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ORIGINAL RESEARCH

Identification of Regulatory *cis*-elements Upstream of *AtNPR1* that are Responsive to Probenazole Treatment in Transgenic Tobacco Plants

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Abstract Probenazole (PBZ) is a highly effective chemical inducer of systemic acquired resistance (SAR). We found that the transcript level of *NPR1*, a key regulator of SAR, was significantly up-regulated upon PBZ treatment in *Arabidopsis*. To identify *cis*-elements involved in this response, a series of 5′ deleted fragments in the upstream region of *NPR1* were fused to the *GUS* gene, and the resultant constructs were then introduced into tobacco plants. We have shown that even the shortest of these fragments was able to drive the expression of *GUS* at a similar level to that of the largest fragment after PBZ treatment. Further mutation analysis within the fragment showed only when both of the two W-boxes present at -128 and -123 were mutated could the responsiveness to PBZ treatment be completely abolished. These results suggest that these two W-boxes are necessary for the full responsiveness of *AtNPR1* to PBZ treatment in tobacco plants. This requirement implies that one or more of WRKY transcription factors may play a key role in the positive regulation of PBZ-induced SAR, mediated by *AtNPR1*. Moreover, the characteristic cross-species responsiveness of the *AtNPR1* upstream region to PBZ treatment demonstrates that a conserved regulatory mechanism of PBZ-induced SAR may exist in diverse plant species.

Keywords *Arabidopsis thaliana* · Tobacco · NPR1 upstream fragment · GUS gene · *cis*-elements · Probenazole

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Abbreviations

CaMV	cauliflower mosaic virus
GUS	β-glucuronidase
MS	Murashige and Skoog
PBZ	probenazole
SA	salicylic acid

Introduction

Plants have developed a battery of active defense mechanisms to protect themselves from being attacked by different microbial pathogens. One common mechanism is the hypersensitive response, which is characterized by the formation of necrotic lesions at local sites of invasion that either kill or restrict the growth and spreading of the pathogens. After the formation of hypersensitive response lesions, another plant defense response, called the systemic acquired resistance (SAR), is activated throughout the whole plant (Ryals et al. 1996), which enables it to acquire resistance to a broad spectrum of pathogens.

SAR development in dicotyledonous plants such as tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* is mediated by salicylic acid (SA). Studies have shown that SAR is induced in several plant species by treatment with chemical inducers such as SA and its analogs, methyl-2,6-dichloroisonicotinic acid or benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (Gorlach et al. 1996; Lawton et al. 1996; Ward et al. 1991; White 1979). Probenazole (3-allyloxy-1, 2-genzisothiazole-1, 1-dioxide, or PBZ), however, is a distinct kind of SAR chemical inducer that, unlike methyl-2,6-dichloroisonicotinic acid or benzo (1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester, acts upstream of SA and induces its synthesis (Yoshioka et al.

2001). PBZ was initially developed by Meiji Seika Kaisha, Ltd. as a rice blast controlling agent; it shows only weak antimicrobial activity and is therefore a low toxic chemical inducer (Kessmann et al. 1994). It can also protect rice from other diseases, including bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (Shimura et al. 1981; Watanabe et al. 1979). More importantly, PBZ can prevent pathogen infection in several other plant species, such as *Arabidopsis* and tobacco (Koganezawa et al. 1998; Yoshioka et al. 2001).

Plant cells express a set of genes that are involved in defense reaction to protect themselves from being infected by different pathogens. In *Arabidopsis*, significant progress has been made in the isolation and characterization of key factors involved in the signal transduction of defense response. The NPR1/NIM1 protein, identified as a key regulator in the SA-dependent signal transduction pathway that regulates the expression of a set of defense-related genes, is an example of one such factor (Cao et al. 1994; Cao et al. 1997; Delaney et al. 1995). The expression of *AtNPR1* is very low under normal conditions, but it is induced to a significantly higher expression level by pathogen infection (Cao et al. 1997; Ryals et al. 1997). Transgenic plants overexpressing the *AtNPR1* gene exhibit enhanced resistance to a broad spectrum of microbial pathogens, whereas those plants underexpressing the *AtNPR1* gene are more susceptible to pathogen infection (Chern et al. 2005; Fitzgerald et al. 2004; Lin et al. 2004; Yuan et al. 2007).

In this study, we analyzed the transcriptomic changes in PBZ-treated *Arabidopsis* plants by microarray and consequently detected a significantly induced transcription level of *NPR1*. To elucidate the mechanism of the induced *AtNPR1* transcription, we made progressive deletions of its upstream region to locate the putative *cis*-elements that are responsive to PBZ treatment. By linking a series of upstream fragments to the *GUS* gene and then examining their responsiveness to PBZ treatment in transgenic tobacco plants, we identified two W-boxes that were required to mediate the response of the gene to PBZ treatment.

