

# Rice WRKY45 Plays a Crucial Role in Benzothiadiazole-Inducible Blast Resistance <sup>WJ|OA</sup>

Masaki Shimono,<sup>a</sup> Shoji Sugano,<sup>a</sup> Akira Nakayama,<sup>a</sup> Chang-Jie Jiang,<sup>a</sup> Kazuko Ono,<sup>b</sup> Seiichi Toki,<sup>b</sup> and Hiroshi Takatsujia,<sup>1</sup>

<sup>a</sup> Plant Disease Resistance Research Unit, National Institute of Agrobiological Sciences, Ibaraki 305-8602, Japan

<sup>b</sup> Plant Genetic Engineering Research Unit, National Institute of Agrobiological Sciences, Ibaraki 305-8602, Japan

**Benzothiadiazole (BTH) is a so-called plant activator and protects plants from diseases by activating the salicylic acid (SA) signaling pathway. By microarray screening, we identified BTH- and SA-inducible WRKY transcription factor (TF) genes that were upregulated within 3 h after BTH treatment. Overexpression of one of them, WRKY45, in rice (*Oryza sativa*) markedly enhanced resistance to rice blast fungus. RNA interference-mediated knockdown of WRKY45 compromised BTH-inducible resistance to blast disease, indicating that it is essential for BTH-induced defense responses. In a transient expression system, WRKY45 activated reporter gene transcription through W-boxes. Epistasis analysis suggested that WRKY45 acts in the SA signaling pathway independently of NH1, a rice ortholog of *Arabidopsis thaliana* NPR1, which distinguishes WRKY45 from known *Arabidopsis* WRKY TFs. Two defense-related genes, encoding a glutathione S-transferase and a cytochrome P450, were found to be regulated downstream of WRKY45 but were not regulated by NH1, consistent with the apparent independence of the WRKY45- and NH1-dependent pathways. Defense gene expression in WRKY45-overexpressed rice plants varied with growth conditions, suggesting that some environmental factor(s) acts downstream of WRKY45 transcription. We propose a role for WRKY45 in BTH-induced and SA-mediated defense signaling in rice and its potential utility in improving disease resistance of rice, an importance food resource worldwide.**

## INTRODUCTION

Plants respond to microbial pathogen attack by activating a variety of defense responses that are mediated through multiple signaling pathways. In many dicot plants, salicylic acid (SA) plays a crucial role in mediating one of the signaling pathways leading to defense responses, including induction of *PATHOGENESIS-RELATED (PR)* genes. After pathogen infection, endogenous levels of SA and its conjugates increase markedly in dicots, such as tobacco (*Nicotiana tabacum*), cucumber (*Cucumis sativus*), and *Arabidopsis thaliana*, preceding the induction of *PR* genes and the onset of disease resistance (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991). Exogenous application of SA induces *PR* gene expression and disease resistance in dicots. In contrast with dicots, which contain low basal levels of SA, rice has basal levels of SA two orders of magnitude higher (Raskin et al., 1990). SA levels do not increase after inoculation of rice with either the bacterial pathogen *Pseudomonas syringae* or the fungal pathogens *Magnaporthe grisea* and *Rhizoctonia solani* (Silverman et al., 1995). By contrast, SA levels in the leaves of 28 rice (*Oryza sativa*) cultivars were correlated with general blast

resistance, suggesting that SA may serve as a chemical barrier against pathogen infection (Silverman et al., 1995). Yang et al. (2004) proposed that SA modulates redox balance and protects rice plants from oxidative stress such as that elicited by *M. grisea*.

Benzothiadiazole (BTH) is a functional analog of SA and one of the so-called plant activators that protect various plants from infectious diseases (Görlach et al., 1996; Lawton et al., 1996). In dicots, plant activators induce defense responses by activating the SA signaling pathway. When BTH is applied to plants at high dosages, it induces constitutive activation of defense responses, including several defense-related genes (direct defense; Kohler et al., 2002; van Hulten et al., 2006). By contrast, when BTH is applied at relatively low dosages, plants' defense responses do not activate immediately but become apparent only after pathogen infection (Conrath et al., 2002). This mode of action, which is common among plant activators, is termed potentiation or priming. In rice, BTH enhances resistance to *R. solani* (Rohilla et al., 2002) and *M. grisea* (Schweizer et al., 1999) and is used in the field, but the molecular mechanisms underlying its action remain to be investigated.

In dicots, several regulatory proteins have been implicated in the transcriptional regulation of defense genes under the control of the SA signaling pathway. Among those is *Arabidopsis* NPR1, an important positive regulator in this pathway that is required for transducing the SA signal to downstream *PR* gene activation (Cao et al., 1997). Chern et al. (2001) have reported that overexpression of *Arabidopsis* NPR1 in rice enhances resistance to *Xanthomonas oryzae*. In addition, *Arabidopsis* NPR1 interacts with the bZIP-type transcription factors (TFs) of rice, as in *Arabidopsis* (Chern et al., 2001). Those investigators have shown that

<sup>1</sup>To whom correspondence should be addressed. E-mail takatsuh@nias.affrc.go.jp; fax 81-29-838-8383.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Hiroshi Takatsuji (takatsuh@nias.affrc.go.jp).

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NH1, a rice ortholog of NPR1, also interacts with a bZIP-type TF in rice and that NH1 overexpression in rice confers high levels of resistance to *X. oryzae* (Chern et al., 2005). These observations suggest that rice has a signaling pathway for disease resistance that is similar to the SA-dependent pathway in dicots.

The WRKY family of TFs has been suggested to play a role in controlling the transcription of defense genes through the W-box in their promoters, which is a key *cis*-element for defense-related transcriptional regulation (Rushton et al., 1996; Eulgem et al., 1999; Maleck et al., 2000). In *Arabidopsis*, the WRKY family consists of an estimated 74 members that fall into three major groups on a structural basis (Eulgem, 2005). A large number of WRKY genes are expressed in response to pathogen infection (Kalde et al., 2003), and the function of several WRKY TFs has been implicated in the defense reactions in *Arabidopsis* (Yu et al., 2001; Chen and Chen, 2002; Robatzek and Somssich, 2002). An *Arabidopsis* group III WRKY TF, WRKY70, activates SA-regulated genes downstream of NPR1 and represses jasmonic acid (JA)-responsive genes, integrating SA and JA signaling during systemic acquired resistance (Li et al., 2004). Recent studies have placed several WRKY TFs, including *AtWRKY70*, downstream of NPR1 in the SA signaling pathway on the basis of transcriptional profiling using *Arabidopsis npr1* mutant plants (Wang et al., 2006). Based on chromatin immunoprecipitation experiments in parsley (*Petroselinum crispum*), it has been proposed that WRKY TFs act in a network of mutually competing participants with temporal displacement occurring at defined *cis*-elements preoccupied by other family members in a stimulus-dependent manner (Turck et al., 2004; Ülker and Somssich, 2004). More recent works have shown various roles of WRKY TFs in defense programs of *Arabidopsis* (Journot-Catalino et al., 2006; Wang et al., 2006; Xu et al., 2006). The WRKY TFs form a superfamily in rice, as in dicots (Xie et al., 2005). Several WRKY genes are expressed in response to the rice blast fungal elicitor (Kim et al., 2000), infection with causal agents of bacterial blight and fungal blast diseases (Wen et al., 2003; Ryu et al., 2006), and defense signal molecules SA and JA (Ryu et al., 2006). Os WRKY03 (Liu et al., 2005) and Os WRKY71 (Liu et al., 2006) were functionally characterized, and both were placed upstream of NH1. However, our knowledge of the functions of WRKY TFs in the defense programs in rice is limited.

