Tobacco Transcription Factors NtWRKY12 and TGA2.2 Interact \textit{in vitro} and \textit{in vivo} and Activate \textit{PR-1a} Gene Expression

Marcel C. van Verk, Lyda Neeleman, John F. Bol, and Huub J.M. Linthorst

Manuscript in Preparation
ABSTRACT

The promoter of the salicylic acid-inducible PR-1a gene of *Nicotiana tabacum* contains binding sites for transcription factor NtWRKY12 (WK box at position -564) and TGA factors (as-1-like element at position -592). Transactivation experiments in Arabidopsis protoplasts derived from wild type, npr1-1, and triple and quadruple tga mutant plants revealed that NtWRKY12 alone was able to induce a PR-1a::β-glucuronidase (GUS) reporter gene to high levels, independent of co-expressed tobacco or endogenous Arabidopsis NPR1 or TGAs. In protoplasts derived from Arabidopsis cell cultures transient expression of NtWRKY12 or TGA2.2 each activated expression of the PR-1a::GUS gene at similar levels, whereas expression of TGA2.1 resulted in only low levels of activation. Expression of a combination of NtWRKY12 and TGA2.2 activated expression to levels similar to the sum of the levels obtained with the separate transcription factors. By in vitro pull-down assays with GST and Strep fusion proteins and by Fluorescence Resonance Energy Transfer assays with protein-CFP and protein-YFP fusions in transfected protoplasts, it was shown that NtWRKY12 and TGA2.2 could interact in vitro and in vivo. A possible interaction of NtWRKY12 with TGA1a or TGA2.1 was not detectable by these techniques. Mutations were engineered in the PR-1a promoter to analyze the role of the WK box (-564) and as-1-like element (-592) in the activation of promoter activity by NtWRKY12 and TGA2.2. Although it cannot be excluded that these two factors activate PR-1a expression in an additive way, several findings point to a functional interaction between NtWRKY12 and TGA2.2 in this process..

INTRODUCTION

Upon pathogen attack plants mobilize inducible defense systems. A classic example is the systemic acquired resistance (SAR) effective against a broad range of pathogens. The signal transduction route leading to SAR involves the induced synthesis of the endogenous signal molecule salicylic acid (SA). SAR is accompanied by the de novo synthesis of pathogenesis-related (PR) proteins of which many directly affect pathogen growth and disease proliferation. Although their exact function is still not fully characterized, the plant-wide conserved PR-1 proteins are generally considered as marker proteins for SAR. In most plant species expression of the PR-1 genes is under transcriptional control.

Early work by the group of Chua in tobacco (*Nicotiana tabacum*) has indicated that gene expression controlled by the 35S promoter from Cauliflower mosaic virus is enhanced by SA and that this effect depends on the presence of activation sequence-1 (as-1), a DNA element in the 90 bp core promoter consisting of two TGACG tandem repeats (Qin et al., 1994). The as-1 element specifically binds to tobacco ASF-1, a DNA-binding complex containing the basic leucine zipper (bZIP) transcription factor TGA1a (Katagiri et al., 1989; Qin et al., 1994). More recently, the structurally related TGA2.2 was identified as the major DNA-binding component of ASF-1, while homolog TGA2.1 was present
at lower amounts (Niggeweg et al., 2000a).

Also promoters of several PR genes, such as *Arabidopsis thaliana* PR-1 and tobacco PR-1a contain as-1-(like) elements in promoter regions important for SA-induced expression. In tobacco the as-1-like element in the PR-1a promoter consists of a set of inverted TGACG motifs which were found to bind TGA transcription factors, while mutation of the element in a PR-1a-promoter::GUS reporter gene affected SA-induced GUS expression (Strompen et al., 1998; Niggeweg et al., 2000b; Grüner et al., 2003). Likewise, a linker scanning analysis of the region of the Arabidopsis PR-1 promoter responsible for induced expression by the SA analog INA revealed the presence of an as-1 element with two TGACG direct repeats of which one is a positive regulatory element (LS7), while the other (LS5) mediates negative regulation of PR-1 expression (Lebel et al., 1998). Through knock-out analyses it was shown that the Arabidopsis bZIP transcription factors TGA2, TGA3, TGA5 and TGA6 act as redundant but essential activators of PR-1 expression and SAR (Zhang et al., 2003; Kesarwani et al., 2007).

The ankyrin repeat protein NPR1 plays a central role in defense responses and is required for induction of PR gene expression and the establishment of SAR (Cao et al., 1997; Delaney et al., 1995; Wang et al., 2006). Upon pathogen induced accumulation of SA, the redox state of the cell changes, resulting in release of reduced NPR1 monomers from cytoplasmic complexes and subsequent translocalization to the nucleus where it interacts with TGA transcription factors to activate gene expression (Mou et al., 2003; Kinkema et al., 2000; Després et al., 2000; Zhang et al., 1999; Zhou et al., 2000). Recently it was shown that coactivation by NPR1 occurs in a pulse-wise manner and is regulated by degradation of NPR1 via the proteasome (Spoel et al., 2009).

In addition to TGAs, WRKY transcription factors are important for transcriptional programs induced in response to environmental signals (Eulgem and Somssich, 2007; Pandey and Somssich, 2009). Unlike the TGA transcription factors that are present at steady state levels (Johnson et al., 2003), many of the WRKY genes are transcriptionally activated upon biotic and non-biotic stress. Of the 73 WRKY genes in Arabidopsis, 49 were found to be differentially expressed upon *Pseudomonas syringae* infection or treatment with SA (Dong et al., 2003). Many WRKY proteins bind to the W-box, a DNA motif with the core sequence TTGAC(T/C) and the overrepresentation of this motif in several WRKY genes suggests their expression is regulated by WRKY transcription factors. However, for several WRKY genes, SA-induced expression is dependent on NPR1 and TGAs, suggesting a similar activation strategy as for PR-1 (Dong et al., 2003; Wang et al., 2006).