Materials and Methods

Plant Materials and Growth Conditions

Tobacco (*N. tabacum* L., cv. k326) seeds were grown in sterilized potting soil [v(peat soil)/v(vermiculite)/v(pearlite)= 3:9:0.5; Shanghai Institute of Landscape Science] in pots (6-cm diameter×9 cm) inside a growth chamber under a 16:8-h light/dark regimen at 22°C. The *A. thaliana* ecotype used was Columbia-0, and its growth conditions were identical to those of the tobacco plants.

Fusion of *AtNPR1* Upstream Fragment with *GUS* Gene

According to the sequence available from GenBank (accession no. At1g64280), a pair of primers (P1/P6) (Table 1) was initially designed to amplify a 1,228-bp fragment upstream of *AtNPR1*, from -1,228 to -1 (ATG, +1). This promoter fragment was inserted into the pGEM-T vector for sequencing and then subcloned into the *Pst*I and *Nco*I restriction sites of the binary vector plasmid pCAMBIA1301, to eventually replace the CaMV 35S promoter. The resultant construct was designated as -1,228/GUS. A series of primers (Table 1) were subsequently designed to amplify an additional four fragments that began at -936, -615, -479, and -252bp, using -1,228/GUS as the template. With a similar cloning strategy, these four promoter fragments were cloned into the plasmid pCAMBIA1301 to replace the CaMV 35S promoter. These four constructs were accordingly designated as -936/GUS, -615/GUS, -479/GUS, and -252/GUS. A negative control construct, named –GUS, was constructed by simply removing the CaMV 35S promoter of the plasmid pCAMBIA1301.

A two-step overlap extension polymerase chain reaction (PCR) method was used to obtain mutated promoter constructs. For construct -252a/GUS, we designed two primers (P7/P8) (Table 1) in which the original TTGAC sequence of the first W-box was changed to TTAAA. Two pairs of primers (P5/P7 and P8/P6) were used to obtain two PCR products, using construct -252/GUS as the template. These two PCR products were mixed and used as a new template to amplify the final mutated promoter fragment with another pair of primers (P5/P6). After verification by sequencing, this mutated promoter fragment was cloned into the plasmid pCAMBIA1301. Other mutated constructs were constructed in the same manner and were designated accordingly as -252b/GUS, -252c/GUS, -252d/GUS, and -252e/GUS. All primers used are listed in Table 1.

Plant Transformation

Plant expression constructs harboring the fusions of the *NPR1* promoter fragments and the *GUS* gene, as well as the control construct -GUS, were introduced into *Agrobacterium tumefaciens* LBA4404 by the electroporation method. *Agrobacterium*-mediated transformation of tobacco was conducted using the leaf disk infection method (Horsch et al. 1985). Primary transformants were allowed to self-fertilize, and T1 seeds were collected and germinated on MS solid medium with 40 μg/mL hygromycin. Transformants were also verified by PCR using primers specific for the *GUS* gene and the respective promoter sequences. Transgenic seedlings were then transferred to soil.

Table 1 Oligonucleotide primers used in constructing fragment::*GUS*

Construct name	Primer name	Primer sequence (5′-3′; underlined are restriction sites of *Pst*I and *Nco*I)
-1,228/GUS	P1	TACTGAATTCACTTAAAATCATACAAATCTTA
	P6	TGCTCCATGGCAACAGGTTCCGATGAATTGA
-936/GUS	P2	TACTGAATTCCACCAATCAAGTGAAGG
	P6	
-615/GUS	P3	TACTGAATTCTAAACATGCTGAACACTG
	P6	
-479/GUS	P4	TACTGAATTCTGTAACTTTCACATGTAA
	P6	
-252/GUS	P5	TACTGAATTCGCTTCCTGTTATATCTTT
	P6	
-252a/GUS	P7	GAGCAGAGCCAATTTAAGTCAACGAGAGTGGTGG
	P8	TTGACTTGGCTCTGCTCGTCAATGGTTATCTTCG
-252b/GUS	P9	GAGCAGAGCCAAGTCAATTTAACGAGAGTGGTGG
	P10	TTAAATTGGCTCTGCTCGTCAATGGTTATCTTCG
-252c/GUS	P11	GAGCAGAGCCAAGTCAAGTCAACGAGAGTGGTGG
	P12	TTGACTTGGCTCTGCTCTTTAATGGTTATCTTCG
-252d/GUS	P13	GAGCAGAGCCAATTTAATTTAACGAGAGTGGTGG
	P14	TTAAATTGGCTCTGCTCGTCAATGGTTATCTTCG
-252e/GUS	P15	GAGCAGAGCCAATTTAATTTAACGAGAGTGGTGG
	P16	TTAAATTGGCTCTGCTCTTTAATGGTTATCTTCG

PBZ Treatment of Plants

Two-week-old *Arabidopsis* or tobacco seedlings in pots were treated by both root drenching and leaf spraying with the agricultural chemical Oryzemate (Meijiseika), which contains 10% (wt/wt) PBZ, at 0.558 g/L. Three days after the treatment, the aerial parts of the plants were taken for GUS activity analysis or RNA extraction.

