A Novel WRKY Transcription Factor Is Required for Induction of PR-1a Gene Expression by Salicylic Acid and Bacterial Elicitors

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ABSTRACT

PR-1a is a salicylic acid-inducible defense gene of tobacco (Nicotiana tabacum). One-hybrid screens identified a novel tobacco WRKY transcription factor (NtWRKY12) with specific binding sites in the PR-1a promoter at positions -564 (box WK1) and -859 (box WK2). NtWRKY12 belongs to the class of transcription factors in which the WRKY sequence is followed by a GKK rather than a GQK sequence. The binding sequence of NtWRKY12 (WK box TTTTCCAC) deviated significantly from the consensus sequence (W box TTGAC[C/T]) shown to be recognized by WRKY factors with the GQK sequence. Mutation of the GKK sequence in NtWRKY12 into GQK or GEK abolished binding to the WK box. The WK box is in close proximity to binding sites in the PR-1a promoter for transcription factors TGA1a (as-1 box) and Myb1 (MBSII box). Expression studies with PR-1a promoter::β-glucuronidase (GUS) genes in stably and transiently transformed tobacco indicated that NtWRKY12 and TGA1a act synergistically in PR-1a expression induced by salicylic acid and bacterial elicitors. Cotransfection of Arabidopsis thaliana protoplasts with 3SS::NtWRKY12 and PR-1a::GUS promoter fusions showed that overexpression of NtWRKY12 resulted in a strong increase in GUS expression, which required functional WK boxes in the PR-1a promoter.

INTRODUCTION

R-gene-mediated recognition of pathogens by plants typically results in a hypersensitive response (HR) mediated by generation of reactive oxygen species and the increased production of salicylic acid (SA). The HR is accompanied by the induction of local and systemic expression of numerous genes involved in defense. The N-gene-mediated resistance of tobacco (Nicotiana tabacum) to infection with Tobacco mosaic virus (TMV) represents a classical model to study expression of pathogenesis-related (PR) proteins and development of systemic acquired resistance (SAR) in plant-pathogen interactions (van Loon and van Strien, 1999). Tobacco PR proteins of classes 1 to 5 are subdivided into acidic, extracellular proteins and basic, vacuolar proteins. Generally, TMV-induced expression of acidic PR proteins is mediated by SA, whereas expression of basic PR proteins is mediated by ethylene (Bol et al., 1990; Brederode et al., 1991; Linthorst, 1991). Although the function of tobacco PR-1 proteins is not clear, these proteins are highly conserved in the plant kingdom and are widely used as markers in studies of signal transduction processes involved in plant pathogenesis and induced resistance.

Studies on expression of PR genes in Arabidopsis (Arabidopsis thaliana) and tobacco revealed the central role of protein NONEXPRESSER OF PR GENES1 (NPR1). NPR1 also mediates cross talk between the SA signaling pathway and the jasmonic acid and ethylene signaling pathways, and interacts with members of the TGA family of transcription factors that bind to activator sequence-1 (as-1) or as-1-like elements that have been identified in promoters of PR-1 genes (Durrant and Dong, 2004). Two as-1-like elements in the Arabidopsis PR-1 promoter were shown to bind several of
the 10 TGA factors in Arabidopsis with different affinity (Lebel et al., 1998; Johnson et al., 2003). The two as-1-like elements in the promoter of the tobacco gene encoding the acidic PR-1a protein bind TGA1a. Mutation of these elements affected SA-induced expression of a GUS reporter gene in transgenic plants (Strompen et al., 1998; Grüner et al., 2003). In addition to TGA1a, the Myb1 protein has been shown to bind to the PR-1a promoter in tobacco. Expression of the Myb1 gene was enhanced by TMV infection and application of exogenous SA, and the Myb1 protein preferentially bound to the MBSII sequence in the PR-1a promoter (Yang and Klessig, 1996). Silencing of Myb1 gene expression attenuated N-gene-mediated resistance to TMV (Liu et al., 2004).

Accumulating evidence indicates that WRKY proteins are involved in differential responses to biotic stresses, either as transcriptional activators or as repressors in Arabidopsis (Asai et al., 2002; Dong et al., 2003; Journot-Catalino et al., 2006; Kim et al., 2006; Li et al., 2006; Wang et al., 2006; Eulgem and Somssich, 2007) and other plants (for review, see Ülker and Somssich, 2004). For instance, silencing of the Nicotiana benthamiana homologs of the tobacco WRKY factors NtWRKY1, NtWRKY2, and NtWRKY3 compromised N resistance. These WRKY proteins share highest similarity at the amino acid level with Arabidopsis WRKY20, WRKY4, and WRKY70, and particularly expression of NtWRKY3 is rapidly induced upon infection with TMV (Liu et al., 2004). WRKY proteins bind to the W box (TTGAC[C/T]) in promoters of various pathogen-responsive genes, including genes encoding the basic, ethylene-responsive tobacco PR-1, PR-2, PR-3, and PR-5 proteins (Eulgem et al., 2000; Kim and Zhang, 2004; Yamamoto et al., 2004).

We have shown that a fragment of 902 bp upstream of the transcription start site of the tobacco PR-1a gene confers inducibility to the GUS reporter gene by TMV infection and SA treatment. This inducibility involved multiple elements in the promoter fragment (van de Rhee and Bol, 1993). The PR-1a promoter was found to contain a number of sites that bind GT-1-like factors with different affinity. The observation that the level of GT-1 decreased after infection of tobacco with TMV suggested a negative role of GT-1 in regulation of PR-1a expression. However, mutation of the GT-1 binding sites did not affect promoter activity (Buchel et al., 1996). In this article, we used the yeast one-hybrid system to identify tobacco proteins interacting with fragments of the PR-1a promoter. One of the proteins obtained turned out to be a novel WRKY protein, named NtWRKY12. Similar to PR-1a, expression of the NtWRKY12 gene was strongly induced by TMV infection, SA treatment, or infiltration of tobacco leaves with a suspension of Agrobacterium tumefaciens. Two binding sites for NtWRKY12 were identified in the PR-1a promoter with a surprisingly low similarity to the consensus W box sequence. Wild-type and mutant PR-1a promoter sequences were fused to the GUS reporter gene and these fusions were expressed in transgenic tobacco to assay induction by SA and expressed from a T-DNA vector in agroinfiltrated leaves to assay induction by bacterial elicitors. The results indicated that NtWRKY12 acts synergistically with TGA1a in the SA-mediated and pathogen-associated molecular pattern (PAMP)-mediated expression of the PR-1a gene. In addition, transactivation assays in Arabidopsis protoplasts provided evidence that NtWRKY12 is a transcriptional activator of PR-1a gene expression.
RESULTS