In an attempt to elucidate the molecular mechanisms underlying BTH-induced disease resistance, we identified WRKY45, which is transcriptionally upregulated by BTH and SA. Induction of rice blast resistance by BTH was abolished in WRKY45 knockdown rice plants, and overexpression of WRKY45 markedly enhanced resistance, indicating that this WRKY TF plays a crucial role in the BTH-inducible defense program of rice. Epistasis analysis revealed that WRKY45 is involved in a signaling pathway downstream of SA but independent of NH1. The presence of two apparently independent pathways was further supported by the fact that different genes were regulated by the two transcriptional regulators. These findings distinguish WRKY45 from any known WRKY TFs in *Arabidopsis* and suggest that the signaling pathways downstream of SA are evolutionally divergent between rice and *Arabidopsis*. To our interest, defense gene expression in these transformants varied between growth conditions. Similar growth condition dependence of PR gene

expression also occurred in BTH-treated wild-type rice plants. On the basis of these results, we propose a role of WRKY45 in the BTH-induced and SA-mediated defense signaling in rice and its potential utility in improving disease resistance of rice, an important food resource worldwide.

## RESULTS

### Identification of BTH-Responsive WRKY TFs

To screen for BTH-responsive genes in rice, we performed microarray analysis of ~44,000 rice genes in BTH-treated rice plants using a 60-mer oligo DNA microarray. We treated plants with 0.5 mM BTH, extracted RNAs from leaves of BTH- and mock-treated plants at 24 h after treatment, and compared the gene expression between the two groups with four biological replicates. Statistical analysis using analysis of variance false discovery rate (*q* value) < 0.05 (Sharov et al., 2005) identified 326 BTH-responsive genes (see Supplemental Table 1 online), including several WRKY TF genes and many defense-related genes. Several studies have implicated WRKY TFs in plant defense responses, so we chose four WRKY genes for which full-length cDNAs were available for further characterization: Os WRKY62 and -76 (group II) and Os WRKY19 and -45 (group III) (Xie et al., 2005), as well as other defense-related genes (Table 1). We examined early-phase patterns of BTH response of the WRKY genes and some defense-related genes by RNA gel blot hybridization (Figure 1). The expression of three of the four WRKY genes (WRKY45, Os WRKY62, and Os WRKY76) was upregulated within 3 h after BTH application. Early-phase BTH response of Os WRKY19 was not clear in this experiment because of substantial signal levels in mock-treated plants, but RT-PCR confirmed the BTH inducibility of this gene (data not shown). BTH-induced upregulation was also confirmed with *PR-1b* and *PBZ1* at 24 h after treatment and with an SA-GTase homolog and *lipoxygenase* at 12 h (Figure 1A). The WRKY genes were upregulated before all other defense-related genes examined.

It is generally thought that BTH, a functional analog of SA, triggers defense responses in plants similar to those triggered by SA. Our microarray analysis in rice supported this notion (data not shown), and we verified SA-responsive upregulation of WRKY45, -62, and -76, as shown in Figure 1B. SA response of Os WRKY19 was again unclear because this gene substantially responded to mock treatment (data not shown).

### Overexpression of WRKY45 Enhances Blast Resistance in Rice

As an initial step to functionally characterize the four BTH-inducible WRKY TFs, we generated transgenic rice lines that express their cDNAs under the control of the constitutive maize (*Zea mays*) *ubiquitin* promoter and tested the transformants for resistance to a compatible race of blast fungus. The transformant rice plants were grown in a growth chamber and inoculated with blast fungus and then disease symptoms were characterized 7 d later. The transformants overexpressing Os WRKY62, -76, and -19 were as susceptible as wild-type plants under our assay conditions (data not shown). By contrast, those overexpressing

**Table 1.** Summary of Microarray Data for the BTH-Inducible Genes Characterized in This Study

Accession Number	Locus ID	Annotation	Fold Change (BTH/Mock)	FDR(Q Value)	References
AK068337	Os09g0417600	Os WRKY76	313.0	<0.00001	
AK067834	Os09g0417800	Os WRKY62	425.0	<0.00001	
AK066255	Os05g0322900	WRKY45	35.8	<0.00001	
AK108389	Os05g0571200	Os WRKY19	14.2	<0.00001	
AK064395	Os09g0518200	SA-glucosyltransferase	60.2	<0.00001	
AK072241	Os12g0559200	Lipoxygenase	12.1	<0.00001	Schaffrath et al. (2000)
AK107926	Os01g0382000	PR-1b	5.5	<0.00001	Agrawal et al. (2000b)
AK071613	Os12g0555500	PBZ1	4.2	0.04300	Midoh and Iwata (1996)
AK103453	Os10g0528300	GST (Tau class)	97.8	<0.00001	
AK072220	Os07g0418500	Cytochrome P450	38.6	<0.00001	
AF251277	Os07g0129200	PR-1a	10.1	0.01200	Agrawal et al. (2000a)

Results of differential expression between BTH- and mock-treated rice plants 24 h after treatments are shown with fold changes and q values. A data set for all the BTH-responsive genes with q values < 0.05 can be found in Supplemental Table 1 online. A complete set of microarray data was deposited to the Gene Expression Omnibus repository under accession number GSE7567. FDR, false discovery rate.

*WRKY45* (*WRKY45-ox*) showed remarkable decreases in susceptible-type lesions compared with wild-type plants (Figures 2A and 2B).

#### BTH-Inducible Blast Resistance Is Compromised in *WRKY45* Knockdown Rice

To characterize the loss-of-function phenotype conferred by *WRKY45*, we generated RNA interference (RNAi) transgenic rice lines by expressing an inverted-repeat sequence of the 3' region of *WRKY45* cDNA, the sequence of which is conserved only at a very low level among members of the *WRKY* family in rice. BTH induction of *WRKY45* expression was reduced in the T1 (second generation) plants of four RNAi lines that had inherited the transgene but not in T1 segregants that had lost the transgene; these results indicate successful transgene-dependent knockdown of *WRKY45* (*WRKY45-kd*; Figure 3A). BTH-induced upregulation of Os *WRKY19*, a group III BTH-inducible *WRKY* gene, was not affected in these transformants (Figure 3A), thus demonstrating the specificity of gene silencing. We tested the BTH-inducible blast resistance associated with *WRKY45* (Figure 3B). Compared with untreated wild-type plants, BTH-treated wild-type plants showed marked reduction in the number of susceptible-type lesions. By contrast, BTH-treated *WRKY45-kd* plants exhibited a comparable number of blast lesions to that of untreated wild-type plants. These results indicate that *WRKY45* plays an essential role in BTH-induced blast resistance in rice.

#### *WRKY45* Activates Transcription through W-Boxes

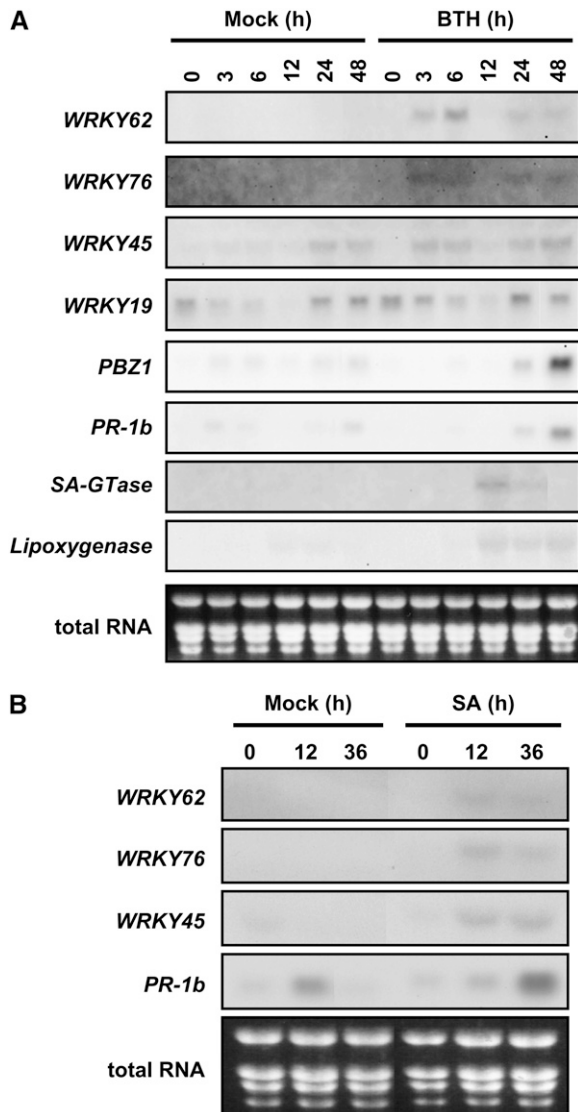
*WRKY* TFs generally act as transcriptional regulators through W-boxes, *cis*-elements that are enriched in the promoters of several defense-related genes (Maleck et al., 2000). To test whether *WRKY45* also modulates transcription through W-boxes and whether it activates or represses transcription, we performed transactivation experiments in rice protoplasts by cotransfecting them with an effector gene to drive *WRKY45* expression and a reporter construct containing W-boxes derived from the tobacco *CHN48* promoter (Yamamoto et al., 2004) located upstream of the *luciferase* gene. We detected increases in luciferase activity

dependent on the presence of the *WRKY45* effector gene (Table 2). In addition, the transcriptional activation was reduced, although not completely, when the W-boxes were mutated (Table 2). Because similar levels of reporter gene activity were observed with the mutated W-boxes in the absence of the effector gene (Table 2), the residual activity is not due to the effector gene-derived *WRKY45* but is presumably due to some endogenous activity that acts through the sequence fortuitously generated in the mutated W-boxes. These results indicate that *WRKY45* is a transcriptional activator and acts through W-boxes.