RNA Extraction and Real-time PCR Analysis

Total RNA was extracted from the leaves of control and PBZ-treated *Arabidopsis* or tobacco transgenic lines using Trizol Reagent (Invitrogen, Carlsbad, CA), followed by chloroform extraction and isopropanol precipitation. The RNA sample was digested with RNase-free DNase (Promega, Madison, WI) and then quantified with a spectrophotometer. Five micrograms of total RNA was reverse transcribed with Superscript reverse transcriptase (Stratagene, La Jolla, CA). The product was subsequently used as the template for real-time PCR analysis. The real-time PCR was performed using SYBR Green I (TOYOBO Co., Osaka, Japan) on an iCycler (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. We used *AtACT2* in *Arabidopsis* and *Nt-ACT9* in tobacco as reference genes. The specific primers for these genes were as follows: *AtACT2* (forward: 5′-GGCTCCTCTTAACCCAAAGGC-3′ and reverse: 5′-CACACCATCACCAGAATCCAG-3′), *Nt-ACT9* (forward: 5′-CTATTCTCCGCTTTGGACT-TGGCA-3′ and reverse: 5′-AGGACCTCAGGACAACGGAAACG-3′) (Thangavelu et al. 1993), *AtNPR1* (forward: 5′-CAACTTTGGAAGGTAGAACCGCACT-3′ and reverse: 5′-GCCACTGC-AAAAGAGGGAGGAACA-3′), and *GUS* (forward: 5′-CGGTCAGTGGCAGTGAAGGG-3′ and reverse: 5′-CGAGGTACGGTAGGAGTTGG-3′).

Quantitative Determination of GUS Activity

β-Glucuronidase activity was determined using a fluorometric assay. Leaf disks were first homogenized in 0.5 mL lysis buffer and then centrifuged for 10 min at 4°C, and the GUS activity of the supernatant was measured as described by Jefferson et al. (1987). The final GUS activity was normalized to the protein concentration for each crude extraction and calculated as the pmol of 4-methyl umbelliferone (4-MU) produced per minute per milligram of soluble protein. The amount of protein was determined by the BCA (Bicinchoninic Acid) method, using BSA (Bovine Serum Albumin) as a standard.

Results

Putative *cis*-elements Upstream of *AtNPR1*

The transcriptional start site for *AtNPR1* has been shown to be located at position 199 upstream of the translational start site (Ryals et al. 1997; Fig. 1.). We analyzed the upstream

region of *AtNPR1* using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al. 2002). The analysis revealed a putative TATA box residing at -239-bp upstream of the translational start site. In addition, several putative *cis*-acting regulatory elements were found in this region. Two WUN-like-motifs, responsible for wound-responsiveness (Pastuglia et al. 1997), were observed at -562 and –596. Three typical W-boxes (TTGAC), the binding site of the WRKY transcription factor for the fungal elicitor response (Rushton et al. 1996), were found at -106, -123, and -128, respectively. A sequence of CAGGAAAGGA, similar to the TCA element that was found to be responsive to SA treatment in tobacco (Goldsbrough et al. 1993), was found at -210. An MBS (MYB binding site) sequence (CAACTG), the binding site of the MYB (v-myb avian myeloblastosis viral oncogene homolog) transcription factor for drought responsiveness in *Arabidopsis*, was found at -74 (Uno et al. 2000). An ethylene-response element (ERE)-like motif (ATTTAAAA) at -448 shared a high homology with an ethylene-responsive element (Itzhaki et al. 1994). A similar gibberellin-responsive element (GARE) was found at -488 (Sutliff et al. 1993). In addition, many other *cis*-elements involved in light responsiveness, such as G-box and I-box, were also found throughout the promoter sequence. Most of the abovementioned motifs are involved in the signal transduction of environmental stresses, suggesting that the *AtNPR1* gene may be regulated by diverse plant stress signals.