A Novel WRKY Factor Binds to the PR-1a Promoter

Previous studies have indicated that elements in the 902-bp tobacco PR-1a promoter are important for SA and TMV-induced expression (van de Rhee et al., 1990; van de Rhee and Bol, 1993; Strompen et al., 1998). Here, we used the yeast one-hybrid system to identify transcription factors binding to the PR-1a promoter. Tetramers of various fragments of the 902-bp promoter sequence were inserted in front of the yeast (Saccharomyces cerevisiae) His reporter gene and integrated into the genome of his yeast strain Y187. TMV-infected tobacco was used as a source for construction of a library of cDNAs fused to the GAL4 activation domain in vector pACT. This library was used to transform yeast strains harboring the various PR-1a promoter fragments. Screening of the cDNA library with fragment IV (bp -605 to -513 of the PR-1a promoter in yeast strain Y187-IV) yielded 37 independent transformants growing on His-free medium (pACT/IV clones). Of the cDNA inserts in these clones, 22 cross-hybridized with each other. Clone pACT/IV-80 was selected for further analysis.

Sequencing of the cDNA insert of pACT/IV-80 revealed that it corresponded to the 610 3’-terminal nucleotides of a mRNA, excluding a poly(A) track of 54 residues probably representing the 3’-terminal poly(A) tail. The cDNA corresponding to the missing 5’-part of the mRNA was obtained using RACE on total RNA from TMV-infected tobacco plants. This resulted in a stretch of 415 additional nucleotides at the 5’-end of the mRNA. The combined 5’- and 3’-sequences revealed an open reading frame for a protein of 220 amino acid residues. The insert in pACT/IV-80 encoded the C-terminal 107 amino acids of this protein. The presence of WRKY and zinc (Zn)-finger domains in the C-terminal half indicates that the protein is a member of the large group of DNA-binding WRKY proteins. Upstream of the WRKY domain, the amino acid sequence contains a stretch of basic residues, reminiscent of nuclear targeting signals. The N-terminal region is relatively rich in acidic residues and has low similarity to WRKY51 from Arabidopsis. Based on the criteria described by Eulgem et al. (2000), the novel tobacco WRKY protein appears to be a member of subgroup 2c of the WRKY superfamily of plant transcription factors. Currently, 11 different tobacco WRKY genes are described in the EMBL/GenBank database. In line with the tobacco WRKY nomenclature, the novel protein identified in our study was named NtWRKY12. The accession number of the full-length cDNA is DQ460475. DNA-blot analyses of restriction enzyme digests of genomic DNA using a probe corresponding to the cDNA insert from pACT/IV-80 showed that the amphidiploid tobacco varieties Samsun NN and Samsun nn contain two to four NtWRKY12-related genes (Fig. 1).

Expression of the full-length NtWRKY12 protein in yeast strain Y187-IV rendered the strain independent of exogenous His (data not shown). This indicates that NtWRKY12 contains an activation domain that is able to replace the GAL4 activation domain fused to the DNA binding region of NtWRKY12 in pACT/IV-80 and to activate transcription of the His reporter gene in yeast.
This strongly supports a role for NtWRKY12 as a transcription factor in tobacco. Expression of an NtWRKY12/GFP fusion construct using an alfalfa mosaic virus-based expression system (Sánchez-Navarro et al., 2001) resulted in specific fluorescence of tobacco nuclei. Similar expression of non-fused GFP showed a more diffuse fluorescence of the cytoplasm and nuclei (Fig. 2). This indicates that the NtWRKY12 sequence contains a nuclear localization signal, which targets the fusion protein to the nucleus. The reverse transcription (RT)-PCR results shown in Figure 3 indicate that, like PR-1a, expression of the NtWRKY12 gene was induced in tobacco leaves by salicylate treatment and by infiltration of leaf tissue with A. tumefaciens strain LBA4404. The last type of induction probably corresponds to a PAMP-type response, similar to responses triggered by peptide patterns of conserved elicitors like bacterial flagellins or elongation factor (EF)-Tu (Felix et al., 1999; Kunze et al., 2004). Indeed, also infiltration with Escherichia coli resulted in induced expression of NtWRKY12 and PR-1a (data not shown).

The time course of expression of the NtWRKY12 gene was studied in TMV-infected Samsun NN tobacco plants. Figure 4 shows northern blots with RNA isolated at various time points after inoculation. It is evident that in noninfected plants the gene was expressed at relatively low levels (top, lane 0). After infection with TMV, expression increased in the inoculated leaves (local) and reached a transient maximum after 1 h. At 2 h postinoculation (hpi), expression was back to the low basal level and remained low until 8 hpi. Subsequently, NtWRKY12 mRNA accumulation was slightly increased at 12 and 24 hpi and became very high at 48 h and later. The strong increase in NtWRKY12 expression coincided with the development of local lesions that first appeared at 36 hpi. Also, in the noninoculated leaves, expression increased, although with some delay and to lower levels. We have not investigated mRNA accumulation in the systemic tissues at later time points. The second image shows the TMV-induced expression pattern of the gene encoding transcription factor Myb1 (Yang and Klessig, 1996). Like NtWRKY12, the Myb1 gene is transiently expressed until 1 hpi and at high levels at 48 and 72 hpi. The timing of NtWRKY12 and Myb1 expression corresponded to that of the PR-1a gene (middle), although PR-1a was not transiently expressed immediately after inoculation.