In the same linker scanning study that identified the two as-1-like regulatory elements in the Arabidopsis PR-1 promoter, a consensus W-box motif with a strong negative effect was identified, suggesting WRKY factors to be important for SA-mediated PR-1 gene expression (Lebel et al., 1998). The tobacco PR-1a promoter does not harbour a consensus W-box, however, NtWRKY12, a WRKY protein with a variant DNA binding domain, was found to bind to WK-boxes (TTTTCCAC) in the PR-1a promoter. Mutations in the WK box at position -564 of the PR-1a promoter reduced SA-mediated PR-1a::GUS expression in transgenic tobacco or bacterial elicitor-mediated expression in agroinfiltrated leaves by 50 to 60%. In these assays, mutations in the as-1-like element at position -592 to -577 of the PR-1a promoter had little or no effect on PR-1a::GUS expression. However,
combined mutation of the WK and as-1-like elements completely abolished inducible expression, suggesting that NtWRKY12 and TGA transcription factors interact in the regulation of PR-1a promoter activity (van Verk et al., 2008).

In this study we used pull down assays and Fluorescence Resonance Energy Transfer (FRET) analysis to identify protein-protein interactions between NtWRKY12 and TGA factors in vitro and in vivo, respectively. In addition, we performed transactivation experiments in Arabidopsis protoplasts to study the effect of combinations of NtWRKY12 and TGAs on PR-1a gene expression. Our findings revealed that NtWRKY12 alone was able to induce PR-1a expression to high levels independent of co-expressed tobacco or endogenous Arabidopsis NPR1 or TGAs. Furthermore, TGA2.2 was shown to specifically interact with NtWRKY12 and enhance PR-1a::GUS expression. The role of the WK box and as-1-like element in PR-1a promoter activity was analyzed.

RESULTS

Protein-Protein Interactions Between NtWRKY12, TGAs and NPR1

Our previous work pointed to a cooperation between NtWRKY12 and TGA transcription factors in the activation of the PR-1a promoter. To analyze a possible protein-protein interaction between NtWRKY12 and tobacco TGA factors in vivo and in vitro, we used FRET analysis and in vitro pull-down assays, respectively.

To elaborate the cellular localization of NtWRKY12, TGA1a, TGA2.1, TGA2.2 and NtNPR1 for the FRET analyses we transfected Arabidopsis protoplasts with plasmids in which the corresponding cDNAs were cloned upstream of the YFP or CFP coding sequence. Examples of imaging of the fusion proteins in living protoplasts by confocal laser scanning microscopy are shown in Figure 1. Whereas fluorescence of unfused CFP and YFP was dispersed throughout the cytoplasm and nucleus, NtWRKY12:CFP, TGA2.1:YFP and TGA2.2:YFP fluorescence localized mainly in the nucleus. The same results were obtained when the proteins were fused to the other chromophore (data not shown). Interestingly, the signals of both NtNPR1:CFP and NtNPR1:YFP were always concentrated in small nuclear spots (data not shown). Furthermore, it is noteworthy that we never detected fluorescence in protoplasts transformed with constructs containing TGA1a fused to either CFP or YFP. These results show that tobacco TGAs 2.1 and 2.2 localize to the nucleus, similar to what has previously been reported for the Arabidopsis homologs (Pontier et al., 2002; Johnson et al., 2003). Due to the extreme brightness of the uneven distributed small nuclear spots of the NtNPR1 chromophore fusions, these could not be used for FRET analysis.

FRET analysis is based on overlapping emission/excitation spectra of donor fluorophore CFP and acceptor fluorophore YFP. Emitted fluorescence from CFP can only excite YFP when both fluorophores are in close (less than 10 nm apart) spatial proximity (Wu and Brand, 1994). Thus, a close association of two proteins with fusions to the respective fluorophores would result in an increase of acceptor fluorescence and quenching of the donor fluorescence. As a positive control for
FRET, Arabidopsis protoplasts were transfected with an expression plasmid encoding a YFP:CFP tandem fusion, while co-transfection with uncoupled CFP- and YFP-encoding plasmids was used as negative control. The protoplasts were incubated for 24h, after which FRET measurements were performed. The result is shown in Figure 2A. For the negative control, protoplasts were selected that showed both CFP (475nm) and YFP (527nm) emission after excitation of the respective fluorophores to confirm transfection with both CFP and YFP plasmids. Excitation of CFP with 457nm UV light in these protoplasts resulted in an emission spectrum with a maximum at 475nm and a certain level of bleeding at 527nm. CFP excitation of the YFP:CFP fusion protein in the positive control protoplasts resulted in quenched emission at 475nm, as part of the emission energy was used to excite the YFP fluorophore of the fusion protein, which was subsequently emitted at 527nm. Thus, the slope of the line connecting the normalized emission intensities at 475nm and 527nm is a measure of the amount of FRET. Similarly, FRET assays were performed on protoplasts cotransfected with combinations of plasmids encoding NtWRKY12 and TGA chromophore fusion proteins.

The control experiments with combinations of NtWRKY12:YFP, TGA2.1:YFP or TGA2.2:YFP with unfused CFP did not result in increased 527nm emission (dashed lines in Fig. 2B, 2C and 2D, respectively), showing that neither NtWRKY12 nor the TGAs interacted with the CFP chromophore, which would preclude the use of FRET for analyzing interactions between these proteins. The angles of the solid lines in Fig. 2B, 2C and 2D indicate the amount of FRET obtained between the various YFP and CFP fusion proteins. In addition to providing the control that the YFP chromophore does not interact with NtWRKY12, the lack of raised 527nm emission with the combination of NtWRKY12:YFP / NtWRKY12:CFP indicates that NtWRKY12 is not able to form homodimers.