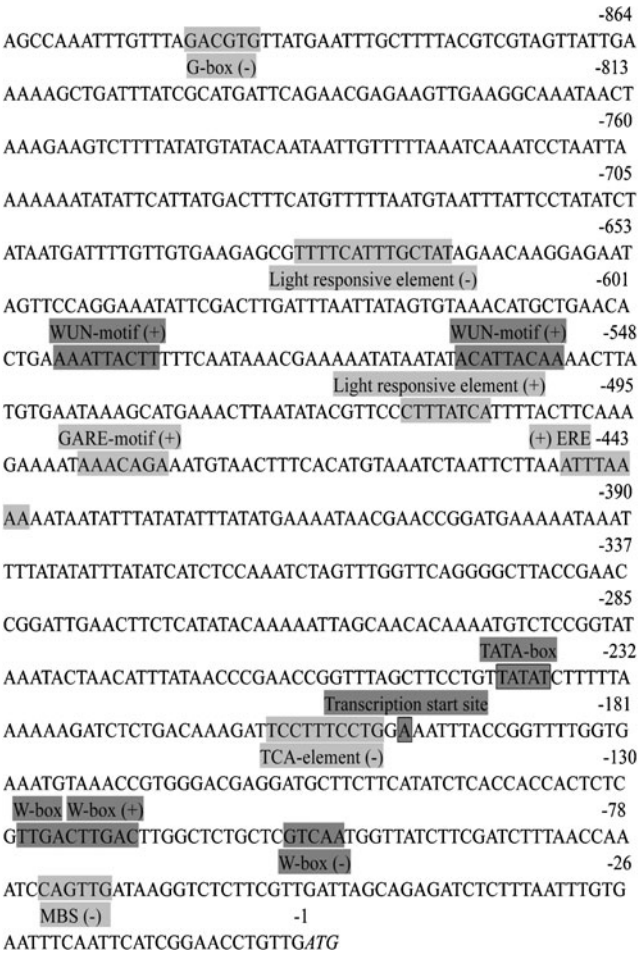

Fig. 1 Predicted 5′-upstream *cis*-acting regulatory elements of *AtNPR1*. Numbering is relative to the first base of the ATG codon, which is shown in *italics*. The transcription start site and the putative TATA-box are indicated with a *dark border*. The predicted regulatory *cis*-elements are shaded in *gray*, and their orientations are shown in *parentheses*

PBZ-induced Expression of *AtNPR1*

Preliminary experiments showed that applying overconcentrated PBZ (higher than 2 mM) could cause obvious damage to *Arabidopsis* leaves. We used real-time PCR to optimize the applied PBZ concentration and found that 0.1 or 0.2 mM PBZ was sufficient to induce *AtNPR1* expression without producing any obvious damage to plants (Fig. 2a). To secure the inducibility, 0.2 mM was used for all of the following analyses unless indicated otherwise.

In a microarray analysis, we found that the expression level of *AtNPR1* in *Arabidopsis* plants increased about fourfold after PBZ treatment (data not shown). Here, we used real-time PCR to further determine its expression dynamics during a continuous PBZ treatment (Fig. 2b). We found that *AtNPR1* mRNA was significantly induced at day 2, reached the highest level at day 3, and then gradually declined to about 2.5-fold at day 8, comparable with the level seen at day 2.

Construction of Responsiveness Analysis Vectors with *AtNPR1* Upstream Fragments

To analyze the responsiveness of *AtNPR1* upstream fragments to PBZ treatment, we first amplified five fragments of a 5′ deletion series of the upstream region, which represented the upstream 1,228, 936, 615, 479, and 252 bp. These fragments, upstream of the ATG codon, were amplified by PCR and then inserted into the pGEM-T vector for sequencing. They were finally subcloned into the pCAMBIA 1301 vector to replace the CaMV 35S promoter (Fig. 3).

Stable Responsiveness of the Upstream Fragments to PBZ Treatment in Transgenic Tobacco Plants

To further define the *cis*-elements required for the regulation of *AtNPR1* expression in response to PBZ treatment, a series of fragments that were amplified from the *AtNPR1* upstream region and fused to the *GUS* reporter gene were then introduced into tobacco plants. For each construct, 14

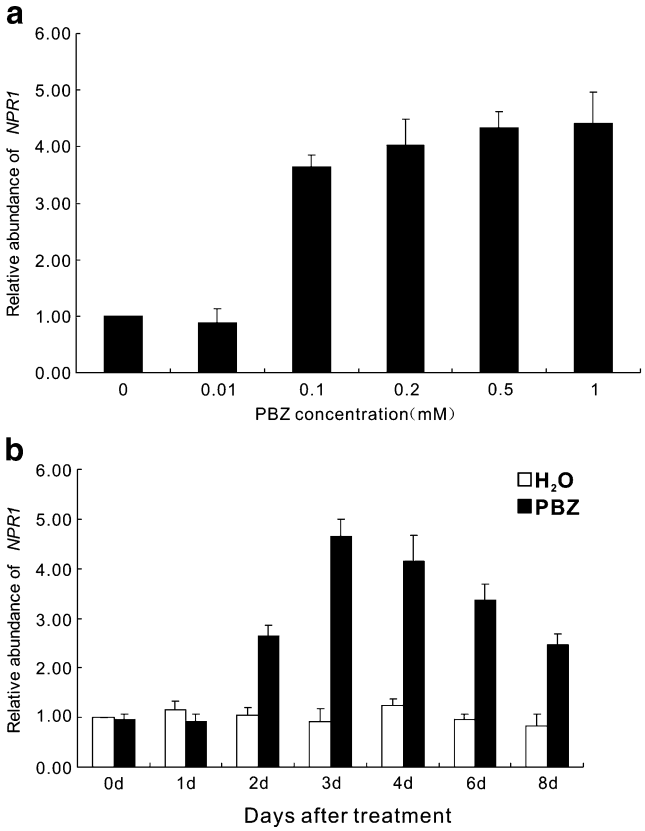

Fig. 2 Induction of *NPR1* expression by PBZ treatment in *Arabidopsis*. **a** *Arabidopsis* plants (ecotype Columbia-0) were treated with PBZ, and leaves were harvested at 3 days posttreatment with the indicated concentrations of PBZ. **b** Time-course analysis of *NPR1* expression in leaves treated with either H_2O or PBZ. The treated leaves were harvested at the indicated days (d). The transcript levels were quantified by real-time reverse transcription (RT) PCR with *ACTIN2* (*ACT2*) as the reference. The expression level of the *NPR1* gene in plants with water treatment for zero days was normalized to one. The values shown are the means of three replicates. The error bars indicate the SD. The experiment was repeated once, with similar results

