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ORIGINAL ARTICLE

Isolation and characterization of the rice *NPR1* promoter

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Abstract NPR1 is a positive regulator of systemic acquired resistance in *Arabidopsis* and rice. Expression of the rice gene *OsNPR1* is induced by salicylic acid (SA). To identify the region of the *OsNPR1* promoter involved in response to SA, we carried out deletion mutagenesis of the region 1005 bp upstream of the *OsNPR1* start codon. *Cis*-element analysis revealed that the *OsNPR1* promoter contains W-boxes and ASF1 motifs, both of which are known to be functional *cis*-elements of the WRKY and bZIP proteins, respectively. The deletion constructs 1005:LUC and 752:LUC, were induced by up to 4.3- and 3.8-fold, respectively, following SA treatment, suggesting that W-boxes and ASF1 motifs may play an important role in the strong induction of these constructs by SA. Using mutation analysis, we also showed that both the W-box and ASF1 motif are necessary for SA-induced expression of *OsNPR1*.

Keywords *cis*-Elements · *OsNPR1* promoter · Salicylic acid · WRKY transcription factor

Abbreviations

JA	Jasmonic acid
SA	Salicylic acid
SAR	Systemic acquired resistance
Xoo	*Xanthomonas oryzae* pv. *oryzae*

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Introduction

Salicylic acid (SA) induces systemic acquired resistance (SAR) in plants, a process that entails the massive activation of defense-related genes and confers broad-spectrum resistance to a variety of pathogens. NPR1 is a key regulator in the SAR signal transduction pathway. Upon SA treatment, NPR1 translocates to the nucleus where it interacts with TGA factors, thereby enhancing the DNA-binding activity of several members of the TGA family to cognate *cis*-elements in promoters of defense-related genes (Zhang et al. 1999; Despres et al. 2000; Kinkema et al. 2000; Niggeweg et al. 2000; Zhou et al. 2000; Yu et al. 2001). The interaction between NPR1 and transcription factors has been shown to be required for the binding activity of these factors to the promoter of SA-mediated pathogenesis-related (PR) genes (Despres et al. 2000; Yu et al. 2001; Fan and Dong 2002).

For the NPR1-mediated activation of the plant defense response, WRKY proteins act upstream of NPR1 and positively regulate its expression during the activation of the plant defense response (Yu et al. 2001). Expression of *NPR1* itself is regulated by WRKY proteins in *Arabidopsis* (Yu et al. 2001). More recently, the expression of several WRKY genes has been shown to be regulated by NPR1 in *Arabidopsis* (Wang et al. 2006), with some WRKY proteins acting upstream of *NPR1* and others acting downstream. Most plant promoters induced by pathogens or SA contain a W-box, GCC box, RAV1 AAT motif, or ASF1 motif (Li et al. 2005; Lee and Hwang 2006; Sohn et al. 2006). The *cis*-elements involved have been identified by serial deletion of the promoters and site-directed mutagenesis of their likely active sites. The W-box [(T)TGACC/T] and the W-box-like element [(T)TGACA] are enriched in the PR1 regulon promoter (Maleck et al. 2000). In a

recently published study, we reported that the W-box-like element 1 (WLE1: TGACA) containing the core sequence TGAC is important in the expression of *OsPR10a* in response to SA (Hwang et al. 2008). The interaction of transcription factors and *cis*-acting elements constitute a key step in defense signaling.

There is very limited information on defense signaling, including SAR. The NPR1-mediated pathway is known to be present in rice (Chern et al. 2001). The *Os*TGA transcription factors interact with *OsNPR1*, as has been reported in *Arabidopsis* (Chern et al. 2001; Yu et al. 2001), and *Os*WRKY12 and *Os*WRKY13 act upstream of *OsNPR1* (Liu et al. 2005; Qiu et al. 2007). However, there has been no report of an in-depth analysis of the regulation of the PR1 promoter in *Arabidopsis* or rice.