Figure 1. Nuclear localization of NtWRKY12 and TGAs. The panels on the middle row show confocal laser scanning microscopy images obtained of Arabidopsis protoplasts transfected with expression plasmids encoding unfused CFP and YFP, and fusion proteins NtWRKY12:CFP (W12:CFP), TGA2.1:YFP, TGA2.2:YFP, and NPR1:CFP. Panels on the bottom row show overlays with bright-field images (top row) of the same protoplasts. Localizations were visualized with a 63x objective. The red rulers indicate 5 µm.
Similarly, although 475 nm emission in the protoplasts transfected with the NtWRKY12:CFP / TGA2.1:YFP plasmids was quenched, 527 nm emission was not significantly higher than in the control protoplasts, showing that no strong interaction occurred between TGA2.1 and NtWRKY12 (Fig. 2C). On the other hand, the large amount of FRET in the protoplasts expressing the combination NtWRKY12:CFP / TGA2.2:YFP demonstrates that NtWRKY12 strongly interacted with TGA2.2.
(Fig. 2D). Although we could not detect the TGA1a:chromophore fusion proteins in our localization experiments (see above), we did perform a cotransfection of protoplasts with TGA1a:YFP and NtWRKY12:CFP. While it was not surprising to find no YFP signal in these protoplasts, what was surprising was the reproducible total absence of protoplasts showing CFP emission.

To confirm the interaction between NtWRKY12 and TGA2.2, in vitro pull-down assays were performed with E. coli-expressed GST and Strep/HIS fusion proteins purified using affinity chromatography. In addition to the interaction between NtWRKY12 and TGA2.2, also interactions with TGA1a, TGA2.1 and NtNPR1 were assayed.

Figure 3 shows the results of in vitro pull-down assays; the data obtained in panels A, B and C are summarized in panel D. Figure 3A shows the interactions between different TGA proteins and NtNPR1. GST:NtNPR1 was incubated with various Strep:TGA:HIS fusion proteins and with a Strep:NtNPR1:HIS fusion, after which the complexes were pulled down using Streptactin beads. The pulled-down proteins were analyzed on Western blots using anti-GST antibody conjugate. Strong NtNPR1-TGA2.2 and NtNPR1-NtNPR1 interactions were observed (Fig. 3A, lanes 3 and 5), whereas no interactions between NtNPR1 and TGA2.1 or TGA1a were detectable (Fig. 3A, lanes 2 and 4). Figure 3A, lanes 6 to 10 show the controls with single fusion proteins. The low background signal obtained with GST:NtNPR1 (Figure 4a, lane 6) was also visible in Figure 3A, lane 4. Homodimer formation as seen with the tobacco NtNPR1 has been reported for Arabidopsis NPR1 (Mou et al., 2003).

In the experiments shown in Figures 3B and 3C, GST fusions of NtWRKY12 and NtNPR1 were incubated with various Strep:TGA:HIS fusions, and protein complexes were bound to Gluthatione-Sepharose 4B beads. The pulled-down proteins were analyzed on Western blots using anti-HIS antibodies. Interactions of NtWRKY12 were observed with TGA2.2 (Fig. 3B, lane 1), but not with TGA1a or TGA2.1 (Fig. 3C, lanes 1 and 5). Moreover, the conclusion from Figure 3A that NtNPR1 interacts with TGA2.2, but not with TGA1a or TGA2.1 was confirmed in this system (Fig. 3B, lane 2; Fig. 3C, lanes 2 and 6).

As a first step towards the characterization of the NtWRKY12 sequence involved in the interaction with TGA2.2, two NtWRKY12 deletion mutants were made. NtWRKY12ΔC lacks the C-terminal 87 amino acids (aa) of the 220 aa long protein; NtWRKY12BD lacks the N-terminal 113 aa. Both mutants were found to interact with TGA2.2 (Fig. 3B, lanes 6 and 7). Either the overlap between the two mutant proteins (aa 114-133) is involved in the interaction of NtWRKY12 with TGA2.2, or NtWRKY12 contains two independent binding sites for TGA2.2, possibly involved in the interaction with a TGA dimer.

**Functional Domains of NtWRKY12**

Previously, yeast-one-hybrid screening for tobacco proteins binding to the PR-1a promoter resulted in the isolation of a protein corresponding to the C-terminal 107 aa of NtWRKY12 fused to the GAL4 activation domain. This protein contained the conserved WRKY and Zn-finger domains and,
apparently, a DNA binding domain (BD) (van Verk et al., 2008). Moreover, it was shown that full-length (220 aa) NtWRKY12 was able to activate \textit{PR-1a::His} gene expression in yeast, indicating that in addition to a BD, NtWRKY12 also contains an activating domain (AD). To further characterize functional domains of NtWRKY12, deletion mutants of NtWRKY12 were assayed in the one-hybrid system in three different ways. First, the mutants were fused to the GAL4 BD and assayed for their ability to activate \textit{GAL4} promoter::\textit{Ade} reporter gene expression (Fig. 4; results summarized in the column with the caption “BD”). Fusions, which activated the reporter gene, were concluded to contain the NtWRKY12 AD. Second, the mutants were fused to the GAL4 AD and assayed for their ability to activate \textit{PR-1a::HIS} gene expression (Fig. 4; results summarized in the column with the caption “AD”). Fusions which activated gene expression were concluded to contain the NtWRKY12 BD. Third, the mutants were expressed as non-fused proteins and assayed for their ability to activate \textit{PR-1a::HIS} expression (Fig. 4; results summarized in the column with the caption “-“). Mutants which
activated gene expression were concluded to contain both the AD and BD domains of NtWRKY12.