Characterization of NtWRKY12 Binding Sites in the PR-1a Promoter

The results of the yeast one-hybrid screening indicated that NtWRKY12 specifically bound to a PR-1a promoter sequence ranging from positions -605 to -513 upstream of the transcription start site. To delineate the binding site in the DNA, this region was further divided into four overlapping subfragments A to D (Fig. 5). With a similar approach as was used above, tetramerized versions of subfragments B and C were able to confer His independence in the yeast one-hybrid system, whereas fragments A and D were not (data not shown). This suggested that the overlap region of fragments B and C contains the NtWRKY12 binding site. This was confirmed in the one-hybrid system with mutants of subfragments B and C of which either the left halves (mutants Blm and Clm) or the right halves (mutants Brm and Crm) were mutated by changing each G to A, A to G, C to T, and T to C (e.g. compare the sequences of B and Blm in...
The results of the yeast one-hybrid assays were confirmed in vitro using electrophoretic mobility shift assays (EMSAs) with complementary oligonucleotides corresponding to regions C and B and a glutathione S-transferase (GST)/NtWRKY12-binding domain (BD) fusion protein expressed in *E. coli*. This fusion protein contained the C-terminal 111 amino acids of NtWRKY12 and was purified by glutathione-Sepharose 4B column chromatography. In the EMSA, the complementary oligonucleotides could anneal to double-stranded structures. Figure 6A, lane 1, shows a band corresponding to the labeled fragment C probe. After incubation with the GST/NtWRKY12-BD protein, part of the probe is shifted to a higher position in the gel (Fig. 6A, lane 2). When only GST protein is used, no band shift is observed (Fig. 6A, lane 3). This indicates that the NtWRKY12-BD is able to form a protein-DNA complex with fragment C. Similarly, lane 5 shows the formation of a complex of GST/NtWRKY12-BD with fragment Blm, but not with Brm (Fig. 6A, lane 7). To determine the exact location of the NtWRKY12 binding site in subfragment Blm, a scanning analysis was performed with a series of complementary oligonucleotides based on Blm, in which two adjacent base pair were changed (Fig. 5; Blm-m1–Blm-m9). The results of EMSAs with these fragments
are shown in Figure 6A, lanes 8 to 27. It is evident that the lanes with mutants Blm-m3 to Blm-m6 lack a band shift and neither did the single mutants Blm-m3’ and Blm-m6’ (Figs. 5 and 6, lanes 29–33). This suggests that the corresponding sequence TTTTCCAC is essential for binding to the NtWRKY12-BD. Complementary oligonucleotides corresponding to fragment Blm-m1, but with the central TTTCCA sequence of the binding site changed into the consensus WRKY box TTGACC (Fig. 5; Blm-m10), were not able to compete with fragment Blm-m1 for binding of GST/NtWRKY12-BD in EMSAs (Fig. 6B, lanes 5 and 6), whereas fragment Blm-m10 alone showed
no binding to GST/NtWRKY12-BD (Fig. 6B, lane 8). This indicates that NtWRKY12 does not bind to the consensus WRKY binding site. As discussed in more detail below (see “Discussion”), in NtWRKY12 the WRKY sequence is followed by the sequence GKK rather than by the sequence GQK found in WRKY factors that have been shown to bind to the consensus W box. We have expressed GST/NtWRKY12-BD with the GKK sequence mutated into GQK or GEK in E. coli (Fig. 6F), but the purified mutant proteins showed no binding in band shift assays to either the WK box in the PR-1a promoter or the consensus W box sequence (Fig. 6, C and D). Apparently, the central Lys in the GKK sequence is essential for binding of NtWRKY12 to the WK box sequence.

We have investigated whether binding to the WK box is a general feature of WRKY proteins with a GKK sequence. Therefore, the full-length GKK-containing AtWRKY51 coding sequence was expressed as a GST fusion protein in E. coli (Fig. 6G). However, this Arabidopsis WRKY was not able to bind to either the WK or the W box sequence (Fig. 6E). The faint bands visible at higher positions in the gel (Fig. 6E, lanes 2–6 and 8–12) are the result of aspecific binding because they cannot be competed by an excess of either unlabeled WK or W box. The same results were obtained with a full-length GST/AtWRKY59 fusion protein (data not shown). These results suggest that the WK box is not a general consensus binding site for GKK WRKYs.

The synthetic oligonucleotides that were used for the above band shift assays contained nonpaired GTAC extensions at the 5’ termini. These sticky ends allowed transient base pairing and formation of multimerized fragments, which greatly facilitated DNA-protein interaction during incubation. Annealed oligonucleotides that did not contain sticky ends at best showed only weak band shifts. Apparently, the multimers remained at least partly intact during electrophoresis and are visible as faint bands above the positions of the monomeric free probes. In several lanes, these oligomers were
apparently stable enough to produce stable multiple free probe bands (Fig. 6A, lanes 14/15, 26) and even double band shifts (Fig. 6A, lane 27).

The sequence TTTTCCAC also occurs at the far upstream position -859 in the PR-1a promoter (Fig. 7). EMSAs with annealed oligonucleotides corresponding to the region -871 to -839 confirmed also that this region of the promoter is able to bind NtWRKY12 (data not shown). The two TTTTCCAC sequences in the PR-1a promoter were named box WK

Role of NtWRKY12 and TGA1a in SA-Induced PR-1a Expression

To determine whether the NtWRKY12 binding site has functional significance for SA-induced expression of PR-1a, stably transformed transgenic tobacco plants were made containing a series of mutant PR-1a promoter::GUS constructs. In close proximity to the WK

Figure 6. Binding of NtWRKY12 to wild-type (WT) and mutant PR-1a promoter fragments. EMSAs were done with the promoter fragments shown in Figure 5: wild-type fragment C (A, lanes 1, 2, and 3), Blm (A, lanes 4, 5, 8, 9, 28, and 29), Brm (A, lanes 6 and 7), and the indicated Blm mutants (A, lanes 10–27 and 30–33; B, C, D, and E, lanes 1–12). In A, B, C, D, and E, plus signs indicate binding mixtures containing 0.5 µg recombinant GST fusion protein purified from E. coli transformed using a pGEX-KG vector with wild-type NtWRKY12-BD (A and B), mutant NtWRKY12-BD with the amino acids GKK mutated to GQK (C), mutant NtWRKY12-BD with the amino acids GKK mutated to GEK (D), and AtWRKY51 (E). In these sections, minus signs above the lanes indicate binding mixtures without recombinant protein. In A and B, the position of the protein-DNA complexes is indicated by an arrow. In B, C, D, and E, lanes 3 to 6 and 9 to 12, a 50- or 250-fold excess of unlabeled fragment Blm-m1 (m1) or Blm-m10 (m10) was added as competitor to the EMSA incubation mixtures. Zero sign (A, lane 3), Control with recombinant GST protein purified from E. coli. F, SDS-PAGE gel containing purified GST fusion proteins of wild-type NtWRKY12-BD with WRKY-GKK (K, lane 1) and mutants NtWRKY12-BDs with WRKYGQK (Q, lane 2)
and Myb1 (box MBSII, -520 to -514; Fig. 7; Yang and Klessig, 1996; Strompen et al., 1998; Grüner et al., 2003). Mutations affecting single transcription factor binding sites in mutants WK₂, as-1, and WK₁ are shown in Figure 7. The double mutant WK₂/WK₁ contains both the WK₂ and WK₁ mutations. In addition, promoter deletions of 85 bp (mutant Δ85) and 32 bp (mutant Δ32) were made. As outlined in Figure 7, the 85-bp deletion removed binding sites as-1, WK₁, and MBSII, whereas the 32-bp deletion removed the WK₁ binding site only. The double mutant WK₂/Δ85 contained both the WK₂ and the Δ85 mutations.