In the study reported here, we analyzed the expression profile of *OsNPR1* and then isolated its promoter. We also identified the *cis*-elements of the NPR1 promoter responsible for the SA-mediated response and verified the identity of these elements by series deletion of the promoter and site-directed mutagenesis of candidate *cis* elements.

Materials and methods

Plant materials and treatments

Plant materials were obtained and treatments carried out as previously described (Hwang et al. 2008). Three-week-old rice seedlings were treated with 1 mM SA and 100 μM jasmonic acid and infected with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Samples were taken at pre-determined times.

Reverse transcription-PCR analysis

Leaf samples and total RNA were prepared for reverse transcription (RT)-PCR as described in Hwang et al. (2008). PCR cycling consisted of 30 cycles at 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 7 min. For the RT-PCR analysis of the *OsNPR1* and *OsActin* in rice, we used primers *OsNPR1* (AK065952; 5′-CCCACAATGCAAAACAG GAGGTTG-3′ and 5′-TGGACTTAACTACTGATATTA CCA-3′) and *OsActin* (XM469569; 5′-TCCATCTTGG CATCTCTCAG-3′ and 5′-GTACCCGCATCAGGCATC TG-3′).

Isolation of OsNPR1 promoter

Based on an annotation of the rice genome, a 1005-bp fragment of the *OsNPR1* promoter was amplified by PCR from rice genomic DNA using the *OsNPR1*-specific primer set. These primers were designed from the genomic clone (accession number AC135792). PCR cycling consisted of 30 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 7 min. The primer sets were 5′-AAAAAGCAGGCTGGGGAGTA TAGTCTTTCACC-3′ and 5′-AGAAAGCTGGGTTGGCG AGGAGAGTCTAGAA-3′, with the underlined sequences matching the attB1 and attB2 sites in the Gateway cloning system (Invitrogen, Carlsbad, CA). Using BP clonase (Invitrogen, Carlsbad, CA), we cloned a 1005-bp PCR product into vector pDONR221 to construct an entry clone; successful insertion was confirmed by sequencing.

Promoter–luciferase (LUC) constructs

Reporter constructs for use in the transient expression assays were constructed. The reporter construct 1005:LUC was created by the LR reaction with the 1005-bp entry clone and promoter destination vector (Hwang et al. 2008). For deletion analysis of the *OsNPR1* promoter, fragments containing a series of 5′ deletions within the region −1005 to −657 bp upstream of the *OsNPR1* start codon were created by PCR using a specific primer set. The sense primers for each deletion construct were −878 (5′-AAAAAGCAGGCTGTACCACTACACCACCTCAC-3′), −752 (5′-AAAAAGCAGGCTCAGAAGAGACGACGAC TCTT-3′) and −657 (5′-AAAAAGCAGGCTGTTCTTG TTTCTTTCCCATC-3′).

We used the antisense primer 5′-AGAAAGC TGGGTTGGCGAGGAGAGTCTAGAA-3′. PCR cycling was performed for 30 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 7 min.

To make the deletion constructs (878:LUC, 752:LUC, and 657:LUC), we amplified promoter fragments by the PCR using the primers described above and then cloned these into the promoter destination vector for the transient expression assays according to the protocol described in Hwang et al. (2008).

The mutagenized reporter constructs used in this study were prepared according to the manufacturer's instructions (Stratagene, La Jolla, CA) and used following the protocol described by Hwang et al. (2008). Primers containing mutations in selected sequences were used to make mutagenized reporter constructs (m1005:LUC and m752:LUC). For the PCR analysis of the mutant strand synthesis reaction, we used the following mutagenic primer pairs: 5′-TTCAATTCAAGGTCGTCGCCTCCC-3′ and 5′-TTTGGGAGGGGTGAGCTGTCGT-3′ for the mutated 1005-bp promoter; 5′-TTCACCCCCGTGAAGTGGGTT GCC-3′ and 5′-GTCGTCGGCGGAAGAATCGTCG-3′ for the mutated 752-bp promoter. Successful insertions were confirmed by sequencing.