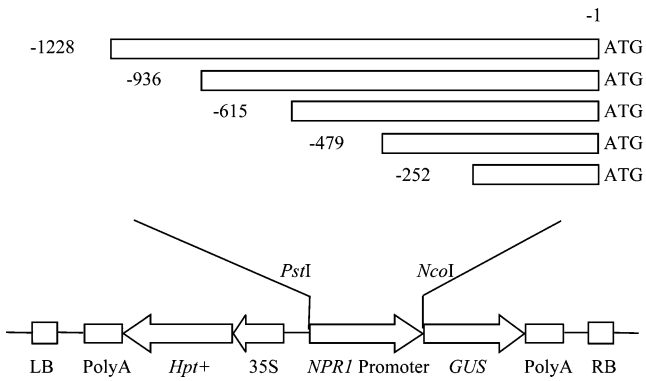

Fig. 3 Schematic map of the pCAMBIA 1301 binary vector and the *NPR1* promoter constructs that were used for plant transformation. Numbering is relative to the first base of the translation start codon of *NPR1*. The indicated *Pst*I and *Nco*I sites were used for cloning into the pCAMBIA 1301 vector. *Hpt*+, hygromycin phosphotransferase; *GUS*, β-glucuronidase gene; 35S, CaMV 35S promoter; LB and RB, the left and right borders of the T-DNA

to 18 independent transgenic lines were identified and self-fertilized. To eliminate the effect of gene copy number on GUS activity, only single-copy insertion T1 progeny of primary transformants, determined by a statistical test on the Hpt^R segregation, were used for further analysis.

For each construct, T1 plants from three to five independent primary transformants were tested for *GUS* responsiveness to PBZ treatment. GUS activity was measured in the leaf tissues of 4-week-old plants, taken after root irrigation for 3 days with water or 0.2 mM PBZ solution. Figure 4 shows the GUS activities that were detected in leaf tissues from plants carrying the different *AtNPR1* upstream fragments. Under normal conditions, transgenic plants containing all five constructs showed a low-basal GUS activity. Upon PBZ treatment, however, a remarkable fourfold to fivefold increase in the GUS activity was consistently detected in the transgenic plants. No significant change in the GUS activity was observed in the negative control transgenic plants, carrying the construct –GUS, after treatment with PBZ. These results indicated that even the shortest fragment of 252 bp was sufficient to promote a response to PBZ treatment, implying that the positive regulatory element(s) should be located within this defined region.

Identification of Specific *cis*-elements Required for Response to PBZ Induction

As the putative TATA-box is located at 239 bp upstream of the translational start site, it was impractical to use the 5′ deletion strategy to further define *cis*-acting elements within the 252-bp upstream fragment. According to a prediction analysis, there were only two kinds of putative *cis*-elements in this upstream region: a putative MYB binding site, which was found to be responsive to drought stress (Uno et al.

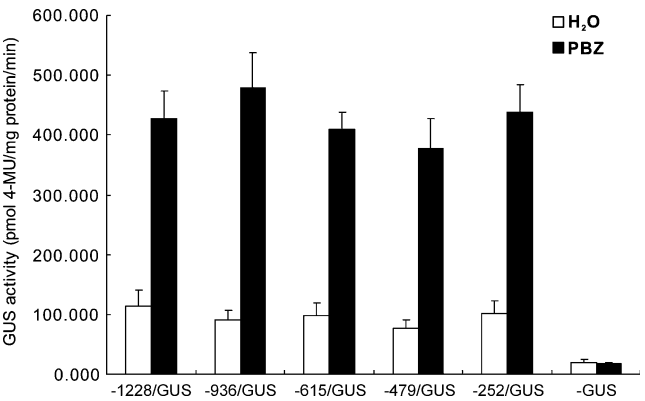

Fig. 4 Effects of 5′ deletion on the PBZ-induced expression of *NPR1* promoter-*GUS* chimeric genes in transgenic tobacco plants. GUS activity was measured in the leaves of T1 progeny for -1,228/GUS, -936/GUS, -615/GUS, -479/GUS, and -252/GUS 3 days after treatment with either PBZ (*dark bars*) or water (*open bars*). The values shown are the means ± SD from three to five independent transgenic lines

2000) and three TTGAC W-box elements within a 28-bp region from position 106 to position 128 upstream of the translational start site (Fig. 5a). One of these three W-box sequences, located at position 106 upstream of the translational start site, contains a reverse TTGACG sequence that is different from the typical W-box sequence (C/TTGACC/T) (Eulgem et al. 2000). Therefore, it is a possible W-box or a TGA factor binding site that has a core sequence (TGACG) (Jakoby et al. 2002). To determine whether these three W-box elements play an important role in the responsiveness of the 252-bp fragment to PBZ treatment, we introduced mutations (TTGAC to TTAAA) into these three W-boxes, sequentially or in combination, as indicated in Fig. 5a, and then linked the resultant fragments to the *GUS* gene and analyzed their responsiveness to PBZ treatment in transgenic tobacco plants, as had been done with the previous five constructs. The fused constructs were as follows: -252a, -252b, and -252c, with a mutation in the first, second, and third W-box, respectively, located at positions -128, -123, and -106; -252d, with mutations in both the first and the second W-boxes; and -252e, with mutations in all the three W-boxes.