Figure 4 (column “BD”) shows that GAL4 BD fusions lacking the C-terminal 37 aa (construct 4) or N-terminal 40 aa (construct 7) of NtWRKY12, and a protein with both these deletions (construct 15) were able to activate GAL4::Ad expression. Apparently, the NtWRKY12 AD function is contained within the aa 41-183 region of the protein. An online search using the ExPASy (www.expasy.org) Nine Amino Acid Transactivation Domain prediction tool revealed that the region from AA 5-70 and AA 209-217 contain six domains that have 7 out of 12 possible prediction criteria. The GAL4 AD fusion of the smallest NtWRKY12 deletion mutant that was able to activate PR-1a::HIS expression was construct 18 (Fig. 4, column “AD”). Thus, the NtWRKY12 BD is localized in the sequence of aa 121-201. This region encompasses both the conserved WRKY and Zn-finger domains. Apparently, aa upstream of the WRKY domain are also necessary for DNA binding, as a deletion mutant with only seven aa in front of the WRKY domain (Fig. 4, construct 13, aa 132-220) was not able to activate HIS gene expression. Construct 17 (aa 41-201) combined the minimal sequences with NtWRKY12 AD and BD activity (construct 15, aa 41-183, and construct 18, aa 121-201). However, the non-fused protein encoded by construct 17 was not able to activate PR-1a::HIS expression (Fig. 4, column “-”). To permit both AD and BD activity, the sequence of construct 17 had to be extended by either the N-terminal 40 aa of NtWRKY12 (Fig. 4, construct 2, aa 1-201) or the C-terminal 19 aa (Fig. 4, construct 7, aa 41-220). Possibly, the lack of activity of the protein encoded by construct 17 (aa 41-201) was due to instability or misfolding of the polypeptide.

**PR-1a::GUS Gene Expression in Arabidopsis Protoplasts Cotransfected with Plasmids Encoding NtWRKY12, NtNPR1 and TGAs**

In our previous paper we showed that cotransfection of Arabidopsis protoplasts with 35S::NtWRKY12 and PR-1a::GUS constructs resulted in a strong increase in GUS expression. To further investigate the role of NtWRKY12, TGA and NPR1 on activation of PR-1a driven expression, additional transactivation assays were set up in protoplasts isolated from leaves of Arabidopsis seedlings grown on MS medium. To avoid interfering effects of NtWRKY12 binding to the far upstream WK binding site (-859), this WK site in the PR-1a::GUS reporter gene used in these experiments was mutated (TTTTCCAC into TCCCTTGC). Fig. 5A shows the effects of overexpression of NtWRKY12, TGA2.1, TGA2.2 and NPR1 on PR-1a::GUS expression in wild type Arabidopsis protoplasts. Obviously, NtWRKY12 greatly enhanced beta-glucuronidase expression from the PR-1a promoter (6-fold over background level). Overexpression of TGA2.1, TGA2.2 or NtNPR1, or combinations of the TGAs with NtNPR1 did not result in enhanced GUS expression. Neither did TGA2.2, alone or in combination with NtNPR1, affect the level of NtWRKY12 enhanced PR-1a::GUS expression, whereas overexpression of TGA2.1, alone or together with NtNPR1, slightly reduced NtWRKY12 activated GUS expression.

In Arabidopsis, PR gene expression is dependent on NPR1 and there is accumulating evidence that NPR1 orthologs similarly effect expression of PR genes in other plant species (Rayapuram and
We wondered whether the lack of effects of overexpressed NtNPR1 on PR-1a::GUS expression in the cotransfection experiments could be due to the presence of saturating levels of functionally equivalent Arabidopsis NPR1. However, the results of transactivation assays in protoplasts from npr1-1 mutant plants were virtually identical to those of the wild type protoplasts (compare Figs. 5A and 5B). This implies that PR-1a expression in Arabidopsis protoplasts is independent of NPR1.

On the basis of sequence homology, tobacco TGA2.2 belongs to the group II TGA proteins together with Arabidopsis TGAs 2, 5 and 6 (Xiang et al., 1997). To exclude the possibility that the absence of effects of overexpressed tobacco TGA on PR-1a::GUS expression in the Arabidopsis protoplasts was caused by functionally similar Arabidopsis TGAs, cotransfection experiments were performed in Arabidopsis protoplasts derived from tga2-1 tga5-1 tga6-1 (tga256) and tga2-1 tga3-1 tga5-1 tga6-1 (tga2356) mutant plants (Fig. 6). Also in these mutant backgrounds, overexpression of
NtWRKY12 led to activation of PR-1a::GUS expression (9-fold over background level in the triple mutant, Fig. 6A), although the enhancement in the quadruple mutant was greatly reduced (2-fold, Fig. 6B). Likely, this reduced GUS expression is the result of reduced production of NtWRKY12 from the transfected 35S::NtWRKY12 (W12), 35S::NtNPR1 (NPR1), 35S::TGA2.1 (TGA2.1), 35S::TGA2.2 (TGA2.2), a combination, or with empty expression vector, as indicated by the plus and minus signs. A, Expression in protoplasts isolated from seedlings of WT Col-0 Arabidopsis, B, expression in npr1-1 protoplasts. The bars represent the percentage of GUS activity from triplicate experiments relative to that of the protoplasts cotransfected with the corresponding PR-1a::GUS construct and empty vector control. Error bars represent the SEM.