Particle bombardment and transient expression assays

Particle bombardment was performed in a Biolistic PDS-1000/He particle delivery system using 1100-p.s.i. rupture disks (BioRad, Hercules, CA). Plasmid DNAs containing promoter–LUC constructs or particle bombardment were prepared according to the manufacturer's instructions. Construct 35S:RLUC was used as an internal control to normalize LUC activities of the samples after bombardment. The procedures followed were as described in Hwang et al. (2008). The luciferase activity of protein extracts were measured using a dual luciferase system (Promega, Madison, WI) and a luminometer (Aureon Biosystems, Vienna, Austria).

Results

Expression pattern of *OsNPR1* in response to various stimuli

Arabidopsis plants subjected to pathogen infection or treatment with SA or JA show a two- to threefold increase in *NPR1* expression compared to healthy uninfected plants (Cao et al. 1997; Ryals et al. 1997). We investigated the possibility that *OsNPR1* expression is also induced by exposure to a pathogen or to SA or JA and found that *OsNPR1* is indeed induced in rice seedlings following infection with *Xoo* and exposure to the pathogen elicitors SA and JA (Fig. 1). The transcript level of *OsNPR1* had increased 6 h after SA or JA treatment or post-inoculation (hpi) with *Xoo* (Fig. 1). These results are in agreement with those reported for *NPR1* in *Arabidopsis* (Cao et al. 1997).

The OsNPR1 promoter is induced by SA treatment

To investigate the mechanism underlying the transcriptional regulation of *OsNPR1* by SA, the *OsNPR1* promoter was amplified by PCR using primers derived from its genomic sequences and confirmed by sequencing. To determine whether the 1.0-kb *OsNPR1* promoter was involved in the SA-induced increase in *OsNPR1* expression seen in treated seedlings, we carried out a transient reporter assay following particle bombardment. The 1.0-kb fragment of the *OsNPR1* promoter was used to make a reporter construct (*OsNPR1:LUC*) (Fig. 2a). The *OsNPR1*:*LUC* construct was introduced into rice leaves by particle bombardment, followed by the treatment of leaf segments with either buffer or SA. Protein extracts were prepared from samples at 24 h post-treatment, and their relative LUC activities were measured. *OsNPR1* promoter activity was expressed as LUC activity relative to buffer-treated control samples (Fig. 2b). The relative luciferase activity in the SA-treated sample was approximately 4.3-fold higher than that in control samples. These findings clearly demonstrate that the *OsNPR1* promoter was induced by SA, as expected based on the increase in expression seen in treated rice seedlings.

Analysis of *cis*-element of OsNPR1 promoter

In order to identify the *cis*-acting elements of the *OsNPR1* promoter involved in the response to SA, we analyzed the activity of *cis*-elements using PLACE, a database for

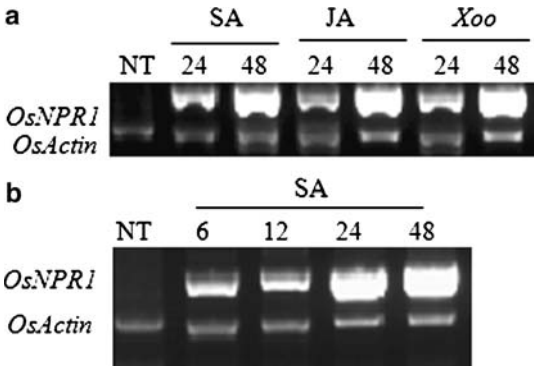

Fig. 1 Expression pattern of *OsNPR1* in rice leaves treated with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and biotic elicitors. **a** Rice seedlings were infected with *Xoo* or treated with salicylic acid (*SA*) and jasmonic acid (*JA*), and *OsNPR1* expression was assayed by PCR at 24 and 48 h post-treatment. **b** Rice seedlings were treated with SA and harvested at 0 (*NT*), 6, 12, 24, and 48 h. Reverse transcriptase-PCR was performed using the *OsNPR1*-specific primer pair. Transcript levels of *OsActin* show that equal amounts of RNA were used in the RT-PCR samples