#### *WRKY45* Acts in the SA Signaling Pathway Independently of NH1

To investigate whether *WRKY45* expression is responsive to only SA and BTH, we examined the effects of various signaling compounds on *WRKY45* expression in leaf discs. As shown in Figure 4A, *WRKY45* transcript levels responded strongly to SA and BTH. In this experimental condition, the levels of *WRKY45* transcript accumulation in response to these chemicals were much higher than those observed in soil-grown plants (Figure 1), presumably because of better accessibility of the chemicals. *WRKY45* transcripts responded only weakly to 1-aminocyclopropane 1-carboxylic acid and to barely detectable levels to the other chemicals tested (Figure 4A). These results indicate that the accumulation of *WRKY45* transcripts is regulated mainly by the SA signaling pathway.

In *Arabidopsis*, NPR1 plays a major role in the SA signaling pathway downstream of SA. A recent article by Wang et al. (2006) reported that >99% of BTH-responsive genes in *Arabidopsis* are under the regulation of NPR1, including several *WRKY* genes. Five of the NPR1-regulated *WRKY* genes (At *WRKY70*, -53, -54, -38, and -66) belong to group III, as does Os *WRKY45*. To comparatively characterize the signaling pathway downstream of SA in rice with that in *Arabidopsis*, we investigated the epistatic relationship between *WRKY45* and NH1 (AK120715), a rice ortholog of NPR1 (Chern et al., 2005), using *NH1* knockdown (*NH1-kd*) rice plants in which BTH-dependent blast resistance is impaired (S. Sugano, C.-J. Jiang, and H. Takatsuji, unpublished results). In *NH1-kd* plants, the levels of *NH1* transcripts were markedly



**Figure 1.** BTH- and SA-Induced Expression of *WRKY* and Defense-Related Genes in Rice.

**(A)** BTH-induced expression. Total RNA (5  $\mu$ g per lane) from the third and fourth leaves of BTH- and mock-treated plants at the indicated time points after BTH application were hybridized with an RNA probe to the 3'-untranslated regions of the gene of interest, as described in Methods. The experiments were performed in two replicates with similar results.

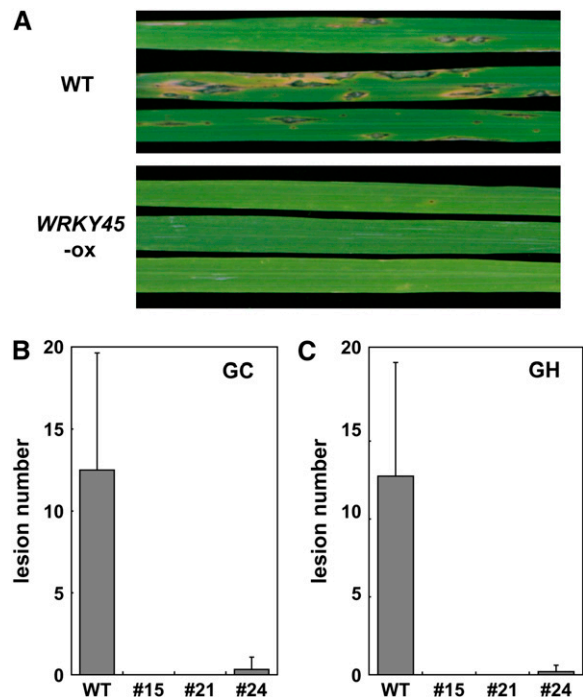
**(B)** SA-induced expression. RNA samples were prepared from SA- and mock-treated rice plants at 12 and 36 h after SA application and hybridized as in **(A)**.

reduced (Figure 4B), while those of its two closest paralogs (AK065952 and AK067198) were unaffected (data not shown). BTH-induced upregulation of *PR-1b* was severely suppressed in these plants, indicating that *PR-1b* is regulated downstream of *NH1* (Figure 4C). By contrast, *NH1*-kd plants were indistinguishable from wild-type plants with respect to BTH-induced upregulation of *WRKY45* (Figure 4C). These results indicate that *WRKY45* is not downstream of *NH1* at the level of transcription,

clearly distinguishing *WRKY45* from the *Arabidopsis* *WRKY* TFs that have been placed downstream of *NPR1*. Yu et al. (2001) have suggested that *NPR1* is transcriptionally regulated by a *WRKY* TF in *Arabidopsis*, so we also examined the expression of *NH1* in response to BTH and the effects of *WRKY45* knockdown on this regulation to see whether *WRKY45* is upstream of *NH1*. As shown in Figure 4D, BTH upregulated *NH1* expression in wild-type rice plants, and this upregulation was scarcely affected by *WRKY45* knockdown. Thus, it appears that *WRKY45* acts independently of *NH1* in the SA signaling pathway.

#### Different Sets of Genes Are Differentially Regulated Downstream of *WRKY45* and *NH1*

The results of epistasis analysis shown above prompted us to compare the genes regulated by *WRKY45* and *NH1*. Among candidate *WRKY45*-dependent BTH-regulated genes selected by a

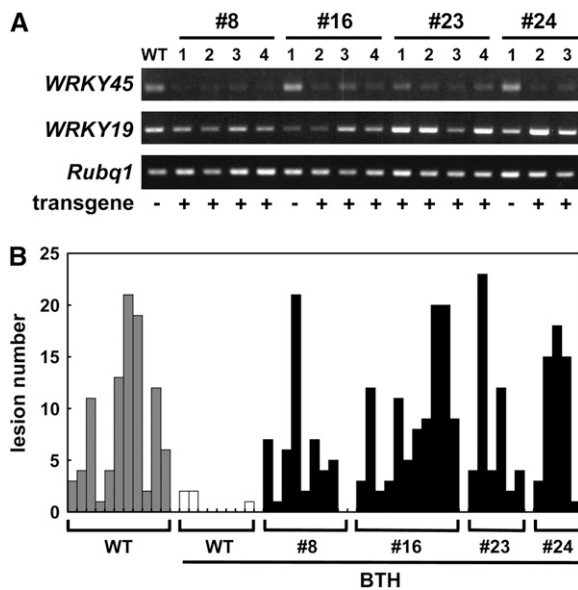


**Figure 2.** Blast Resistance of *WRKY45*-ox Plants.

*WRKY45*-ox homozygote T3 (fourth generation) and control wild-type plants grown in a growth chamber **(A)** and **(B)** or in a greenhouse **(C)** were inoculated with *M. grisea* (race 007.0) at the five- to six-leaf stage, and disease symptoms were characterized 7 d after inoculation.

**(A)** Disease symptoms on the sixth leaves of wild-type and *WRKY45*-ox (line 15) plants grown in a growth chamber.

**(B)** and **(C)** Numbers of lesions on plants grown in a growth chamber **(B)**; GC) and a greenhouse **(C)**; GH). The bars represent the number of susceptible-type lesions in the 10-cm central region of the sixth leaves of individual T3 *WRKY45*-ox (lines #15, #21, and #24) and wild-type rice plants. Averages of 5 to 10 plants with standard deviations are shown. Greenhouse conditions were 28/23°C (day/night) with natural light in December. Essentially similar results were obtained when the plants were grown in March (data not shown).