The number of independent, phenotypically normal transformants obtained with wild-type and mutant PR-1a promoter::GUS constructs ranged between 2 and 16. Primary transformants were analyzed at the six- to eight-leaf stage for noninduced GUS expression and for GUS activity after floating leaf discs on water and SA. The results are presented in Figure 8. As expected, the reporter gene was constitutively expressed in 35S::GUS plants, whereas the wild-type PR-1a promoter conferred strong SA-inducibility to the GUS gene. In agreement with the results of Strompen et al., (1998), we noticed that mutation of the as-1 box resulted in a modest reduction of SA inducibility of the PR-1a promoter. Mutation of the upstream binding site for NrWRKY12 (mutant WK₂) did not reduce SA inducible reporter gene expression. However, mutation (mutant WK₁) or deletion (mutant Δ32) of the downstream NrWRKY12 binding site reduced the SA inducibility of the PR-1a promoter by approximately 60% to 70%. Although the number of transgenic lines with the WK₁ mutation (two lines) or the Δ32 mutation (three lines) were relatively low, the results with these two mutants demonstrate that mutation or deletion of the WK₁ box only partially affects PR-1a promoter activity. The combined mutation of both the WK₂ and the WK₁ binding site (mutant WK₂/WK₁; six lines) further reduced expression. Mutant Δ85 (16 lines) lacks the WK₁, as-1, and MBSII boxes and showed no significant SA-inducible expression. Probably, NrWRKY12 is able to bind to the WK₂ box of mutant Δ85, but this binding is not sufficient for SA-inducible expression. Mutation of the WK₁ box in mutant Δ85 (mutant WK₂/Δ85; seven lines) did not affect the phenotype of mutant Δ85. Binding of TGA1a and/or Myb1 factors to the PR-1a promoter may be responsible for the approximately 20% level of SA inducibility observed with mutantWK₂/WK₁. Together, the results indicate that full SA inducibility of the PR-1a promoter requires synergistic interactions between NrWRKY12 and TGA1a or Myb1 factors.

Role of NrWRKY12, TGA1a, and Myb1 Factors in Elicitor-Induced PR-1a Expression

As shown in Figure 3, infiltration of tobacco leaves with A. tumefaciens results in induction of NrWRKY12 and PR-1a gene expression. Probably this expression is induced by bacterial elicitors and WRKYGEK (E, lane 3), respectively, which were used in the EMSAs of A to E. Lane 5 of F was loaded with a purified extract from empty GST expression vector (G). G, SDS-PAGE gel of extract from uninduced (minus sign, lane 2) or induced (plus sign, lane 3) E. coli containing pGEX-KG vector with AtWRKY51-GST fusion protein. In F and G, the position of the full-length induced fusion proteins is indicated by asterisks, whereas lanes labeled L were loaded with size markers of 94, 67, 43, 30, 20, and 14 kD. NrW12, NrWRKY12; AtW51, AtWRKY51.
To study a possible role of NtWRKY12 in elicitor-induced expression of the PR-1a gene, tobacco leaves were agroinfiltrated with A. tumefaciens suspensions harboring PR-1a promoter::GUS fusions in the T-DNA vector. For these experiments, the collection of promoter mutants used in the plant transformation experiments was extended with a mutant containing an altered Myb1 binding site (mutant MBSII; see Fig. 7) and a series of double and triple mutants. In double mutants as-1/WK₁, as-1/MBSII, and WK₁/MBSII, two of the boxes as-1, WK₁, and MBSII contain the point mutations specified in Figure 7. In the triple mutant as-1/WK₁/MBSII, all three boxes are mutated.

The results are shown in Figure 9, A and B. The relatively low GUS expression of the 35S::GUS constructs can be ascribed to the much lower density of the 35S::GUS Agrobacterium inoculum obtained in comparison to that of the PR-1a::GUS strains (approximately A₆₀₀ = 0.2 versus A₆₀₀ = 1, respectively). To enable a comparison of different experiments, GUS activity in leaves expressing the wild-type PR-1a::GUS construct was taken as 100%. The effects of mutations WK₂, WK₁, WK₂/WK₁, Δ32, Δ85, and WK₁/Δ85 on elicitor-mediated GUS expression in tobacco plants (Fig. 9A) were largely similar to their effects on SA-mediated expression of GUS in plants with PR-1a::GUS transgenes (Fig. 8). Expression of mutants WK₁, WK₂/WK₁, and Δ32 was reduced by 40% to 60%, whereas mutants Δ85 and WK₂/Δ85 did not support significant levels of elicitor-mediated GUS expression (Fig. 9A).

In mutant Δ85, the as-1, WK₁, and MBSII boxes are deleted. To analyze the role of these boxes in the Δ85 phenotype, we made mutants with two or all three boxes mutated. Figure 9B shows that elicitor-mediated expression of the double mutant as-1/WK₁ is as low as that of the Δ85 mutant. The effect of the double mutation in mutant as-1/WK₁ (<5% of wild-type induction) is much stronger than the combined effects of the two single mutations as-1 (no significant reduction of wild-type induction; Fig. 9A) and WK₁ (40%–60% of wild-type induction; Fig. 9, A and B). This demonstrates that a synergistic action of factors binding to the as-1 and WK₁ boxes is essential for elicitor-induced PR-1a promoter activity. The additional mutation of the MBSII box in triple mutant
as-1/WK\textsubscript{1}/MBSII did not alter the phenotype of the as-1/WK\textsubscript{1} mutant.

Elicitor-mediated induction of the double mutants as-1/MBSII and WK\textsubscript{1}/MBSII is about 40\% of the induction driven by the wild-type PR-1\textsubscript{a} promoter (Fig. 9B). The observation that expression by these double mutants is modestly reduced when compared to the single mutants as-1, WK\textsubscript{1}, and MBSII indicates that MBSII contributes to some extent to the expression driven jointly by the as-1 and WK\textsubscript{1} boxes.