Figure 5. Activation of PR-1a::GUS in WT Col-0 and npr1-1 mutant lines. Leaf protoplasts were cotransfected with PR-1a::GUS constructs together with expression plasmids containing 35S::NtWRKY12 (W12), 35S::NtNPR1 (NPR1), 35S::TGA2.1 (TGA2.1), 35S::TGA2.2 (TGA2.2), a combination, or with empty expression vector, as indicated by the plus and minus signs. A, Expression in protoplasts isolated from seedlings of WT Col-0 Arabidopsis, B, expression in npr1-1 protoplasts. The bars represent the percentage of GUS activity from triplicate experiments relative to that of the protoplasts cotransfected with the corresponding PR-1a::GUS construct and empty vector control. Error bars represent the SEM.

Together, the results of the cotransfection assays in Arabidopsis leaf protoplasts suggest that NtWRKY12 is the main transcriptional activator of PR-1a::GUS expression and that TGA2.1, TGA2.2 or NtNPR1, alone or in combination, do not positively effect activation.
Role of the WK and *as-I*-like Boxes in the Activation of the *PR-1a* Promoter by NtWRKY12 and TGA2.2

Arabidopsis protoplasts isolated from leaf usually had very low levels of *PR-1a::GUS* expression in the absence of co-transfected NtWRKY12 expression plasmid, a situation comparable to that of leaves from non-induced plants that do not express the *PR-1a* gene. In contrast, protoplasts prepared from Arabidopsis cell cultures usually had much higher basal levels, suggesting that in these protoplasts the *PR-1a::GUS* reporter gene was already expressed, apparently mediated by endogenous transcription factors. We checked whether the different media in which the two types of protoplasts were incubated after cotransfection were responsible for this difference in *PR-1a* promoter activity. Therefore, protoplasts prepared from cell cultures were transfected with combinations of *PR-1a::GUS* and empty expression construct, or expression vectors for NtWRKY12 and/or TGA2.2, upon which equal numbers of protoplasts were incubated overnight in the “rich” Protomedium normally used for incubation of cell culture protoplasts, or in the minimal W5 medium used for leaf protoplasts, before GUS activity was measured. As can be seen in Fig. 7A, GUS expression in the absence of NtWRKY12 or TGA2.2 was 32-fold higher in the protoplasts incubated in the Protomedium than in the minimal W5 medium. While the W5 protoplasts were more sensitive to NtWRKY12, resulting in 17.2-fold increased GUS activity, NtWRKY12 further increased GUS expression in the protoplasts from the Protomedium only 2.7-fold over the basal level. Intriguingly, while there was no effect of TGA2.2 in the W5 protoplasts, TGA2.2 enhanced GUS expression in the Protomedium protoplasts to a similar level as did NtWRKY12 (3.1-fold).

To further investigate the involvement of TGA proteins in activation of *PR-1a* expression under these experimental conditions, cell culture protoplasts were cotransfected with NtWRKY12, TGA2.1 or TGA2.2, together with the *PR-1a::GUS* reporter construct and incubated in Protomedium overnight. The results of these transactivation assays are shown in Figure 7B. Similar to the results shown in Fig. 7A, the presence of the NtWRKY12 plasmid increased *PR-1a* promoter-directed GUS expression approximately 3-fold in comparison to the basal level obtained with the empty expression vector, while again addition of plasmid expressing TGA2.2 led to enhanced GUS expression, to similar levels (2.5-fold) as by NtWRKY12. However, plasmid expressing TGA2.1 could not significantly enhance transcription of the GUS reporter gene. Co-expression of NtWRKY12 together with TGA2.1 did not result in higher GUS expression than NtWRKY12 alone, indicating that TGA2.1 is not involved in activation of *PR-1a*. The combination of NtWRKY12 and TGA2.2 led to an additive enhancement of *PR-1a::GUS* expression to a 5-fold increased level over the background. Noteworthy, while expression of TGA1a alone did not enhance *PR-1a::GUS* expression over the basal level, the combination of TGA1a and NtWRKY12 resulted in expression levels similar to those of the TGA2.2/NtWRKY12 combination (Results not shown).

Previous studies have shown that NtWRKY12 activates *PR-1a::GUS* expression in Arabidopsis protoplasts by binding to the WK box (TTTTCCAC) at position -564 in the *PR-1a* promoter.
The binding site involved in the induction of PR-1a::GUS expression by TGA2.2 has not been determined yet, but the as-1-like element (CGTCA[N]₆TGACG) at position -592 is a possible candidate for binding this TGA factor. This raises the possibility that NtWRKY12 and TGA2.2 bind to the PR-1a promoter in close proximity, and binding of NtWRKY12 and TGA2.2 might be stabilized by interactions between the two factors that were observed in vivo and in vitro (Figs. 2 and 3). To investigate protein-protein and protein-DNA interactions involved in the activation of the PR-1a promoter by NtWRKY12 and TGA2.2 in Arabidopsis protoplasts, we analyzed GUS expression driven by the PR-1a promoter with mutations in the WK box, the as-1-like element or both these boxes (Fig. 7C, D and E). The controls with the WT PR-1a promoter are shown in Fig. 7A (panel Protomedium) and Fig. 7B. In these experiments the protoplasts were incubated in Protomedium.

As shown before (van Verk et al., 2008), mutation of the WK box abolished induction of GUS expression by NtWRKY12 (Fig. 7C). GUS expression induced by TGA2.2 was slightly reduced by the WK mutation, and co-expression of NtWRKY12 did not further enhance the expression level (Fig. 7C). This indicates that possible protein-protein interactions between TGA2.2 and NtWRKY12 do not compensate for the loss of NtWRKY12-DNA interactions. Unexpectedly, GUS expression driven by the PR-1a promoter with a mutated as-1-like element (Fig. 7D) was very similar to the
expression driven by the wild-type promoter (Fig. 7A, Protomedium). Mutation of the \textit{as-1}-like element caused no significant reduction of GUS expression by NtWRKY12, TGA2.2 or both these
factors. This indicates that the as-1-like element at position -592 is dispensable for TGA2.2-mediated PR-1a gene expression. This conclusion is further corroborated by the observation that GUS expression driven by the WK/as-1 double mutant (Fig. 7E) is similar to the expression by the WK single mutant (Fig. 7C). The role of TGA2.2 and as-1-like elements in activation of the PR-1a promoter is discussed below.