**Figure 3.** Effects of *WRKY45* Knockdown on Blast Resistance.

**(A)** Expression of *WRKY45* in response to BTH in *WRKY45*-kd transgenic rice plants. Wild-type rice plants and T1 *WRKY45*-kd transgenics were grown in growth chambers, treated with 0.5 mM BTH, and analyzed by RT-PCR for *WRKY45* expression at 48 h after treatment. We also evaluated expression of Os *WRKY19* and *Rubq1*. T1 segregants containing the transgene show reduced transcript levels of *WRKY45* but not Os *WRKY19* or *Rubq1*. Presence (+) or absence (-) of the transgene in each plant is indicated.

**(B)** BTH-induced blast resistance in *WRKY45*-kd transgenic rice plants. *WRKY45*-kd T1 and wild-type rice plants were grown in growth chambers, treated with 0.5 mM BTH at the four-leaf stage, and inoculated with *M. grisea* (race 007.0) 2 d after treatment. Disease symptoms were characterized 7 d after inoculation. Inheritance of the transgene in individual T1 segregants was examined by PCR, and the data for those containing the transgene only are shown. The bars represent the number of susceptible-type lesions in a 10-cm region of the fourth leaves of individual T1 segregants having the transgene. Average lesion numbers in the *WRKY45*-kd plants were comparable to those in untreated wild-type plants, whereas BTH-treated wild-type plants had very few lesions. The lesion numbers varied substantially among individual plants. This is presumably due to difficulties in evenly applying BTH and subsequently blast fungi by spraying them on rice leaves.

preliminary microarray screening in *WRKY45*-kd rice (data not shown), we chose two genes encoding a glutathione S-transferase (GST; AK103453) and a cytochrome P450 (AK072220) (Table 1) and examined their regulatory patterns. *WRKY45*-kd and wild-type rice plants were BTH and mock treated, and the transcript levels of those genes were determined by quantitative RT-PCR 24 h after treatments (Figure 5A). The results demonstrated that BTH-responsive upregulation of these two genes was evidently reduced in *WRKY45*-kd plants compared with wild-type plants, indicating that these genes are under the regulation of WRKY45 either directly or indirectly. By contrast, the upregulation of these genes by BTH was not abolished in *NH1*-kd plants (Figure 5B), indicating that NH1 is not involved in the BTH-responsive

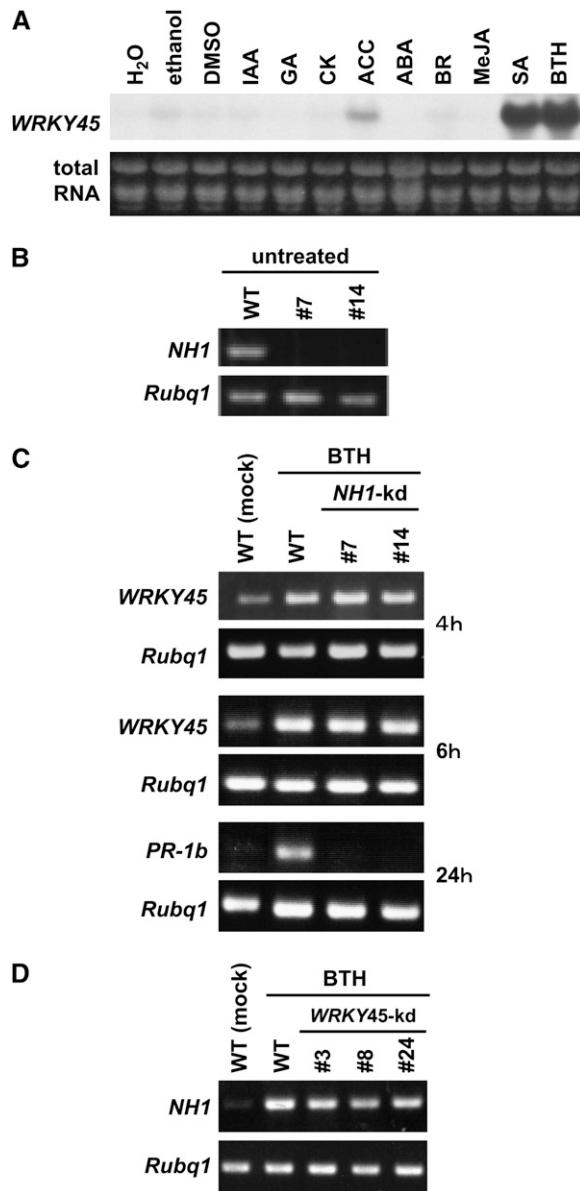
regulation of these genes. These results further support the conclusion that WRKY45 and NH1 are involved in apparently independent signaling pathways. *PR-1a* and *PR-1b* showed coregulated patterns of expression that are obviously different from those of the GST and cytochrome P450 genes. BTH-responsive upregulation of *PR-1a* and *PR-1b* was consistently abolished in *NH1*-kd plants, indicating that these genes are under the regulation of NH1. The WRKY45 dependence of these genes varied in different experiments: the BTH responses of these genes were not affected by *WRKY45* knockdown in some experiments (Figure 5A) but were affected in other experiments (see Supplemental Figure 1 online). The variation of the results was not dependent on the lines used (data not shown). These results suggest that the BTH responses of *PR-1a* and *PR-1b* are conditionally dependent on WRKY45, and some minor differences in experimental conditions can influence their apparent WRKY45 dependence. The BTH response of a *lipoxygenase* gene (AK072241) appeared unaffected by the knockdown of either WRKY45 or NH1 (Figures 5A and 5B; see Supplemental Figure 1 online), suggesting that the BTH-responsive regulation of this gene is dependent on neither transcriptional regulator. Collectively, these results reveal at least three classes of coregulated genes among BTH-responsive genes: WRKY45-dependent and NH1-independent ones, NH1-dependent and conditionally WRKY45-dependent ones, and those independent of either transcriptional regulator.

A BLAST search revealed that rice *PR1b* is one of the closest homologs of *Arabidopsis PR1* (At2g14610), which is BTH inducible and NPR1 regulated (Cao et al., 1997). As for other BTH-responsive rice genes described above (AK103453, AK072220, and AK072241), BLAST searches revealed several *Arabidopsis* homologs with similar levels of homology to the corresponding rice genes, some of which are BTH responsive, while others are not (Wang et al., 2006). In this situation, it is difficult to specify counterpart relationships between the rice and *Arabidopsis* genes without further experiments.

**Table 2.** Transactivation by *WRKY45* in the Rice Protoplast Transient System

Reporter	Effector	Relative LUC Activity
-ELI	+W45	3.7 ± 1.6
+ELI	-W45	1.8 ± 0.8
+ELI	+W45	50.0 ± 19.2
+ELIm	+W45	20.2 ± 3.5
+ELIm	-W45	21.9 ± 5.6

Reporter plasmids (5 μg) with (+ELI) or without (-ELI) wild-type W-boxes and 3 μg of effector plasmids with (+W45) or without (-W45) *WRKY45* coding sequence were cotransfected into rice protoplasts, and luciferase activities were determined. Reporter plasmids with mutant W-boxes (ELIm) were also tested. Sequences of ELI and ELIm are 5'-TTGGTCA-GAAAGTCAGTCGTCGAGTTGGTCAGAAAGTCAGTC-3' (W-box underlined) and 5'-TTGGGAAGAAAGGAAGTCGTCGAGTTGGGAAGAAAGG-AAGTC-3' (mutated nucleotides boxed), respectively. LUC activities relative to those of *Renilla* LUC, the internal control, are shown. Data are shown as the mean of six independent experiments with SD.