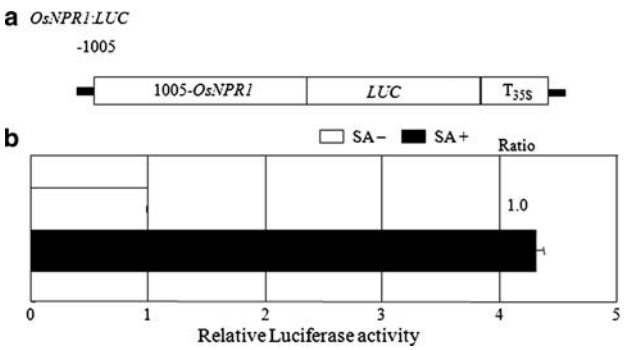

Fig. 2 *OsNPR1* promoter activity in response to SA. **a** Schematic representation of the *OsNPR1* promoter in the 1005:LUC reporter construct. **b** Results of the transient assay to measure the promoter activity of the 1005:LUC in response to SA. Relative luciferase activity is the ratio of the value obtained with the SA-treated 1005:LUC divided by the value obtained with the buffer-treated 1005:LUC construct. Values represent the mean plus/minus the standard deviation (SD) of triplicate measurements. *LUC* Luciferase

PLAnt *Cis*-acting Elements (http://www.dna.affrc.go.jp/cDNA/place) (Fig. 3). The 1.0-kb promoter region upstream of the *OsNPR1* start codon contains various putative *cis*-elements. We analyzed only *cis*-elements in boxes known to be related to defense signaling (Shinshi et al. 1995; Eulgem et al. 1999; Kagaya et al. 1999; Sohn et al. 2006) (Fig. 3). The *OsNPR1* promoter contains two W-boxes and one W-box-like element 1 (WLE1) (Hwang et al. 2008); there are also three ASF1-motif elements and two RAV1 AAT elements (Fig. 3). The W-box, ASF1 motif, and the RAV1 AAT sites are known to be *cis*-acting elements of the WRKY, bZIP, and RAV1 proteins, respectively (Abe et al. 1997; Chen and Chen 2002; Yamamoto et al. 2004). It is therefore possible that the WRKY, bZIP, RAV1 proteins are involved in the response of the *OsNPR1* promoter to SA.

Deletion analysis of the *OsNPR1* promoter to identify the regions responsible for induction by SA

To identify the regions of the *OsNPR1* promoter active in the response to SA, we created serial deletions of the *OsNPR1* promoter using PCR (Fig. 4a). Deletions, beginning at positions −878, −752, −657, and an intact promoter fragment extending to position −1005 were fused to LUC coding sequences. These four constructs were tested for SA induction of the *OsNPR1* promoter by introducing them into rice leaves by particle bombardment followed by treatment with either SA or buffer for 24 h. Protein extracts were made from the bombarded leaves, and their LUC activities were then measured (Fig. 4b). With the 1005:LUC construct, LUC activity increased up to fourfold higher than that in the control following SA treatment; however, in the presence of construct 878:LUC, LUC activity increased less than twofold, indicating that there is a positive *cis*-element in region I between positions −1005 and −878 bp of the *OsNPR1* promoter (Fig. 4b). Two W-boxes were identified in region I. LUC activity in the 752:LUC construct increased by about fourfold with SA treatment, similar to the increase seen with 1005:LUC, indicating that there is a negative element in region II (between positions −878 and −752 bp) of the *OsNPR1* promoter. There is only one WLE1 in region II. In construct 657:LUC, there was only an approximately twofold increase in LUC activity with SA treatment, indicating that there is at least one positive element in region III (between positions −752 and −657 bp) and another one in region IV (between positions −657 and −1 bp) of the *OsNPR1* promoter. There are only two W-boxes and none of the three other types of elements examined here in region I, suggesting that WRKY proteins may be responsible for the dramatic induction of the 1005:LUC construct by SA. Three ASF1 motifs were identified in region III and two RAV1 AAT motifs were found in region IV of the *OsNPR1* promoter, at least one of which might act as a positive element.