**NtWRKY12 Activates PR-1\textsubscript{a}::GUS Gene Expression in Arabidopsis Protoplasts**

The above results indicate that NtWRKY12 plays a role in inducible PR-1\textsubscript{a} gene expression. To more directly demonstrate that NtWRKY12 functions as a positive transcriptional activator of PR-1\textsubscript{a} gene expression, Arabidopsis protoplasts were cotransfected with a plasmid containing the NtWRKY12 coding region under the control of the cauliflower mosaic virus (CaMV) 35S promoter together with a plasmid containing the GUS reporter gene cloned either behind the full-length (902 bp) wild-type PR-1\textsubscript{a} promoter or behind PR-1\textsubscript{a} promoters with mutations in the WK\textsubscript{1} box or in both the WK\textsubscript{1} and WK\textsubscript{2} boxes. Similar cotransfections with a plasmid lacking the NtWRKY12 coding sequence were performed as controls. The results of these transactivation assays are shown in Figure 10. In the presence of the NtWRKY12 plasmid, PR-1\textsubscript{a} promoter-directed
GUS expression was increased approximately 4-fold in comparison to the basal level obtained in protoplasts cotransfected with the empty vector. Apparently, NtWRKY12 produced in the protoplasts activates transcription of the GUS reporter gene by the Arabidopsis transcriptional machinery. Obviously, NtWRKY12 does so, at least partly, by binding to the WK$_1$ box because mutation of the WK$_1$ box resulted in a reduction of GUS activity to approximately 45% of that directed by the wild-type promoter. Upon mutation of both the WK$_1$ and the WK$_2$ box, NtWRKY12 no longer activated reporter gene expression.

**DISCUSSION**

**DNA Binding Site of NtWRKY12**

Among the first WRKY-type DNA binding proteins that were identified was a parsley (*Petroselinum crispum*) transcription factor involved in expression of the *Phytophthora megasperma*-induced gene encoding protein PR1 (Rushton *et al.*, 1996; Eulgem *et al.*, 1999). As a PR protein of class 10, parsley PR1 is not related to the classical PR-1 proteins originally characterized in tobacco and conserved in many other plant species. Induction of parsley PR1 is not mediated by SA and the protein accumulates in the cytoplasm as opposed to the classical PRs that accumulate either extracellularly or in the vacuole.
A number of recent studies have suggested the involvement of Arabidopsis WRKY transcription factors in induced PR gene expression, although no direct evidence has been presented for specific WRKY-PR promoter interactions (Chen and Chen, 2002; Robatzek and Somssich, 2002; Kim et al., 2006). In a screen of genes coexpressed with the Arabidopsis PR-1 gene under SAR-inducing conditions, Maleck et al. (2000) found the consensus WRKY binding site TTGAC(C/T) to be present in the promoters at twice the statistically expected frequency, whereas the as-1 element TGACG, the consensus binding site of TGA transcription factors, occurred only at one-half the statistically expected frequency.

In this article, we have identified NtWRKY12 as a WRKY-type DNA binding protein that specifically recognizes the sequence TTTTCCAC. This DNA element is located at two positions in the upstream region of the tobacco PR-1a promoter that was previously found to be important for inducible gene expression (van de Rhee et al., 1990; van de Rhee and Bol, 1993; Grüner and Pfitzner, 1994; Strompen et al., 1998). The NtWRKY12 binding box at position -564 is located between binding sites for transcription factors TGA1a (-592) and Myb1 (-520), which have been implicated in SA- and TMV-induced gene expression (Yang and Klessig, 1996; Strompen et al., 1998).

**NtWRKY12 Contains a Variant WRKY Domain**

NtWRKY12 is the first WRKY protein to be identified that interacts with a DNA binding site different from the consensus WRKY binding site TTGAC(C/T). As far as the sequence of the conserved
WRKY domain is concerned, NtWRKY12 is different from most other WRKY proteins in that it contains a Lys (K) residue instead of a Gln (Q) in the conserved domain (WRKYG[Q/K]K). This variation of the WRKY domain is conserved among other plant species. A BLASTP (http://www.ncbi.nlm.nih.gov/BLAST) search of all 796 eukaryote proteins containing one or two WRKY domains of which sequence data were present in the National Center for Biotechnology Information databases resulted in 131 sequences with high protein-protein similarity to NtWRKY12. Of these, the 28 proteins with highest similarity to NtWRKY12 all contained the WRKYGGKK variant domain. The 10 most similar WRKYGGKK proteins (61%–86% similarity) were from both dicotyledonous (Vitis vinifera, Brassica rapa, Glycine max) and monocotyledonous (rice [Oryza sativa]) plants. All WRKY factors shown to bind the W box element contain the GQK sequence.

Of all 72 Arabidopsis WRKY genes, the three closest homologs of NtWRKY12 are AtWRKY50, AtWRKY51, and AtWRKY59 (68%, 64%, and 59% similarity, respectively; Supplemental Fig. 11). Although the similarity between NtWRKY12 and these Arabidopsis WRKYs is mainly limited to the C-terminal halves of the proteins, they share the variant WRKYGGKK domain, have approximately similar sizes, and are all induced by SA and pathogenesis (Dong et al., 2003). It was suggested that AtWRKY59’s lack of W box binding activity might be due to the Q to K change (Dong et al., 2003). Although an Ala scanning study showed that mutation of the Q residue had only a minor effect on binding of NtWRKY9 to the consensus W box (Maeco et al., 2001), NMR spectroscopy measurements have revealed that the Q residue is one of the four amino acids in the WRKYGQKK sequence of AtWRKY4 that contacts the bases in the major groove of the DNA and therefore is highly significant for sequence-specific recognition (Yamasaki et al., 2005). Recently, an extensive mutational analysis of the region containing the C-terminal WRKY domain of AtWRKY1 confirmed that the Q to K mutation affected its binding to the consensus Wbox (Duan et al., 2007). NtWRKY12 mutant proteins in which the GKK sequence was changed to GQK or GEK (another WRKY domain sequence variation occurring, for example, in WRKY proteins of rice) were not able to bind to either the WK box-containing Blm-m1 probe or the Blm-m10 probe with the consensus W box (Fig. 6B). This suggests that, in addition to the WRKYG[Q/K]K domain, other regions in the WRKY proteins are probably also involved in the specificity of DNA binding.

**Role of NtWRKY12 in PR-1a Gene Expression**

PAMPs are universally conserved in a class of microbes. As in animals, plants recognize elicitors derived from pathogens, such as viruses, bacteria, fungi, or oomycetes. Well-characterized elicitors that induce defense responses in plants are represented by bacterial flagellin and EF-Tu or peptides from these proteins. In Arabidopsis, the flagellin-derived peptide flg22 binds to a Leu-rich repeat-type receptor-like kinase (FLS2), which activates a mitogen-activated protein kinase (MAPK) pathway and expression of WRKY transcription factors (Gómez-Gómez, 2004; Boller, 2005). We observed that agroinfiltration of tobacco leaves with a suspension of *A. tumefaciens* induced the expression of NtWRKY12 and PR-1a. The components of *A. tumefaciens* responsible for
FAI stands for Fertilizer Application and Integration. It is a strategy that integrates various forms of fertilizers to optimize soil nutrient availability and plant growth under varying soil and climatic conditions. The technique aims to improve crop yield and reduce environmental impact by carefully managing fertilizer input.