**Figure 4.** WRKY45 Acts Downstream of SA in an Apparently NH1-Independent Pathway.

**(A)** Responses of *WRKY45* expression to various signal compounds. Fully expanded fourth leaves of rice at the four-leaf stage were cut into pieces 2 cm long and immersed in aqueous solutions containing 0.01% Silwet L-77 and the following signal compounds: indole-3-acetic acid (IAA; Sigma-Aldrich), gibberellin G<sub>3</sub> (GA; Wako), kinetin (CK; Sigma-Aldrich), 1-aminocyclopropane 1-carboxylic acid (ACC; Sigma-Aldrich), abscisic acid (ABA; Sigma-Aldrich), brassinolide (BR; Wako), methyl jasmonate (MeJA; Wako), 100  $\mu$ M BTH, or 1 mM sodium salicylate (Nakalai) for 8 h at 30°C. Total RNAs (5  $\mu$ g) were extracted, and *WRKY45* transcript levels were analyzed by RNA gel blot analysis.

**(B)** Expression of *NH1* in *NH1*-kd rice plants. Transcript levels of *NH1* in untreated wild-type and *NH1*-kd plants (lines #7 and #14 and T2 homozygote) were examined by RT-PCR.

**(C)** Responses of *WRKY45* and *PR-1b* expression to BTH in *NH1*-kd rice plants. Transcript levels of *WRKY45* were analyzed by RT-PCR at 4 and

### Plant Growth and *PR* Gene Expression in *WRKY45*-ox Rice Are Dependent on Growth Conditions

We fortuitously observed that the growth of *WRKY45*-ox rice plants was dependent on growth conditions. As shown in Figure 6A, wild-type rice plants grew even better in a growth chamber than in a greenhouse. *WRKY45*-ox rice plants exhibited some growth retardation compared with wild-type plants under both conditions. However, the degree of growth retardation was obviously more severe in the growth cabinet than in the greenhouse. We reasoned that this growth condition dependence might be correlated with the expression of defense-related genes, so we examined transcript levels of *PR-1b* and *PR-2* in *WRKY45*-ox lines grown under the two conditions (Figure 6B). These *PR* genes are BTH inducible and are known to be responsive to blast fungus infection (Kim et al., 2001) and a fungal elicitor (Romero et al., 1998). When T1 *WRKY45*-ox plants were grown in the growth chamber, both *PR* genes were upregulated but not in the sibling T1 plants that lacked *WRKY45* overexpression (Figure 6B). When the *WRKY45*-ox plants were grown in the greenhouse, however, transcripts of neither *PR* gene were detectable, even in the plants expressing *WRKY45* at levels comparable to those in growth chamber-grown transformants (Figure 6B). Quantitative PCR revealed that the two *WRKY45*-regulated genes encoding GST and cytochrome P450 also exhibited similar growth condition dependence (Figure 6C). Thus, growth conditions have a profound influence on the expression of *PR* genes during concurrent *WRKY45* overexpression, which may in turn affect the growth of *WRKY45*-ox plants.

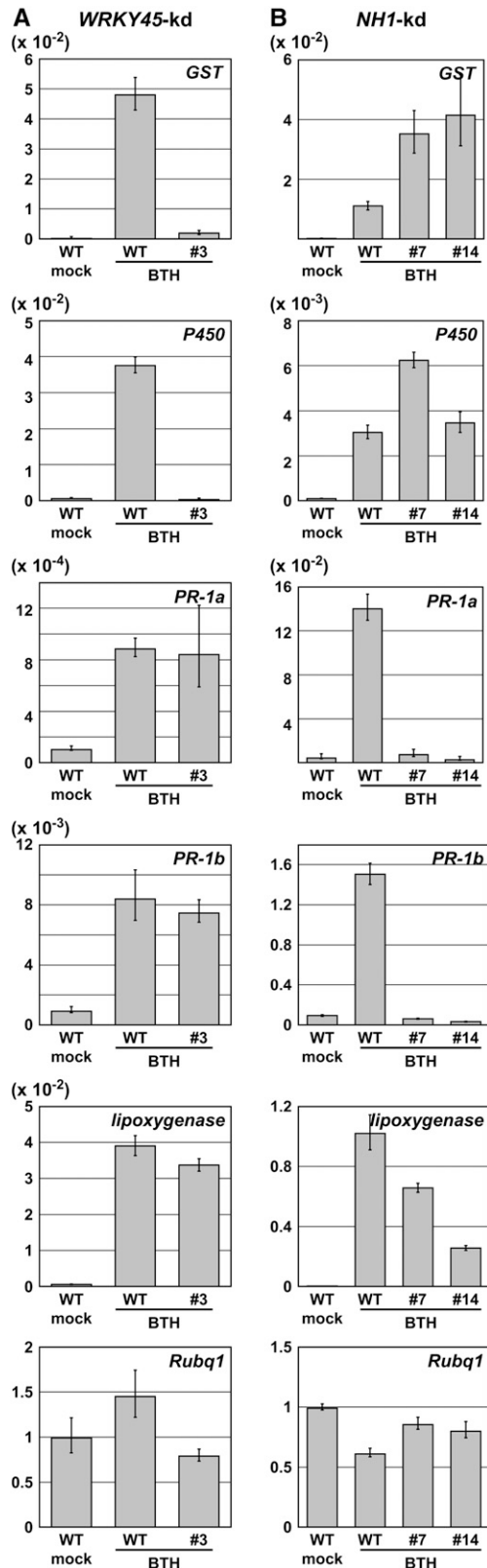
This finding led us to test blast resistance of the *WRKY45*-ox plants that had been grown in a greenhouse to see whether the lack of the defense gene expression before fungus infection influences the resistance of these plants. As shown in Figure 2C, however, greenhouse-grown *WRKY45*-ox plants showed high blast resistance comparable to that of growth chamber-grown *WRKY45*-ox plants (Figure 2B). These results indicate that constitutive upregulation of those defense genes before fungus infection is not a prerequisite for the blast resistance of *WRKY45*-ox plants.

### BTH Induction of *PR* Gene Expression Is Also Dependent on Growth Conditions

The growth condition dependence of the *PR* gene expression in *WRKY45*-ox rice prompted us to examine whether the growth conditions also affect BTH-induced regulation of defense genes. Wild-type rice plants were grown under the two conditions and were BTH and mock treated. RT-PCR analysis showed that *WRKY45* was upregulated by BTH regardless of the growth condition (Figure 7). By contrast, *PR-1b* was upregulated in the

6 h, and those of *PR-1b* at 24 h, after BTH application to wild-type and *NH1*-kd (lines #7 and #14 and T2 homozygote) rice plants.

**(D)** Responses of *NH1* expression to BTH in *WRKY45*-kd rice plants. Transcript levels of *NH1* were analyzed 24 h after BTH application to wild-type and *WRKY45*-kd (lines #3, #8, and #24 and T2 homozygote) rice plants.