Mutations in W-boxes and ASF1 motifs of the OsNPR1 promoter abolish promoter activity in response to SA

To determine whether the two W-boxes in region I and the ASF1 motif elements in region III are involved in the

Fig. 3 Putative *cis*-acting elements in the 1.0-kb *OsNPR1* promoter. Predicted *cis*-elements (W-box, WLE1, RAV1AAT, and ASF1 motif) are indicated in *boxes*. *Arrows* indicate the direction of the *cis*-element. *WBOX* WRKY transcription factor binding site, *WLE1* putative WRKY transcription factor binding site, *RAV1AAT* RAV transcription factor binding site, *ASF1* bZIP factor binding site

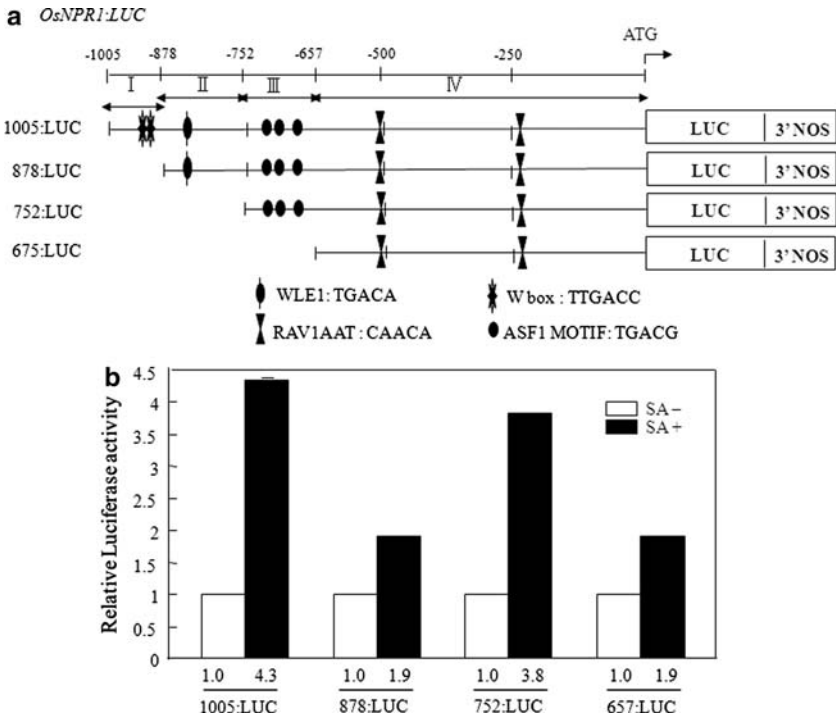

Fig. 4 Deletion analysis of the *OsNPR1* promoter. **a** Schematic diagram of serial deletion constructs of the *OsNPR1* promoter. The *numbers* in the names of the *constructs* indicate the distance in basepairs from the start codon ATG. The predicted *cis*-elements (W-box, RAV1AAT, ASF1motif, and WLE1) and the ATG start codon are indicated. **b** LUC activity in deletion constructs of the *OsNPR1* promoter. Values represent mean plus/minus the standard error (SE) of three replicates

promoter response to SA, a core sequence in the two elements was mutagenized from TGAC to TGAA (Fig. 5), based on the report of Eulgem et al. (1999) that the WRKY protein does not bind to the TGAA. Basal promoter activity of the mutated m1005:LUC was similar to that of 1005:LUC (Fig. 5c); however, m1005:LUC did not respond to SA, indicating that the W-boxes are involved in the response of 1005:LUC to SA. Promoter activity of m752:LUC was also similar to that of the 752:LUC in terms of basal promoter activity; however, the responsiveness seen for 752-LUC to SA was completely abolished in mutated m752:LUC.