The five constructs carrying mutated W-box sequences were introduced into tobacco plants to analyze their responsiveness to PBZ treatment. The construct containing three normal W-boxes was used as a positive control and was called as -252. GUS activity was measured in the same manner as that described above. Figure 5b shows the average GUS activities in leaf tissues from plants carrying the constructs with a mutated W-box(es). As expected, the control transgenic plant, containing construct -252, showed a significant responsiveness to the PBZ treatment, with an average threefold to fourfold increase in GUS activity. A similar response was observed with the construct -252c. In sharp contrast, the transgenic plants containing constructs -252d or -252e did not show any obvious responses to PBZ treatment. This observation implies that the first and/or second W-box(es) might be responsible for responsiveness to the PBZ treatment. In transgenic plants carrying either the -252a or -252b construct, a significantly lower response, as compared with that in the control transgenic plant, was detected. However, the response was still dramatically higher than those in the plants containing either -252d or -252e, indicating that both of the W-boxes were responsible for the responsiveness. Additionally, it was noticed that water-treated (and PBZ-treated) transgenic plants that were transformed with the -252d or -252e construct showed a dramatic decrease in the basal level of GUS activity, when compared with the water-treated control transgenic plants.

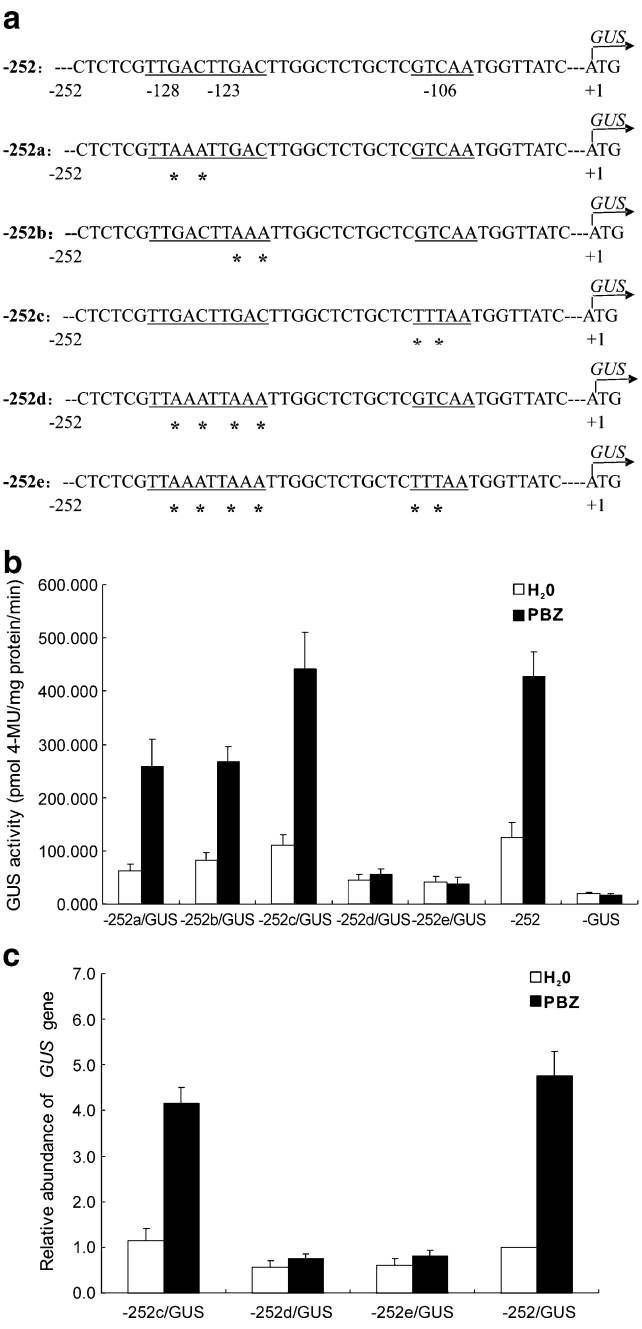

Fig. 5 Importance of two W-box elements for the responsiveness of the *NPR1* promoter to PBZ treatment in tobacco. **a** The constructs -252a/GUS, -252b/GUS, -252c/GUS, -252d/GUS, and -252e/GUS. W-box sequences are *underlined*, and *asterisks* indicate the mutated bases in the W-box sequences. Numbering is relative to the first base of the translation start codon of *NPR1*. **b** GUS activity was measured in leaves of T1 progeny for -252/GUS, -252a/GUS, -252b/GUS, -252c/GUS, -252d/GUS, and -252e/GUS 3 days after treatment with either PBZ (*dark bars*) or water (*open bars*). The values shown are the means ± SD from three to five independent transgenic lines. **c** Chimeric *GUS* gene expression in transgenic tobacco plants carrying construct -252/GUS, -252c/GUS, -252d/GUS, or -252e/GUS 3 days after treatment with either PBZ (*dark bars*) or water (*open bars*). The transcript levels were quantified by real-time reverse transcription (RT) PCR with Nt-ACT9 as the reference. The expression level of the *GUS* gene in plants containing -252/GUS with the water treatment was normalized to one. The values shown are the means ± SD from three independent transgenic lines for each construct

This dramatic decrease may be explained by the disturbance incurred by the point mutations introduced into the 5′-untranslated region of *AtNPR1*.

To verify if the GUS activity is an authentic reflection of the *GUS* transcript level, we used real-time PCR to quantify *GUS* transcript abundance in water- or PBZ-treated transgenic plants. Figure 5c shows that the *GUS* transcript levels in transgenic plants containing construct -252d or -252e were not obviously responsive to PBZ treatment, whereas there was a dramatic increase (fourfold to fivefold) in transgenic plants carrying constructs -252c and -252 after PBZ treatment. These kinetic changes were consistent with those of the GUS activities. Therefore, the results described above clearly indicated that the first and second W-box elements, located at positions -123 and -128 in the upstream region, were essential for its full responsiveness to PBZ treatment in transgenic tobacco plants. The third W-box, however, was not necessary for this response.