**Figure 5.** Quantitative RT-PCR Analysis of the Dependence of the BTH-Responsive Genes on WRKY45 and NH1.

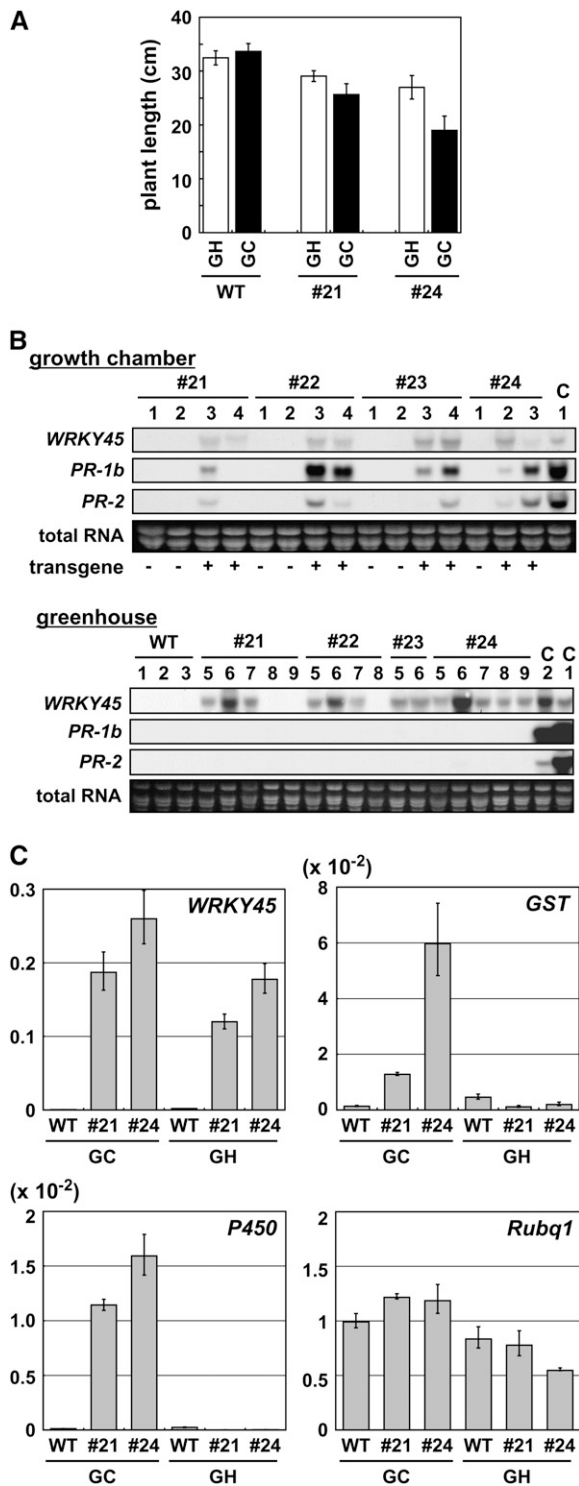
growth chamber-grown plants but not in the greenhouse-grown plants (Figure 7). This growth condition dependence of *PR* gene expression is strikingly similar to that observed in the *WRKY45*-ox plants. Presumably, the same environmental factor(s) is responsible for the similar phenomena observed in BTH-treated wild-type plants and *WRKY45*-ox plants.

## DISCUSSION

### WRKY45 Plays an Essential Role in BTH-Induced Disease Resistance in Rice

BTH induces resistance to various pathogens in plants. In an attempt to elucidate the molecular mechanism underlying the BTH-induced disease resistance, we identified BTH-responsive WRKY TF genes and found that one of them, WRKY45, plays a crucial role in BTH-induced defense reactions in rice. Several studies have highlighted the importance of WRKY TFs in disease resistance in dicots. For example, enhanced resistance to bacterial pathogens has been observed in *Arabidopsis* in which WRKY TFs, such as *AtWRKY29* (Asai et al., 2002) and *AtWRKY18* (Chen and Chen, 2002), are overexpressed. *AtWRKY70* exhibits opposite functions against different types of pathogens consistent with its proposed role in mediating SA-dependent signaling that antagonizes JA-mediated signaling (Li et al., 2004, 2006). Evidence of the loss-of-function phenotype for WRKY genes has been limited because functional redundancy obscures such phenotypes. Recently, however, characterization of double and triple mutants has revealed various functions of different WRKY TFs in defense reactions (Wang et al., 2006; Xu et al., 2006). We have shown in this study that constitutive overexpression of rice *WRKY45* markedly enhanced blast resistance, whereas overexpression of three other BTH-inducible WRKY genes did not. BTH induction of blast resistance was markedly compromised in *WRKY45*-kd plants, being exempted from major functional redundancy. On the basis of these results, we conclude that rice *WRKY45* plays a crucial and predominant role in BTH-inducible defense responses that strongly enhances resistance to blast infection.

T2 homozygotes of *WRKY45*-kd and *NH1*-kd rice plants and wild-type plants were treated with 0.5 mM BTH. The fourth leaves from four plants were harvested and pooled 24 h after treatment, and the expression of selected BTH-responsive genes was analyzed by quantitative RT-PCR to examine the dependence of the expression on WRKY45 (A) and NH1 (B). Averages of three determinations relative to those of *Rubq1* are shown with SD. The expression level of *Rubq1* is relative to that in mock-treated wild-type plants. The experiments were done four or five times with similar results except for those with *PR1a* and *PR1b* in *WRKY45*-kd (see Supplemental Figure 1 online). The magnitudes of BTH responses varied substantially between the experiments in (A) and (B), presumably due to variable penetration of BTH into tissues and/or minor variations in the temporal patterns of expression between the experiments. After repeated experiments, however, we confirmed that the variations in the magnitudes of responses did not influence our conclusions regarding the signaling pathways. The genes analyzed are those listed in Table 1. *WRKY45*-kd line #3 and *NH1*-kd lines #7 and #14 were used.



**Figure 6.** Effects of Growth Conditions on Plant Growth and Gene Expression in *WRKY45*-ox Plants.

**(A)** Effects of growth conditions on growth of *WRKY45*-ox rice plants. Lengths of wild-type and T2 homozygote *WRKY45*-ox rice plants grown in a growth chamber and a greenhouse were measured 15 d after sowing.

### **WRKY45 Acts in an SA Signaling Pathway Apparently Independent of NH1**

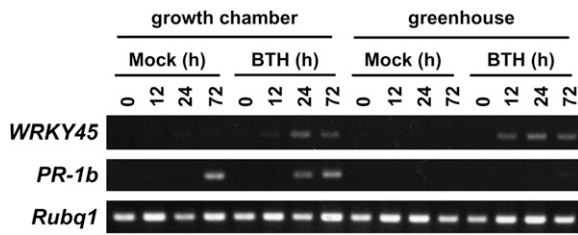
Our results have shown that *WRKY45* is neither downstream nor upstream of *NH1*, a rice homolog of *NPR1*; therefore, *WRKY45* appears to be involved in a pathway independent of *NH1* downstream of SA (Figure 8). Expression analysis of BTH-responsive defense-related genes in *WRKY45*-kd and *NH1*-kd plants supports this notion: two BTH-responsive genes coding for a GST and a cytochrome P450 were found to be regulated by *WRKY45*, either directly or indirectly, but were not regulated by *NH1*. The dependence of *PR-1a* and *PR-1b* on the two regulators is not straightforward. BTH-responsive upregulation of these genes was consistently dependent on *NH1*. However, its dependence on *WRKY45* in *WRKY45*-kd plants varied in different experiments. Moreover, *PR-1b* was upregulated in *WRKY45*-ox plants in a growth condition-dependent manner. Thus, this class of genes is dependent on both *WRKY45* and *NH1* under certain conditions. These observations suggest that the *WRKY45*-dependent and *NH1*-dependent pathways are not completely independent. As illustrated in Figure 8, a conditional signal flow from *WRKY45* to *NH1*, which presumably does not involve transcriptional regulation of *NH1*, would account for these observations, but this hypothesis remains to be experimentally examined. Our results also suggest the presence of a pathway that involves neither *WRKY45* nor *NH1* leading to the regulation of *lipoxygenase*.

It has previously been reported that rice has high SA levels and that those levels do not increase after pathogen infection (Raskin et al., 1990; Silverman et al., 1995), making the role of the SA signaling pathway in rice disputable. Our results, however, demonstrate that the SA signaling pathway mediated by *WRKY45* or *NH1* plays an active role in defense responses in rice. What is the biological significance of having (at least) two signaling

**(B)** Effects of growth conditions on BTH-induced expression of *PR* genes in *WRKY45*-ox plants. Individual T1 plants of each *WRKY45*-ox line with or without *WRKY45* overexpression were analyzed for the expression of *PR-1b* and *PR-2* (*Gns5*) by RNA gel blot hybridization. Rice plants were grown in growth chambers (top panels) or a greenhouse (bottom panels); greenhouse conditions were 28/23°C (day/night) with natural light in March. A mutant rice plant homozygous for *Tos17*-inserted *Os SSI2*, a rice homolog of *Arabidopsis SSI2* (Kachroo et al., 2001), that constitutively expresses *WRKY45* and *PR* (our unpublished results) was used as a positive control (C1). In the bottom panels, an RNA sample from growth chamber-grown *WRKY45*-ox rice plants was also run as a control (C2). Numbers at the top of lanes indicate serial numbers of the individual T1 transformants examined. The presence (+) or absence (-) of the transgene in each transformant as determined by PCR is indicated in the top panels. *PR* genes were expressed depending on *WRKY45* expression in the growth chamber but not in the greenhouse.

