novagen, Madison, WI) and transformed into Escherichia coli strain BL21(DE3). Induction of expression and purification of recombinant His-tagged WRKY proteins were performed according to the protocol provided by Novagen. The purified proteins were dialyzed for more than 6 hr against a nuclear extraction buffer (25 mM Hepes-KOH, pH 7.5, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and 30 μg/mL phenylmethylsulfonyl fluoride) at 4°C.

Preparation of Arabidopsis nuclei and nuclear extracts was performed as described previously (Green et al., 1987). Double-stranded synthetic oligonucleotides were labeled to specific activities of approximately 10^6 cpm/ng using the Klenow fragment of DNA polymerase I. Sequence-specific DNA binding was assayed with electrophoretic mobility shift assays essentially as described previously (Chen and Chen, 2000). Binding reactions contained 12 μL of nuclear extraction buffer, 5 μg of poly(dIdC), 5 μL of proteins (25 μg for nuclear extracts and 1 μg for purified recombinant WRKY proteins), and 2 ng of labeled double stranded oligoDNA. DNA-protein complexes were allowed to form at room temperature for 20 min and resolved on a 10% polyacrylamide gel in 0.5 × TBE (1 × TBE is 0.1 M Tris, 0.09 M boric acid and 0.001 M EDTA) at 4°C.

**Isolation of NPR1 Gene Genomic Clones**

An Arabidopsis genomic library (in EMBL λ vector from Clontech, Palo Alto, CA) of 10^6 phages was screened using ^32P-labeled PCR-amplified products with primers designed from the genomic sequences of the NPR1 gene. The hybridization was performed in a buffer of 5 × SSPE, 0.5% SDS, 5 × Denhardt’s solution, and 100 μg/mL salmon sperm DNA for 16 hr at 55°C. The filters were washed three to four times for 30 min each with 0.5 × SSPE and 0.5% SDS at 55°C. A 5-kb NPR1 gene fragment was subcloned into pBluescript KS II (+) and sequenced partially to confirm its identity.

**RNA Gel Blotting**

For RNA gel blot analysis, total RNA (12 μg) was separated on agarose-formaldehyde gels and blotted to nylon membranes according to standard procedures (Sambrook et al., 1989). Blots were hybridized with ^32P-dATP-labeled gene-specific probes. Hybridization was performed in 1 M NaCl, 50 mM Tris-HCl, pH 7.5, 1% SDS, 5 mM K2PO4, 100 μg/mL denatured salmon sperm DNA, 10% dextran sulfate, 0.2% BSA, 0.2% Ficoll 400, and 0.2% polyvinylpyrrolidone 400 for 16 hr at 65°C. The membrane was then washed for 10 min twice with 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 1% SDS and for 10 min with 0.1 × SSC and 1% SDS at 65°C.

**Construction of the Promoter-GUS Fusion and Arabidopsis Transformation**

A 3373-bp EcoRV–XbaI fragment of the NPR1 genomic clone that includes the 2419-bp promoter sequence and part of the coding region was first cloned into a pBluescript (+) vector to produce pYU12. An Ncol site was introduced into the translation start site through overlapping PCR. The pJL131 plasmid (provided by Dr. Joyti Shah,
Kansas State University, Manhattan) was digested with Ncol and XbaI, and the β-glucuronidase (GUS) reporter gene insert was isolated and cloned into the same sites of pYU12. The mutant promoter of the NPR1 gene in which the TTGAC sequences were changed to TTGAA was generated by overlapping PCR. PCR-amplified fragments were all sequenced to verify the sequences. The resulting recombinant plasmids were digested with Sall and SacI, and the resulting promoter-GUS fusion fragments were cloned into the Arabidopsis transformation vector pOCA28 provided by Dr. Daniel F. Klessig (Rutgers University, New Brunswick, NJ).

Arabidopsis transformation was effected by the vacuum infiltration procedure as described previously (Bechtold and Pelletier, 1998). The seed were collected from the infiltrated plants and selected in Murashige and Skoog (1962) medium containing 50 µg/mL kanamycin. Kanamycin-resistant plants were transferred to soil 9 days later and grown in a growth chamber at 22°C under 180 µE·m⁻²·sec⁻¹ light with a short day photoperiod (8-hr-light/16-hr-dark). For the measurements of GUS activity, 3-week-old transgenic plants were sprayed with 2 mM SA. Leaves of the plants were collected just before the SA spraying to determine basal GUS activity and 24 hr after the treatment to determine SA-induced GUS activity. The leaves were homogenized in ice-cooled extraction buffer and microcentrifuged at 4°C. The GUS activity in the supernatant was measured using 4-methylumbelliferyl-β-d-glucuronide as substrate (Jefferson et al., 1987). The standard curves were prepared with 4-methylumbelliferylone.

Nuclear Run-On Analysis

Nuclei were isolated from 4- to 5-week-old Arabidopsis plants before and after SA spraying (2 mM for 12 hr) as described (Cox and Goldberg, 1988). Nuclear run-on reactions in the presence of [³²P-CTP and extraction of the labeled RNAs were performed as described (Cox and Goldberg, 1988). The radiolabeled transcription products from each reaction were used as probes in filter blot assays. Linearized plasmid DNA (5 µg containing the GUS or APHII sequence were blotted to a nylon membrane. Prehybridization, hybridization, and washing were performed as described (Kasschau and Carrington, 1998). The hybridization intensity for each plasmid was determined by autoradiography.

Antibody Preparation, Purification, and Binding Assays

WRKY antibodies were made against the peptide CWRKYGQK in rabbits (Zymed Laboratory, South San Francisco, CA). A Cys residue was introduced at the N-terminal position to facilitate coupling to the KLH carrier protein and to Affi-Gel 501 for affinity chromatography. A DEAE Affi-Gel blue column was used first to separate the IgG fraction of the rabbit serum as described (Borg et al., 1993). The affinity column matrix for antibody purification was prepared by covalently coupling Affi-Gel 501 gel to the N-terminal Cys of the peptide according to the instructions provided by the manufacturer. After coupling, the column was equilibrated with PBS (0.039 M NaH₂PO₄, 0.061 M Na₂HPO₄, 0.14 M NaCl, and 0.02% NaN₃, pH 8.0). The IgG fraction of the antibodies was loaded onto the column and incubated overnight at 4°C. The column was washed extensively with PBS, and the antibodies were eluted with 0.1 M Gly·HCl, pH 2.5, neutralized immediately with 0.1 volume of 1 M Tris·HCl, pH 8.0, and dialyzed against PBS. Inhibition of W-box binding activities was assayed by preincubating for 30 min at room temperature 1 µg of affinity-purified antibodies to 15 µL of binding reaction, and the samples were then processed as usual. Control experiments were performed by incubating the same amount of IgG fraction of preimmune antiserum in the binding reaction mixtures.

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