The figure shows a diagram illustrating the application of fertilizers through different methods such as band placement, banding, and side-dress, each with its own advantages and considerations. The diagram highlights the importance of timing, placement, and type of fertilizer to achieve optimal results.

**Fertilization Strategies**

- **Band Placement**: Fertilizer is placed in a narrow band around the seed, typically 1-2 cm deep, to ensure direct nutrient availability to the young plant.
- **Banding**: Similar to band placement but with a wider band width, providing a more uniform nutrient supply.
- **Side-dress**: Fertilizer is applied after the seedling stage, typically side-planted or broadcast over the soil surface, providing a more extended nutrient supply.

**Benefits of FAI**

- **Improved Nutrient Use Efficiency**: By applying fertilizers more precisely, FAI reduces the risk of nutrient leaching and runoff, leading to more efficient nutrient use.
- **Optimized Plant Growth**: Tailored nutrient inputs support optimal growth and development of crops, leading to increased yields.
- **Environmental Benefits**: Reduced fertilizer application rates and better nutrient use efficiency can lead to decreased environmental impacts, such as reduced greenhouse gas emissions and improved water quality.

**Challenges and Considerations**

- **Cost**: The initial investment in precision equipment and management can be high.
- **Varietal Adaptation**: Not all crops respond equally well to FAI, and selection of appropriate fertilization strategies may vary by crop type.
- **Environmental Interactions**: The impact of FAI on soil ecosystems and biodiversity requires careful monitoring and management.

In conclusion, FAI offers a strategic approach to fertilizer management that can enhance crop productivity while reducing environmental impacts, making it a valuable tool for sustainable agriculture.

**References**

Our as-1 mutant was impaired in SA-mediated expression of a reporter gene (Fig. 8), but the effect was less pronounced than that observed by Strompen et al. (1998) and Grüner et al. (2003). The PAMP-mediated expression of the as-1 mutant showed no repression in comparison to the wild-type promoter. This may be due to differences in mutations that were engineered in the as-1 box. Our observation that single mutations in the as-1 or WK₁ box are insufficient to completely knock out SA-induced expression driven by the PR-1a promoter indicates that multiple factors are required for promoter activity. A complete knock out (<5% of the wild-type activity) was obtained with the Δ85 promoter deletion, which removes the as-1, WK₁, and MBSII boxes. This mutational analysis of the PR-1a promoter revealed that similar elements are involved in SA-mediated and elicitor-mediated expression of the reporter gene (Figs. 8 and 9).

The finding that point mutations in the as-1 and WK₁ boxes in double mutant as-1/WK₁ fully knocked out elicitor-mediated expression (Fig. 9B) demonstrates that TGA1a and NtWRKY12 are the major players in the regulation of PR-1a promoter activity. A comparison of the activity of this double mutant with the single mutants as-1 and WK₁, revealed that TGA1a and NtWRKY12-like factors act synergistically in PR-1a gene expression. In contrast to mutant as-1/WK₁, the double mutants as-1/MBSII and WK₁/MBSII showed significant levels of elicitor-mediated PR-1a promoter activity (Fig. 9B). A comparison of this activity with that of the single mutants as-1, WK₁, and MBSII indicates that, in addition to the major effectors TGA1a and NtWRKY12, Myb1 plays a modest role in expression of the PR-1a gene. Recently, it was shown that several structurally related WRKY proteins are able to physically interact to form homologous and heterologous complexes (Xu et al., 2006). The synergistic effect of NtWRKY12 and TGA1a on PR-1a gene expression provokes a study of their possible direct or indirect interaction.

**NtWRKY12 Is a Transcriptional Activator of PR-1a Gene Expression**

The effect of NtWRKY12 overexpression on PR-1a promoter activity was studied by transactivation experiments in Arabidopsis protoplasts. These clearly demonstrated that NtWRKY12 acts as a transcriptional activator of PR-1a gene expression in vivo. GUS activity resulting from the expression of the wild-type PR-1a promoter::GUS gene was greatly enhanced in the presence of NtWRKY12 (Fig. 10). When the WK₁ box in the promoter was mutated, GUS expression was reduced, albeit still higher than in the absence of NtWRKY12. The results presented in Figures 8 and 9 indicated that the WK₁ box was less important for induction of the PR-1a promoter than the WK₁ box. However, in the transactivation assay (Fig. 10), the difference in GUS expression obtained with the WK₁ and WK₁/WK₁ mutants clearly points to a role of WK₁ in NtWRKY12-mediated expression.

In nonstressed tobacco, the PR-1a gene is not expressed (Figs. 3 and 4). The basal level of GUS expressed in the absence of NtWRKY12 in transfected Arabidopsis protoplasts (Fig. 10) indicates that the tobacco PR-1a promoter is recognized by the Arabidopsis transcriptional machinery. It is arguable that protoplast preparation and transfection result in a stress response that triggers a certain level of expression of stress-inducible genes, including the transfected tobacco PR-1a::GUS gene.
The observation that mutation of the WK$_1$ box results in reduced GUS expression in the absence of NtWRKY12 (Fig. 10) suggests that the WK$_1$ box is also involved in stress-induced expression by Arabidopsis transcription factors. Whether in Arabidopsis protoplasts NtWRKY12 activates expression of the tobacco PR-$1a$ gene alone or in combination with Arabidopsis TGA, Myb, or other transcription factors is presently unknown. Experiments are under way to further investigate this.

**Occurrence of the WK Box in Other Promoters**

The NtWRKY12 binding site TTTTCCAC is remarkably similar to that of the *E. coli* protein DnaA (TTTTCCACA; Weigel *et al.*, 1997). DnaA is involved in DNA replication and binds to single-stranded DNA. Our band shift results were not caused by contaminating DnaA from the *E. coli* extract because no band shift was produced with a similarly isolated unfused GST protein preparation from *E. coli* (Fig. 6A, lane 3).

In tobacco, a TTTTCCAC box is also found 249 bp upstream of the transcription start site in the SA-inducible PR-$2d$ gene (EMBL/GenBank accession no. X69794) and 1,012 bp upstream of the initiation codon in *Sar8.2b* (U64816). We have checked the occurrence of the WK box in the Arabidopsis genome. Whereas Maleck *et al.* (2000) found the W box to be overrepresented at 2.5-fold the statistically expected level in the promoters of a set of 25 PR-$1$ coregulated genes, we found the WK box to be overrepresented 3.3-fold in this set. Moreover, in the 1,000-bp upstream promoter regions of a set of 372 BTH-induced genes (Bülow *et al.*, 2007), the WK box is found at twice the expected level, whereas the W box is present at 1.4-fold. Interestingly, in both sets the as-$1$ element is present at exactly the statistically expected level.