DISCUSSION

Our previous studies pointed to NtWRKY12 as the major regulator of PR-1a gene expression (van Verk et al., 2008). Mutations in the NtWRKY12 binding site (WK box) in the PR-1a promoter reduced the SA-induced expression of PR-1a::GUS fusions in transgenic tobacco by 60%, whereas mutations in the as-1-like element resulted in a 30% reduction. Transient expression of PR-1a::GUS fusions, induced by bacterial elicitors in agroinfiltrated tobacco leaves, was not affected by mutations in the as-1-like element, but was reduced by 50% when mutations were made in the WK box. Interestingly, when both the WK box and as-1-like element were mutated, elicitor induced expression was reduced by 95%. This result pointed to synergistic interactions between factors binding to the WK box and the as-1-like element (van Verk et al., 2008). In the present work we further analyzed the role of NtWRKY12 and TGA transcription factors in PR-1a gene expression.

FRET analysis of possible interactions between NtWRKY12 and the tobacco transcription factors TGA2.1 and TGA2.2 revealed a strong and specific interaction between NtWRKY12 and TGA2.2 in the nucleus of transfected Arabidopsis protoplasts (Fig. 2). This interaction was confirmed by in vitro pull-down assays. In vitro, no interaction between NtWRKY12 and TGA1a, TGA2.1 or NtNPR1 was observed (Fig. 3). Pull-down assays and studies with the yeast one-hybrid system permitted an initial localization of domains in NtWRKY12 involved in the interaction with TGA2.2, in DNA binding and in transcription activation (Figs. 3 and 4). The role of NtWRKY12 and TGA2.2 in PR-1a gene expression was further investigated by transactivation studies in Arabidopsis protoplasts co-transfected with one vector expressing a transcription factor and another vector containing the PR-1a::GUS reporter construct. The major advantage of this system over SA induced or elicitor induced gene expression in whole plants is the possibility to analyze the response induced by well-defined single transcription factors.

We noticed that results obtained with transactivation assays are affected by the medium used for incubation of the protoplasts. Previously, we showed that in protoplasts incubated in Protomedium expression of PR-1a::GUS was increased about 4-fold by NtWRKY12 (van Verk et al., 2008). In the present study, we noticed that in protoplasts incubated in Protomedium the basal GUS expression is about 30-fold higher than in protoplasts incubated in W5 medium. In these W5 protoplasts, GUS expression was induced by NtWRKY12 to much higher-fold levels than in Protomedium protoplasts (Fig. 7A). Mutations in the WK box abolished NtWRKY12 mediated GUS expression, but did not affect the basal level of GUS in Protomedium protoplasts (Fig. 7C). Similarly, this basal level was not affected by mutations in the as-1-like element.
(Fig. 7D). Probably, Arabidopsis homologs of NtWRKY12 or factors binding to the as-1-like element are not responsible for the high basal level of PR-1a promoter activity in the Protomedium protoplasts. It could be that the presence of the synthetic auxin naphthalene acetic acid in the Protomedium and/or differences in Ca<sup>2+</sup> concentration in the two media are responsible for differences in basal expression of the PR-1a promoter.

In W5 protoplasts, PR-1a::GUS expression was activated by co-expression of NtWRKY12, but not by co-expression of NtNPR1, TGA2.1 or TGA2.2. This expression pattern was not affected in protoplasts from npr1-1 or tga Arabidopsis mutants (Figs. 5 and 6). These results indicate that NtWRKY12 activates GUS expression independently of exogenously or endogenously expressed NPR1 or TGA factors. In Protomedium protoplasts, NtWRKY12 and TGA2.2 each induced a 3-fold increase in GUS activity (Fig. 7A, right panel). The activity obtained by co-expression of both factors equalled the sum of the activities of the separate factors (Fig. 7B). As shown previously (van Verk et al., 2008), mutation of the WK box in the PR-1a promoter abolished NtWRKY12-mediated GUS expression (Fig. 7C). Contradictory to our expectation, mutation of the as-1-like element in the PR-1a promoter did not affect TGA2.2 mediated GUS expression (Fig. 7D). Recently we observed that in EMSA experiments a 47bp fragment of the PR-1a promoter, harbouring the as-1-like element at position -592, showed a specific band-shift with TGA2.2. Mutation of the as-1-like element abolished this band-shift (unpublished data). This indicates that TGA2.2 binds specifically to the as-1-like element at position -592 in the PR-1a promoter. The finding that, in the absence of mutations in the WK box, mutations in this as-1-like element have little or no effect on PR-1a promoter activity in SA-treated transgenic tobacco (van Verk et al., 2008), agroinfiltrated tobacco (van Verk et al., 2008) or TGA2.2-transfected Arabidopsis protoplasts (this study), suggests that TGA2.2 can mediate PR-1a expression by binding to a second as-1-like element in the PR-1a promoter that has yet to be identified. If the PR-1a promoter contains indeed two as-1-like elements, both could be functional under in vivo conditions.

Similar to TGA2.2, TGA1a has been shown to bind to the as-1-like element at position -592 in the PR-1a promoter (Strompen et al., 1998). In our experiments TGA1a did not activate expression of the PR-1a::GUS reporter when expressed alone, but did so when expressed together with NtWRKY12 (unpublished results). TGA1a has been shown to act as a transcriptional activator in yeast (Pascuzzi et al., 1998; Niggeweg et al., 2000b). Several studies have shown that TGAs are involved in PR-1a gene expression, either acting as positive or negative regulators (Strompen et al., 1998; Niggeweg et al., 2000b; Pontier et al., 2001).