Discussion

Systemic acquired resistance is a crucial defense response in plants. It induces the expression of defense-related genes and confers lasting broad-spectrum resistance to a variety of pathogens. SA induces SAR in *Arabidopsis*. However, SA levels are generally high in rice plants compared to *Arabidopsis* (Silverman et al. 1995). The analysis of transgenic rice expressing NahG, which encodes salicylate hydroxylase, suggests that SA may also play a role as a defense signal in rice (Yang et al. 2004). NPR1 is a central regulator of the SA-mediated defense-signaling pathway in *Arabidopsis* (Pieterse and van Loon 2004). Chern et al. (2001) suggested that an NPR1-mediated defense pathway may also exist in rice. In this study, we focused on the question of how the expression of the rice homolog of NPR1, NH1, is regulated in rice.

NPR1 is induced by treatment with SA, 2, 6-dichloroisonicotinic acid, or benzothiadiazole in the model plant *Arabidopsis* (Cao et al. 1997; Ryals et al. 1997). *OsNPR1* is also known to be induced by the pathogen *Xanthomonas oryzae pv. oryzae* and the biotic elicitors SA and JA at high levels in rice, similar to what has been shown in *Arabidopsis*, suggesting that *Os*NPR1 has a function similar to that of NPR1. It has also been demonstrated that *OsNPR1* complements the *Arabidopsis* npr1-1 mutant. Yu et al. (2001) reported that mutations of the W-box in the *NPR1* promoter inhibits the induction of *NPR1* by SA. WRKY proteins are known to be involved in the regulation of *NPR1* expression in *Arabidopsis*. However, the *NPR1* promoter has not been extensively studied in *Arabidopsis* or rice. Here, we isolated the *OsNPR1* promoter and demonstrated that the promoter was activated by SA, as was expected based on its expression profile in seedlings. To identify the elements responsible for the induction of the *OsNPR1* promoter by SA, we analyzed *cis*-acting elements of the promoter. The 1-kb *OsNPR1* promoter contains two W-boxes, one WLE1, three ASF1 motifs, and two RAV1 AAT motifs. Based on *cis*-elements found in the *OsNPR1* promoter, we constructed and analyzed three different deletion constructs (878:LUC, 752:LUC, and 657:LUC). The results of the transient reporter assay of the promoter deletion constructs show that there is at least one positive element in region I, one negative element in region II, and one or more positive elements in region III to induce