PBZ-induced Transcription of WRKY Genes in *Arabidopsis*

Many W-box *cis*-elements have been found to be recognized by different WRKY DNA-binding proteins (Eulgem et al. 2000). Our results suggest a potential role of WRKY proteins in the responsive step of the *AtNPR1* gene to PBZ treatment in transgenic tobacco plants. So far, there have been no reports addressing the relationship between PBZ and WRKY proteins in *Arabidopsis*. By using an expression microarray, we analyzed the global gene expression pattern in PBZ-treated *Arabidopsis* plants. About 15 WRKY family genes were found to be up-regulated at least two-fold after PBZ treatment for 72 h. Figure 6 lists the names of these genes and the fold changes that were found in two independent experiments.

Gene ID	Annotation	Fold Change (Log2) 72h-1 VS 0h-1	Fold Change (Log2) 72h-2 VS 0h-2
AT2G23320	WRKY15	1.6	1
AT4G31800	WRKY18	3.2	2
AT2G30250	WRKY25	2.2	1.7
AT5G24110	WRKY30	5.9	4.5
AT2G38470	WRKY33	2.5	1.3
AT5G22570	WRKY38	7.1	5.8
AT3G04670	WRKY39	1.9	1.4
AT1G80840	WRKY40	3.9	2.7
AT2G46400	WRKY46	4.2	3
AT4G01720	WRKY47	1.2	1.5
AT4G23810	WRKY53	5.3	3
AT2G40750	WRKY54	8	6.3
AT2G40740	WRKY55	1.7	2.9
AT3G01080	WRKY58	3.9	1.6
AT2G25000	WRKY60	2.7	3.2

Fig. 6 PBZ-induced WRKY genes in *Arabidopsis*. A list of PBZ-induced WRKY genes that were identified in the microarray experiments. Data from two independent replicates are presented. 72 h-1 versus 0 h-1 and 72 h-2 versus 0 h-2 represent two independent experiments in which RNA samples from *Arabidopsis* plants treated with PBZ for 72 h were compared with RNA samples from plants treated with PBZ for 0 h. Note that the fold change values are log base 2 transformed. The twofold difference in the expression level between the 72-h and 0-h samples was set as the threshold for considering a gene to be PBZ-inducible

Discussion

Studies have shown that PBZ treatment induces resistance to a broad spectrum of pathogens in many plant species (Shimura et al. 1981; Watanabe et al. 1979). Several genes from rice have been shown to be induced after treatment with PBZ (Midoh and Iwata 1996; Minami and Ando 1994; Sakamoto et al. 1999). In our experiment, we found that the transcript level of *AtNPR1* could be significantly induced by PBZ treatment in *Arabidopsis*. To further explore the general mechanism of PBZ-induced resistance, the responsiveness of *AtNPR1* to PBZ treatment was analyzed ectopically in diverse plant species. In transgenic tobacco plants, a significant responsiveness, in terms of GUS activity, was detected with all five constructs after a 3-day PBZ treatment (Fig. 4). These results, together with the observation that PBZ-induced transient *GUS* expression was driven by the 1,228-bp upstream fragment in onion epidermal cells (data not shown), indicate that the examined upstream fragments, even the shortest 252-bp fragment, contained the major element(s) that are sufficient for PBZ treatment responsive expression of *NPR1* across species. In addition, a low-background GUS activity was consistently detected with each of the five constructs (Fig. 4). This observation is in agreement with the previously reported low-background expression of the endogenous *AtNPR1* in *Arabidopsis* (Cao et al. 1997; Ryals et al. 1997). Considering the consistency of the across-species responsiveness, as well as the higher transformation efficiency and larger leaves of tobacco, we carried out all subsequent transgenic analyses in tobacco for convenience.