Recently, Sun *et al.* (2003) characterized the region of the promoter of the sugar-responsive iso$1$ gene from barley (*Hordeum vulgare*) that bound to barley transcription factor SUSIBA2. The 573-amino acid protein SUSIBA2 contains two WRKY and Zn-finger domains, which classifies it as a member of group 1 of the WRKY superfamily. Interestingly, SUSIBA2 bound to a region of the iso$1$ promoter lacking the consensus TTGAC(C/T) W box. Although the authors have not further delineated the exact SUSIBA2 binding box, we noticed that the region contains the sequence TTTTCCA and that mutations in this sequence affected the formation of band shifts with SUSIBA2 protein. Our results with NtWRKY12 suggest that it could be this sequence that determines the SUSIBA2 binding site. If so, the occurrence of two such similar WRKY binding sequences in promoters of genes involved in different physiological processes and in different plant species would indicate that the consensus TTGAC(C/T) WRKY box is not the only conserved cis-element involved in binding-WRKY transcription factors. However, it must be noted that neither of SUSIBA2’s WRKY domains contains the WRKYGKK sequence present in NtWRKY12.
CONCLUSION

In WRKY transcription factors, the WRKY consensus sequence is followed by the amino acid sequences GQK, GKK, or GEK. Factors of the GQK type have been shown to bind to the W box element (TTGAC[C/T]). We identified a tobacco WRKY factor (NtWRKY12) of the GKK type, which specifically recognized two WK boxes (WK₁ and WK₂; TTTTCCAC) in the promoter of the SA-inducible tobacco PR-1a gene, but failed to bind to the W box element. The central K residue in the GKK sequence was crucial for binding of NtWRKY12 to the WK box. Overexpression of NtWRKY12 in protoplasts strongly stimulated PR-1a promoter activity via functional WK₁ and WK₂ boxes. Synergistic interactions between NtWRKY12 and other transcription factors, particularly TGA1a, appeared to be required for maximal induction of the PR-1a promoter in planta by SA or bacterial elicitors.

MATERIALS AND METHODS

Plants and Plant Treatments

Tobacco (Nicotiana tabacum ‘Samsun NN’) plants were grown in growth chambers at 25°C, 60% relative humidity, with a 16/8-h photoperiod.

For cDNA library cloning, 8-week-old plants were inoculated with 0.1 mL per leaf of an inoculum of 18 ng TMV/mL by rubbing the inoculum on three lightly carborundum-dusted leaves per plant after which the plants were immediately placed in a growth room at 33°C with a 16-h day/8-h night regime. After 2 d, the plants were returned to the 25°C growth room and inoculated leaves were collected after 5 h. For gene expression studies, three leaves of 8-week-old tobacco plants were inoculated with 3 ng TMV/mL and kept at 25°C. Inoculated and noninoculated leaves were sampled at different time points and immediately frozen in liquid nitrogen and stored at -80°C.

Discs of 24 mm were punched out of new, fully expanded leaves of wildtype and transgenic plants and floated on water or on 1 mM sodium salicylate, pH 6.8. After 2 d, the discs were blotted dry and four 12-mm discs were punched out, transferred to Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C.

Transgenic tobacco plants containing 35S::GUS and PR-1a::GUS reporter genes were obtained through Agrobacterium tumefaciens-mediated leaf disc transformation with transgene constructs cloned into the pMOG800 transformation vector and regeneration of kanamycin-resistant shoots (Linthorst et al., 1989). The number of transgenic plants obtained were 14 (35S), 14 (wild type), two (WK₁), two (WK₂), six (WK₁/WK₂), three (Δ32), 16 (Δ85), seven (WK₁/Δ85), and three (as-1).

Tiny punctures were made with a scalpel in the bottom epidermis of new, fully expanded leaves of 8-week-old tobacco plants, through which Agrobacterium infiltration mixtures (A₆₀₀ = 1 for the PR-1a::GUS strains and A₆₀₀ = 0.2 for the 35S::GUS strain) were supplied to the intercellular spaces by gentle pressure using a syringe without needle. After 2 d, 12-mm leaf discs were sampled from fully infiltrated areas adjacent to the puncture hole, transferred to Eppendorf tubes, frozen in liquid nitrogen, and stored at -80°C.

One-Hybrid Screening

mRNA was isolated from TMV-infected tobacco 5 h after the plants were transferred from 33°C to 25°C using the PolyAtract mRNA isolation system (Promega). First-strand cDNA was synthe-
sized on 5 µg poly(A) RNA using an XhoI-oligo(dT) linker primer and M-MLV reverse transcriptase (Promega), after which the second strand was synthesized using RNaseH and Pfu DNA polymerase (Stratagene). After ligation of EcoRI adapters and digestion with XhoI, 150 ng of the sized cDNA fraction longer than 500 bp was ligated into XhoI/EcoRI double-digested λACTII arms. After packaging, the λACTII cDNA library (1.4 x 10^6 independent transformants) was amplified in Escherichia coli XL-1 Blue MRF. The phage library was subsequently obtained as a plasmid expression library in pACTII by in vivo excision using E. coli BNN132.

Fragments of the tobacco PR-1a promoter corresponding to the regions -701 to -612 (region III) and -605 to -513 (region IV) relative to the transcription start site and various mutants of region IV were obtained by PCR using forward primers extended with BamHI and reverse primers extended with BglII restriction sites. This allowed convenient cloning and concatamerization of the fragments in plasmid pIC19H. Collinear tetramers were cloned in front of the His-3 gene of plasmid pHIS3N/X and subsequently the PR-1a promoter tetramer/His-3 bait constructs were cloned into pINT1 for integration into the genome of yeast (Saccharomyces cerevisiae) strain Y187 containing an auxotrophic his3 mutation (Ouwerkerk and Meijer, 2001). This resulted in strains Y187-III and Y187-IV, respectively. Leakiness of the PR-1a promoter tetramer/His-3 genes of the respective strains was virtually absent.

Screening of the cDNA library in the yeast one-hybrid system was performed essentially as described by Ouwerkerk and Meijer (2001). His-independent clones resulting from the transformations with the pACTII cDNA library were named pACT/IV-n.

Tetramerized subfragments and mutations thereof of promoter fragment IV were analyzed in the one-hybrid system for their ability to confer His independent growth in one-hybrid assays with the NtWRKY12 DNA-BD of pACT/IV-80.