NPR1-mediated gene expression in Arabidopsis is largely dependent on its proteasome mediated turnover as shown by Spoel et al. (2009). In this same paper the authors examined whether the proteasome activity affects induction of direct targets of NPR1 like WRKY18, WRKY38 and WRKY62 that lack complete responsiveness in npr1-1 mutants. The SA-mediated induction of these genes is inhibited for 50-60% by a MG115 treatment. Surprisingly the SA-induced expression of PR-1 is only affected for 5-10% by MG115 treatment, indicating that its activation is less dependent on the proteasome. These results could also indicate that SA-mediated induction of PR-1 is mainly achieved via other transcription factors, like Arabidopsis variants of the Tobacco NtWRKY12 that can activate
*PR-1a* gene expression independently of NPR1. Which most likely themselves are direct targets of NPR1, resulting in a NPR1 dependent activation of *PR-1(a)* gene expression.

In our previous paper, transient expression of NtWRKY12 in protoplasts was done in Arabidopsis protoplasts incubated in protomedium (van Verk *et al.*, 2008). In the present work, these studies were extended with transient expression of tobacco TGA factors (Fig. 7A and B). Of the factors tested, TGA2.2 was found to be most active in activation of expression of the *PR-1a::GUS* reporter. Also, TGA2.2 was the only factor that was found to interact with NtWRKY12 in FRET and/or in vitro pull-down assays. However, it is possible that binding sites involved in protein-protein interactions are masked in the fusion proteins used in these assays. Further studies are required to reveal whether or not different TGA factors use different pathways in the activation of the *PR-1a* promoter.

Separately, NtWRKY12 and TGA2.2 activated the *PR-1a* promoter to similar levels. Jointly the two factors activated the promoter rather in an additive way than synergistically. However, several observations suggest that NtWRKY12 and TGA2.2 do interact in the activation of the *PR-1a* promoter. Previously, we showed that mutation of the *as-1*-like element at position -592 had no effect on induction of *PR-1a::GUS* by bacterial elicitors but drastically reduced this induction when the as-1 mutation is made in a Wk<sub>1</sub>-mutant background (van Verk *et al.*, 2008). In the present work, a similar effect was seen on the TGA2.2-mediated expression of *PR-1a::GUS*. TGA2.2-mediated expression of the wild-type *PR-1a::GUS* construct (Fig. 7A, right panel and Fig. 7B) was little affected by mutation of the *as-1*-like element (Fig. 7D). However, TGA2.2-mediated expression was strongly reduced by mutation of the Wk<sub>1</sub> box, either alone (Fig. 7C) or in combination with the as-1 mutation (Fig. 7E).

This reduction suggests that TGA2.2 activity depends on the interaction of this factor with exogenously or endogenously expressed WRKY factors. Also the observation that TGA1a stimulated *PR-1a::GUS* expression when co-expressed with NtWRKY12 supports the notion that TGA and WRKY factors interact. TGA and WRKY transcription factors are known to interact with a variety of proteins. As members of the bZIP class of transcription factors, TGAs bind to DNA as homo- and heterodimers (Deppmann *et al.*, 2006). In addition to their ability to dimerize, there is accumulating evidence that TGAs are able to interact with other interaction partners involved in transcriptional processes. Previously, transcription factors of the Dof and ERF families were isolated as TGA-interacting proteins (Zhang *et al.*, 1995; Büttner and Singh, 1997). Furthermore, TGAs from tomato, tobacco and Arabidopsis were shown to interact with Arabidopsis NPR1, with Arabidopsis TGAs 2, 3, 5, 6 and 7 acting as constitutive interaction partners of NPR1, while interaction with TGAs 1 and 4 was induced by SA-mediated reduction of their intramolecular disulfide bridges (Zhang *et al.*, 1999; Niggeweg *et al.*, 2000b; Zhou *et al.*, 2000; Després *et al.*, 2003; Kesarwani *et al.*, 2007). Recently, glutaredoxin was shown to interact with Arabidopsis TGA2 and tobacco TGA2.2 (Ndamukong *et al.*, 2007), and Arabidopsis TGAs 2, 5 and 6, were found to recruit GRAS protein SCL14 to promoters of genes mediating protection to xenobiotic stress (Fode *et al.*, 2008). In the interactions with NPR1, glutaredoxin and SCL14, the TGAs are considered the DNA-binding partners, bringing the other protein to the promoter to affect transcription. WRKY transcription factors have been found to interact with other proteins involved in transcriptional regulation of stress response genes. In addition
to homo- and heterodimerization as was shown to occur with Arabidopsis WRKYs 18, 40 and 60 (Xu et al., 2006), examples are Arabidopsis WRKY7 interacting with calmodulin (CaM) through a CaM binding domain in the N-terminal half of the protein, which is conserved in other members of the WRKY IId group (Park et al., 2005), WRKY70 interacting with the EAR domain repressor ZAT7 (Ciftci-Yilmaz et al., 2007), WRKY53 interacting with mitogen activated protein kinase kinase MEKK1 (Miao et al., 2007), WRKY33 interacting with mitogen activated protein kinase 4 (MAPK4; Andreasson et al., 2005), and WRKYs 38 and 62 interacting with histone deacetylase19 (Kim et al., 2008). Together, the increasing data on protein-protein interactions between different transcription factors fits well in the concept of evolution of transcription circuits as laid out by Tuch et al. (2008). Further studies on the role of the interaction between NtWRK12 and TGA2.2 observed in our study, requires the identification of the TGA2.2 binding site in the PR-1a promoter.