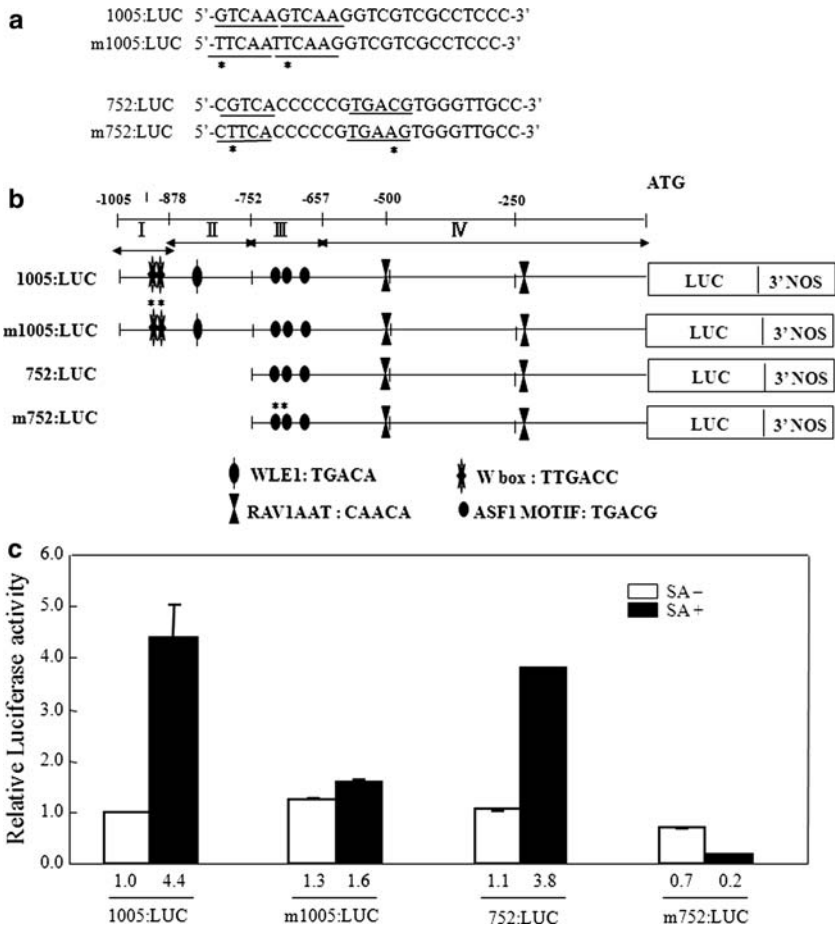

Fig. 5 Effects of mutations in the W-box and ASF1 motif of *OsNPR1* promoter. **a** Region of the W-box of the *OsNPR1* promoter (1005:LUC) where the sequence TGAC was replaced by TGAA (substitutions shown in m1005:LUC). W-box sequence is *underlined* and *asterisks* represent mutated bases. **b** Schematic diagrams of serial deletion constructs of *OsNPR1* promoter. The *numbers* in the name of each *construct* indicates the distance upstream from the start codon ATG. The predicted *cis*-elements (W-box, RAV1AAT, and ASF1motif, and WLE1) and their core sequences, as well as the start codon, are indicated. **c** Relative luciferase activity in 1005:LUC, m1005:LUC, 752:LUC, and m752:LUC in rice leaves. Values represent the mean plus/minus SE of triplicate measurements

OsNPR1 in response to SA. We also confirmed that two consecutive W-boxes in region I (between positions −1005 and −878 bp) and two ASF1 motifs in region III (between −752 and −657 bp) play an important role in regulating the induction of the *OsNPR1* promoter in response to SA. We verified that WRKY proteins and TGA factors are involved in the response of *OsNPR1* to SA. *NPR1* is constitutively expressed in *Arabidopsis* and can be further induced by SA or infection by a pathogen (Cao et al. 1997; Ryals et al. 1997). Previous studies have shown that NPR1 accumulates in the cytoplasm in an oligomeric form. During SA- and pathogen-induced SAR, oligomeric NPR1 is converted into monomers and moves into the nucleus, where it induces PR genes, a process essential for its own function (Kinkema et al. 2000; Mou et al. 2003). In the nucleus, monomeric NPR1 binds to TGA factors, leading to the ultimate activation of downstream genes (Fan and Dong 2002). In addition to TGA factors, WRKY proteins are known to be involved in the SA-mediated defense pathway; however, no direct link has been demonstrated for the functions of NPR1 and WRKY. Wang et al. (2006) recently used microarray analysis to demonstrate that several WRKY genes, such as *At*WRKY18 and *At*WRKY58, are NPR1 targets. Our data suggest that WRKY and TGA factors play essential roles in the response of *OsNPR1* to SA. Verification of this hypothesis, which will require the isolation and characterization of factors that interact with the W-box and the ASF1 motif, are important avenues for future research.

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