**RACE**

The cDNA region matching the 5’-part of the mRNA corresponding to the insert of pACT/IV-80 was obtained using RACE (Boehringer) on total RNA from TMV-infected tobacco plants using primer 5’-CCTTCATATGTTGTTAATAAGCTGG, which is complementary to an internal region starting at position 271 of the insert of clone pACT/IV-80. Resulting clones were characterized and the clone containing the longest insert was sequenced to confirm that it corresponded to pACT/IV-80. The insert was subsequently fused to the insert of pACT/IV-80 using a common BglII site to result in clone pNtWRKY12 containing the full-length coding region of NtWRKY12.

**Bacterial Expression of NtWRKY12 Fusion Proteins**

The C-terminal partial open reading frame of pACT/IV-80 (NtWRKY12-BD), mutants in which the GKK sequence was changed into GQK or GEK, and the full-length coding sequence of AtWRKY51 and AtWRKY59 were cloned in frame behind the GST open reading frame of expression vector pGEX-KG (Guan and Dixon, 1991). These plasmids were transformed into E. coli BL21-DE3. For induction of protein expression, cultures were grown to mid-log phase at 37°C, after which isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM and incubation continued for 3 h at 20°C. The cells were harvested by centrifugation, resuspended in 1/20th volume sonication buffer (1x phosphate-buffered saline containing 2% [v/v] Tween 20, 0.1% [v/v] Triton X-100, 5 mM dithiothreitol [DTT], and 1 mg mL^{-1} lysozyme) and lysed by sonication (Vibracell). The fusion proteins were purified using glutathione-Sepharose 4B columns (Amersham), which were eluted overnight at 4°C with 10mM reduced glutathione, after which 1/50th volume Complete (Roche) protease inhibitors were added. Expressed fusion proteins were analyzed using 12% SDS-PAGE.

**EMSA**

EMSAs were performed essentially as described by Green et al. (1989). DNA probes for the EMSA assays were obtained by slowly cooling down mixtures of equimolar amounts of complementary
oligonucleotides from 95°C to room temperature. Annealed oligonucleotides were subsequently labeled using T4-nucleotide kinase and [γ-32P]ATP after which unincorporated label was removed by Autoseq G-50 column chromatography (Amersham-Pharmacia Biotech).

EMSA reaction mixtures contained 0.5 µg purified protein, 3 µL 5x gel shift binding buffer [20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.25 mg mL–1 poly(dI-dC) x poly(dIdC) (Promega)] in a total volume of 14 µL. After 10-min incubation at room temperature, 1 µL containing 60,000 cpm of labeled probe was added and Novel WRKY Factor in Defense Signaling incubation was continued for 20 min at room temperature. The total mixture was loaded onto a 5% polyacrylamide gel in Tris-borate buffer and electrophoresed at 4°C. After electrophoresis, the gel was dried, autoradiographed, and analyzed using a Bio-Rad Phosphoimager.

RT-PCR and RNA-Blot Analysis

Total RNA was isolated from pulverized frozen tobacco leaf tissue by phenol extraction and LiCl precipitation. Oligo(dT)-primed cDNA for PCR was obtained using M-MLV reverse transcriptase. Subsequently, PCR was performed during 25 cycles with primers corresponding to NtWRKY12 (AACACAGTTTAATCCCTAAACG, AGAACAAAGACCGAGCTTGAGATC), PR-1a (ATCATCTCCATTGTTACACTGAAC, GCTTCCCAATTGGCTGCAG), and tobacco actin (TGCTAGGAGCCAGTGAGTA, GTGATGCTGTCCAGC). The products were analyzed on agarose gel.

For RNA-blot analysis, total RNA was denatured using formamide/ formaldehyde, electrophoresed in 1.5% agarose gel, blotted to Hybond1(Amersham), and hybridized to 32P-labeled cDNA probes as described previously (Brederode et al., 1991). After hybridization, the blots were washed at high stringency with a final wash step in 30 mM NaCl, 3 mM sodium citrate, 0.1% SDS at 50°C for 20 min.

Transactivation Experiments

Protoplasts were prepared from Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 cell suspensions according to Axelos et al. (1992), with some modifications. A 5-d-old cell suspension culture was diluted 5-fold in 50 mL medium (3.2 g/L Gamborg B5 basal medium with minimal organics [Sigma- Aldrich], 3% Suc, 1 µM naphthalacetic acid [NAA], pH 5.8) and incubated overnight at 25°C at 250 rpm. Cells were harvested and cell walls digested with 20 mL of enzyme mix (0.4% macerozyme R-10 [Yakult], 1.5% cellulose Onozuka R-10 [Yakult], 12% sorbitol, pH 5.8) for 3 h at 28°C. The protoplasts were filtered through a 63-µm steel sieve and washed twice in 50 mL of protomedium (Gamborg B5 basalmedium, 0.1 M Glc, 0.25 M mannitol, 1 µM NAA, pH 5.8). The volume of the protoplast suspension was adjusted to 4 x 106 cells/mL. Protoplasts were cotransfected with 2 µg of plasmid carrying one of the PR-1a promoter::GUS constructs (wild type, WK, WK, WK) and 6 µg of effector plasmid pRT101 (Töpfer et al., 1987) carrying 35S::NiWRKY12. As a control, cotransformation of PR-1a promoter::GUS fusions with the empty expression vector pRT101 was carried out. Protoplasts were transformed using polyethylene glycol as described previously (Schirawski et al., 2000). The protoplasts were harvested 16 h after transformation and frozen in liquid nitrogen.

Fluorometric GUS Assays

When the transgenic plants had reached a size of 15 to 20 cm for each transgenic plant for each treatment (untreated, water, and SA), four leaf discs were separately assayed for GUS activity, with each data point being the average of duplicate measurements. Each leaf disc was homogenized in 0.5 mL GUS extraction buffer (Jefferson, 1987), supplied with 20% methanol (Kosugu et al., 1990). After centrifugation for 5 min at 8,000g duplicate samples of 10 µL supernatant were incubated with 90
µL 1 mM 4-methylumbelliferyl-β-D-glucuronide at 37°C for 20 h. The reaction was terminated by adding 300 µL 0.2 M sodium carbonate and 460-nm fluorescence was measured using a Fluoroscan II (Titertek) at 355-nm excitation.

For transient GUS expression measurements, homogenates were made of 10 pooled 12-mm discs from infiltrated areas of leaves of five independently infiltrated plants. GUS activity, normalized against protein concentration, was determined from the average of duplicate measurements per sample.

For protoplast experiments, GUS activity was determined as described (van der Fits and Memelink, 1997), with minor modifications. GUS activities from triplicate experiments were normalized against total protein level.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number DQ460475.