**(C)** Quantitative PCR analysis of the effects of growth conditions on the expression of *WRKY45*-regulated genes in *WRKY45*-ox plants. The same RNA samples as used in **(B)** were analyzed for the expression of *WRKY45*, *GST* (AK103453), and *cytochrome P450* (AK072220). Averages of three determinations relative to those of *Rubq1* are shown with SD. The expression level of *Rubq1* is relative to that in mock-treated wild-type plants.





**Figure 7.** Effects of Growth Conditions on BTH Induction of *PR* Genes.

Wild-type rice plants grown in a growth chamber or greenhouse were BTH or mock treated, and then RT-PCR analysis was used to evaluate the temporal patterns of expression of the *WRKY45* and *PR-1b* genes. *WRKY45* was induced by BTH under both growth conditions, whereas induction of *PR-1b* occurred only in growth chamber-grown plants.

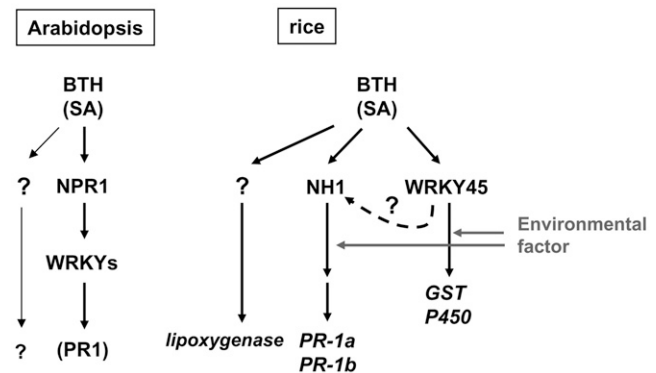
pathways for defense responses downstream of SA/BTH, unlike in *Arabidopsis*, in which the NPR1-dependent pathway is predominant and regulates nearly all BTH-responsive genes? One possibility is that the NH1-dependent pathway has more limited functions in rice than the NPR1-dependent pathway has in *Arabidopsis*, with the WRKY45-dependent pathway playing a crucial role in rice. Alternatively, the WRKY45-dependent pathway may play a role in a defense program unique to rice or monocots. Comprehensive comparative analysis of downstream genes regulated by the two regulators would be of particular use in elucidating the roles of respective regulators in the defense program in rice.

### Does *Arabidopsis* Have a WRKY45 Counterpart?

WRKY TFs are classified into three groups in light of their structures (Eulgem et al., 2000); WRKY45 falls into group III. Systematic characterization of expressional responses of group III WRKY genes in *Arabidopsis* has shown that four subsets of genes are present and show different response patterns to different signaling cues during compatible, incompatible, and nonhost interactions and that these subsets do not reflect phylogenetic relationships (Kalde et al., 2003). According to the phylogenetic analysis by Wu et al. (2005), At WRKY70, -54, -53, -41, -30, and -46 are more closely related to WRKY45 (denoted as Os WRKY50 in Wu et al. [2005]) than other group III WRKY TFs in *Arabidopsis*. Of these genes, At WRKY30 is not SA inducible, whereas the other five are (Kalde et al., 2003). At WRKY70 and -53 play a positive role in SA-mediated resistance to *P. syringae*. These WRKY genes have been placed downstream of NPR1 (Wang et al., 2006), distinguishing them from WRKY45. At WRKY54, which is functionally redundant with At WRKY70 with respect to negative regulation of SA synthesis, has also been placed downstream of NPR1 (Wang et al., 2006). Therefore, these four group III WRKY TFs are not orthologs of WRKY45. At WRKY41 and -46, which have not been well characterized so far, remain candidate WRKY45 orthologs. However, it is plausible that *Arabidopsis* does not have a counterpart to WRKY45, considering that >99% of BTH-responsive genes are under the regulation of NPR1.

### An Environmental Factor(s) Acts on Defense Gene Expression Downstream of WRKY45 Transcription

The WRKY45-regulated defense genes were upregulated in WRKY45-ox plants when the plants were grown in a growth chamber, but transcripts were barely detectable in plants grown in a greenhouse. *PR-1b* expression in BTH-treated wild-type rice plants also showed similar growth condition dependence, whereas WRKY45 was upregulated regardless of the growth conditions. These observations strongly suggest that WRKY45 mediates BTH-induced transcriptional regulation of the defense genes and that an environmental factor(s) acts downstream of the transcription of WRKY45. The growth of WRKY45-ox rice plants was also growth condition dependent, which most likely reflects the expression of WRKY45 downstream genes. Similar growth condition-dependent phenotypes have been seen in transgenic rice plants overexpressing *Arabidopsis* NPR1 (Fitzgerald et al., 2004) and rice NH1 (Chern et al., 2005). These plants show lesion mimic and cell death phenotypes and dwarfed growth when they are grown in a growth chamber but not when grown in a greenhouse. Fitzgerald et al. (2004) suggested that the abundance and quality of the light that plants perceive may affect the phenotype of the NPR1-ox and NH1-ox rice plants. The same could hold for the growth condition-dependent phenotype of WRKY45-ox plants. Identification of the environmental factor and elucidation of the signaling pathway mediating it are of particular interest in understanding the molecular mechanism underlying this growth condition dependence of the phenotypes.



**Figure 8.** Proposed Model for BTH/SA Signaling Pathway in Rice.

In *Arabidopsis*, most BTH-responsive genes are regulated downstream of NPR1, and this regulation is mediated by several WRKY TFs acting downstream of NPR1. In rice, WRKY45 and NH1 constitute apparently independent signaling pathways. *GST* and *cytochrome P450* genes are regulated by WRKY45 but not by NH1. *PR-1a* and *PR-1b* are dependent on NH1 and are conditionally dependent also on WRKY45. Posttranslational signal flow from WRKY45 to NH1 (dashed arrow) would account for the dependence of these genes on both NH1 and WRKY45. The *lipoxigenase* gene does not appear to depend on either NH1 or WRKY45, so it might be regulated by another regulator. An environmental factor acts downstream of WRKY45 transcription and might also act on the NH1-dependent pathway (gray arrows).

### Possible Role of WRKY45 in BTH-Primed Potentiation

When plants are potentiated by BTH, induction of cellular defense responses upon pathogen infection occurs more rapidly or to a greater degree than in untreated plants, thereby accounting for the enhanced disease resistance in potentiated plants. Under potentiated conditions, most defense genes remain silent and become upregulated only after pathogen infection (Conrath et al., 2006). Greenhouse-grown *WRKY45*-ox plants show strong resistance to blast disease in spite of the lack of constitutive expression of the defense-related genes before fungus infection (Figures 2C and 6B). On the basis of these observations, it seems most likely that *WRKY45*-ox plants grown in a greenhouse are under a potentiated state similar to BTH-primed plants. On the other hand, the defense reactions against blast fungus we observed in growth chamber-grown *WRKY45*-ox plants are most likely equivalent to direct defense. To our interest, Kohler et al. (2002) reported that BTH-primed *Arabidopsis* plants exhibit defense responses in response to wounding and water infiltration, which indicates that the manifestation of defense responses occurs in response to abiotic stresses as well as to pathogen infection. These observations seem to parallel ours that an as yet unidentified environmental factor(s) triggers defense gene expression in *WRKY45*-ox and BTH-treated plants. We speculate that the environmental factor(s) could activate the same signaling pathway that pathogen infection does, leading to synergistic interaction of this signaling pathway with the SA signaling pathway downstream of *WRKY45* transcription. This possible synergistic interaction of the two signaling pathways could be a key feature underlying BTH-primed potentiation of plant defense reactions.

In many cases, enhanced disease resistance conferred by overexpression of TF genes accompanies growth defects resulting in reduced productivity. This is presumably due to constitutive activation of defense responses under the regulation of introduced TFs. The overexpression of *WRKY45* imposes relatively small adverse effects on plant growth, although they are dependent on the growth conditions. This is presumably due to the potentiated state established in these plants as discussed above. Our preliminary results have shown that the growth defects can be further reduced without major loss of blast resistance by optimizing the level of *WRKY45* expression. After appropriate optimization, *WRKY45* would serve as an effective tool to improve disease resistance of rice, an important food resource worldwide.