Bioinformatic analysis indicated that there were three W-box elements and one MYB binding site in the 252-bp fragment (Fig. 1). To determine whether the W-box elements were necessary and/or sufficient for the responsiveness of *AtNPR1* to PBZ treatment, we inserted mutations into the sites in transgenic tobacco plants and analyzed their responsiveness to PBZ treatment. The results showed that the two W-box elements located at positions -123 and -128 were indispensable for responsiveness to PBZ treatment in transgenic tobacco plants, based on the measurements of both GUS activity and *GUS* transcription (Fig. 5). It has been reported that the two W-box elements were essential for the inducible expression of *AtNPR1* upon

SA treatment in *Arabidopsis* (Yu et al. 2001). A related study indicated that 1,2-benzisothiazol-3 (2*H*)-one 1,1-dioxide (BIT), an active metabolite of PBZ (Uchiyama et al. 1973), could increase the total SA content (free SA and SA glucoside, SAG) in *Arabidopsis*, although it did not change the level of free SA (Yoshioka et al. 2001). We also measured the free SA content of PBZ-treated *Arabidopsis* plants and found that PBZ treatment could significantly increase the levels of both free SA and SAG in leaf tissues (data not shown). Collectively, these results indicate that PBZ might up-regulate *NPR1* expression by increasing the level of free endogenous SA in *Arabidopsis*, and the key components involved in the regulatory pathway are most likely conserved in tobacco and other plant species, such as onion.

Plant WRKY proteins are plant-specific transcription factors that frequently exert regulatory roles in the plant response to pathogen attacks by binding to promoter regions that contain the consensus sequence TTGAC (W-box elements), which are present upstream of many different defense-related genes (Eulgem et al. 2000). In a microarray analysis, we found that 15 WRKY family genes were up-regulated by PBZ treatment in *Arabidopsis* (Fig. 6). Of the 15 WRKY genes, AtWRKY15, AtWRKY18. and AtWRKY53 were also found to be induced in SA-treated *Arabidopsis* plants, and a gel retardation assay demonstrated that the purified recombinant AtWRKY18 protein could bind to the three W-box sequences in the *AtNPR1* promoter region (Yu et al. 2001). However, there is currently no in vivo data to confirm the role of AtWRKY18 in regulating *AtNPR1*. Nevertheless, we assume that one or more of the 15 up-regulated WRKY genes may play an important role in the positive regulation of PBZ-induced disease resistance that is mediated by *AtNPR1*.

Earlier studies have demonstrated that *cis*-regulatory elements located in both the 5′- and 3′-untranslated region (UTR) can enhance or regulate gene expression in plant and animals genes. For instance, Gallo-Meagher et al. (1992) found that sequences located within the transcribed region of the pea *Fed-1* gene (encoding ferredoxin I) seemed to contribute to its light-regulated mRNA accumulation in transgenic tobacco seedlings (Gallo-Meagher et al. 1992). A sequence motif, ACAAAA, in the 5′UTR of the *Arabidopsis Fed*-A gene was found to contribute quantitatively to its full expression (Caspar and Quail 1993). Recently, Karthikeyan et al. (2009) reported that the 5′UTR of the *AtPht1;4* gene is essential for its expression in root tips and enhances the level of its expression in roots during Pi starvation (Karthikeyan et al. 2009). Cumulative evidence has suggested that sequence-specific *cis*-regulatory elements located within the UTRs or even the coding regions of transcripts can interact with *trans*-acting factors to modulate gene expression (Bolle et al. 1994; Kertesz et al. 2006; Zhang and Mehdy 1994). In this study, two identified W-box elements that are responsive to PBZ treatment also locate within the 5′UTR of *AtNPR1*, according to the likely transcriptional start site of *AtNPR1* as determined by Ryals et al. Specific WRKY proteins induced by PBZ treatment may bind to these two W-box elements to regulate *AtNPR1* gene expression by enhancing the transcriptional efficiency or the post-transcriptional stabilization of the mRNA (Gutierrez et al. 1999). Further analysis is needed to clarify how these *cis*-elements are involved in the positive regulation of *AtNPR1* expression in response to PBZ treatment.

The progress made in this study will certainly facilitate the further exploration of the mechanism of PBZ-induced SAR, particularly the identification of the transcription factor(s) upstream of *AtNPR1*. Additionally, the W-box elements can be exploited to construct chemical-inducible foreign gene expression systems in combination with the application of PBZ, an effective SAR inducer with low toxicity. In these systems, PBZ can play dual beneficial roles by both triggering SAR and inducing foreign gene expression.

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