MATERIALS AND METHODS

Bacterial Expression of Fusion Proteins

The open reading frames of NtWRKY12 and NtNPR1, and mutants encoding the 133 amino acids of the N-terminal half (NtWRKY12ΔC) or 107 amino acids of the C-terminal half (NtWRKY12BD) were cloned in frame behind the GST open reading frame of expression vector pGEX-KG (Guan and Dixon, 1991), expressed and purified according to van Verk et al., (2008).

The full length coding sequence of Nicotiana tabacum TGA1a, TGA2.1, TGA2.2 and NPR1 were cloned in frame of expression vector pASK-IBA45 plus harboring a Strep and HIS tag (IBA). These plasmids were transformed into Escherichia coli XL1. For induction of protein expression, cultures were grown to mid-log phase at 37°C, after which tetracycline was added to a final concentration of 0.2 µg mL-1 and incubation continued for 3.5 h at 29°C. The cells were harvested by centrifugation, resuspended in 1/25th volume lysis buffer (1x PBS containing 1% (v/v) NP40, 2 mM DTT and 1/50th volume Complete (Roche) protease inhibitors) and lysed by sonication (Vibracell, Sonics en Materials inc. USA). Soluble protein fraction was collected by centrifugation, and expressed fusion proteins were analyzed using 12% (w/v) SDS-polyacrylamide gel electrophoresis.

In Vitro Pulldown

For the in vitro pull-down assay, GST-fusion proteins were mixed with Strep-fusion-HIS proteins in binding buffer (1xPBS, 1% (w/v) NP40, 2 mM DTT) and incubated on an orbital shaker for 1h at room temperature. To this mixture Glutathione Sepharose 4B beads (GE Healthcare) or Strep-Tactin Sepharose beads (IBA) in buffer W (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA) were added, and incubation was continued for an additional hour. The beads were washed five times with PBS (with 1% (w/v) NP40 for Glutathione beads) after which the beads were collected, resuspended in Laemmli buffer, and heated at 95°C for 2 min.

The proteins bound to the beads were separated by SDS-PAGE and transferred onto Hybond P membrane (GE Healthcare). Membranes were incubated with the anti-GST antibody (GE Healthcare), or anti-HIS antibody (5 Prime) according to manufacturers instructions and exposed to X-ray film.

One-Hybrid Screening

A tetramer fragment of the tobacco PR-1a promoter corresponding to the region -605 to -513 relative to the transcription start site cloned in front of the His3 gene and integrated in the Saccharomyces cerevisiae genome of strain Y187 (van Verk et al., 2008) was used to screen for the DNA binding
domain (BD) of NtWRKY12 and presence of both an activation (AD) and BD. Deletion mutants of NtWRKY12 were cloned in pACT2 to screen for the presence of an BD or p415GPD-HA to screen for both the BD and AD. Mutants were screened for His-independent growth with addition of 3AT up to 20 mM. To locate the AD, deletion mutants were cloned into pAS2-1 and transformed in yeast strain PJ69-4A containing the Gal4 binding site in front of the Ade gene. Mutants were screened for adenine independent growth.

**Protoplast Preparation and Transactivation Experiments**

For microscopy and transactivation experiments, protoplasts were prepared from Arabidopsis thaliana ecotype Col-0 cell suspensions according to van Verk et al., (2008).

The leaves from approximately 50 four-week-old seedlings (Col-0, npr1-1, tga256, tga2356) grown on sterile medium were cut in small pieces and protoplasts were prepared according to He et al. (2007). In total 1x10^5 protoplasts were transformed per transfection using polyethylene glycol (40% (w/v) PEG 4000, 0.2 M mannitol, 0.1 M CaCl2).

Protoplasts were co-transfected with 2 µg of plasmid carrying PR-1a promoter::GUS construct and 6 µg of 35S::effector plasmid pRT101 (Töpfer et al., 1987). As a control, cotransformation of PR-1a::GUS construct with the empty expression vector pRT101 was carried out. The protoplasts harvested 16 hrs after transformation and GUS activity was determined. GUS activities from triplicate experiments were normalized against total protein level.

**Microscopy and Förster Resonance Energy Transfer (FRET)**

Protoplasts were cotransfected with 10 µg of plasmid carrying protein::CFP and 10 µg of protein::YFP constructs. As controls 2.5 µg of plasmid containing unfused CFP/YFP or 10 µg YFP:CFP fusion was used. Protoplasts expressing the fusion proteins were analyzed with a Leica DM IRBE confocal laser scanning microscope with a 63x water objective, digital zoom and 51% laser intensity. The fluorescence was visualized with an Argon laser for excitation at 457nm with 471-481nm emission filter for CFP and 514nm excitation with a 522-532nm filter for YFP. A transmitted light picture was used as reference. For FRET analysis Lambda scanning was performed by excitation at 457nm and by measuring emission from 468nm to 587nm in a total of 30, 5nm wide intervals using a RSP465 filter. Of every interval the intensity of the whole cell was quantified using ImageJ. The intensity of five protoplasts were averaged and normalized. The slopes between the 475nm and 527nm point were compared for differences in quenched donor emission and increased acceptor emission in comparison to the controls. Similar results were obtained for three independent transfections.

**ACKNOWLEDGEMENTS**

The authors would like to thank dr. Xinnian Dong for kindly providing Arabidopsis tga256 and tga2356 mutant seed, dr. Corné Pieterse for providing Arabidopsis npr1-1 mutant seed, Carlos Galvan-Ampudia for providing pART7CFP, pART7YFP and FRET control plasmids, Roy Baas for assistance with several transactivation experiments, and Gerda Lamers and Ward de Winter for their help with microscopy and tissue culture, respectively.