## METHODS

### Plant Materials, Chemicals, and Pathogen Treatments

All the experiments were performed with rice (*Oryza sativa* cv Nipponbare). Unless otherwise noted, plants typically were grown in growth chambers in soil at 28/23°C (light/dark), 50% humidity, 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity, and 14-h photoperiods. BTH (in 0.5% [v/v] acetone + 0.05% [v/v] Tween 20) and SA (in 0.01% [v/v] Tween 20) were sprayed onto leaves (1 mL/plant). Mock treatments were done by spraying the solvents only. Conidia of blast fungus (*Magnaporthe grisea* Cavara, race 007.0) were suspended in 0.01% Tween 20 at a density of  $10^5/\text{mL}$  and sprayed onto rice plants as described (Watanabe et al., 1977).

### Microarray Analysis

Rice plants were BTH or mock treated at the four-leaf stage and then fourth leaves were harvested from four plants at 24 h after treatments and pooled. Total RNA was isolated from each pool using an RNeasy plant mini kit (Qiagen). The RNAs (400-ng aliquots) were labeled with a Low RNA Input Linear Amplification/Labeling Kit (Agilent Technologies) according to the manufacturer's instructions. Aliquots of Cy3-labeled cRNAs (1  $\mu\text{g}$  each) of the BTH- or mock-treated samples were used for hybridization in an Agilent Rice Oligo Microarray (44K, custom-made; Agilent Technologies). Four biological replicate samples sets (of both BTH and mock) were analyzed. After hybridization, microarray slides were scanned (scanner model G2505B and software G2565BA; Agilent), and data were analyzed using Feature Extraction software (version 9.1; Agilent Technologies) at the default settings. All microarray procedures and data analyses were performed according to the manufacturer's manual (<http://www.chem.agilent.com/scripts/generic.asp?page=11617&indcol=Nandprodcol=Y;G4140-90040>). The data were normalized using GeneSpring GX 7.3 software (Agilent) and statistically analyzed using the National Institutes of Aging array analysis tool (<http://lgsun.grc.nia.nih.gov/ANOVA/>) (Sharov et al., 2005). We used the error model "maximum of averaged and Bayesian error variances" to reduce false positives. The false positives were controlled by measuring the false discovery rate (Benjamini and Hochberg, 1995).

### RNA Gel Blot Hybridization

Total RNA was isolated using Trizol reagent (Invitrogen) and purified with an RNeasy mini kit (Qiagen). The RNAs were separated in agarose gels and transferred onto GeneScreen membranes (NEN Life Science Products) as described by Kapoor et al. (2002). Digoxigenin (DIG)-labeled antisense RNAs prepared with a DIG RNA labeling kit (SP6/T7; Roche Diagnostics) were used as probes. The blotted membranes were hybridized with the DIG-labeled probes using SSC-based buffers according to manufacturer's protocol, and signals were detected by chemiluminescence reaction using a DIG nucleic acid detection kit (Roche Diagnostics) and CDP-STAR (Roche Diagnostics). Chemiluminescence signals were detected by a LumiVision PRO densitometer (Aisin Seiki). Sequences for the probes (according to nucleotide numbers) are as follows: Os *WRKY62*, nucleotides 932 to 1161 (AK067834); Os *WRKY76*, nucleotides 1126 to 1327 (AK068337); *WRKY45*, nucleotides 1126 to 1426 (AK066255); Os *WRKY19*, nucleotides 658 to 1016 (AK108389); *PBZ1*, nucleotides 560 to 822 (D38170); *PR-1b*, nucleotides 577 to 793 (AK107926); *SA-GTase* homolog, nucleotides 1297 to 1710 (AK064395); *lipoxygenase*, nucleotides 2567 to 2747 (AK066737); and *PR-2* (*Gns5*), nucleotides 1095 to 1295 (AK070677).

### RT-PCR Analysis

RT-PCR was performed with 2  $\mu\text{g}$  of total RNA treated with DNase I (Invitrogen). Reverse transcription was done using SuperScript II RNase H<sup>-</sup> (Invitrogen) and oligo(dT)<sub>23</sub> primers (Sigma-Aldrich). PCR was performed using cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 7 min. Quantitative RT-PCR was run on a Thermal Cycler Dice TP800 system (Takara Bio) using SYBR premix Ex Taq mixture (Takara) with cycles of 95°C for 5 s and 60°C for 30 s. *Rice ubiquitin 1* (*Rubq1*; AK121590) was used as an internal standard. Primers for PCR used in this study are listed in Supplemental Table 2 online. These primer sets were tested by dissociation curve analysis and verified for the absence of nonspecific amplification.

### Plasmid Construction and Plant Transformation

To construct a plasmid for *WRKY45* RNAi, part of the *WRKY45* cDNA (nucleotides 1047 to 1535) was amplified by PCR and inserted into the

pANDA vector with the antisense sequence upstream of the sense one as described (Miki and Shimamoto, 2004; Miki et al., 2005), except that the pDONR207 vector (Invitrogen) was used instead of pENTR as an intermediate vector. To construct a plasmid for constitutive expression of *WRKY45*, pUCAP/Ubi-NT was generated by replacing the *HindIII*-*Bam*HI fragment containing the cauliflower mosaic virus (*CaMV*) 35S promoter in pUCAP/35S-NT (Kapoor et al., 2002) with the fragment containing the *Zea mays polyubiquitin* promoter (*Ubi-1*) from pAHC27 (Toki et al., 1992; Christensen and Quail, 1996). *Sfi*I and *Bam*HI sites were generated between the *Ubi-1* promoter and the *NOS* terminator in pUCAP/Ubi-NT by digesting the plasmid with *Bam*HI and *Sac*I and inserting a double-stranded linker (5'-GATCTGGCCAAATCGGCCGGTACCGGATCCGCGGCCGCGAGATC-3' annealed to 5'-CGCGGCCGCGGATCCGGTACCGGCCGATTTGGCCA-3') into this site. A fragment for full-length *WRKY45* cDNA was inserted between the *Sfi*I and *Bam*HI sites of pUCAP/Ubi-NT. Then, a fragment encoding the *Ubi-1* promoter, the *WRKY45* cDNA, and the *Nos* terminator was excised from this plasmid using *Hind*III and *Pac*I and inserted between corresponding sites in the binary vector pZH1, a derivative of pPZP202 (Hajdukiewicz et al., 1994), which contains the *CaMV* 35S promoter, the *hygromycin phosphotransferase* gene, and the *NOS* terminator. Rice was transformed by an *Agrobacterium tumefaciens* (strain EHA105)-mediated procedure as described (Toki et al., 2006), except that rice seeds precultured for 3 d on medium containing 2,4-D were used for inoculation with *Agrobacterium* rather than 2-week-old rice callus derived from scutellum tissue of mature seeds (Toki et al., 2006).

#### Transient Gene Expression in Rice Protoplasts

Luciferase (LUC) reporter plasmids were constructed by inserting a *CHN48*-derived W-box (Yamamoto et al., 2004) or its mutant sequences upstream of the *CaMV* 35S minimal promoter (-46) (Odell et al., 1985) and a *LUC*+*Nos*-terminator fragment in pUC. The effector plasmid 35S:*WRKY45* was constructed by replacing the *Xba*I fragment of the *LUC*+ sequence in 35S:*LUC*+*Nos*/pUC with a PCR-generated fragment of *WRKY45* coding sequence. Protoplasts from the rice suspension-cultured cell line Oc were used for electroporation (Hattori et al., 1994). Electroporation was performed using an Electro Cell Manipulator (ECM 600; BTX) at 875 V cm<sup>-1</sup> with a 550- $\mu$ F capacitor. After incubation overnight at 30°C, cells were collected, and proteins were extracted as described (Kyojuka et al., 1987). Luciferase activities were assayed using the dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. The *Renilla LUC* gene (Promega) under the control of the *CaMV* 35S promoter (0.1  $\mu$ g) was cotransfected as an internal control, and the ratio of LUC activities (firefly LUC/*Renilla* LUC) was calculated to normalize each assay.

#### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers listed in Table 1.

#### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Quantitative RT-PCR Analysis of the Dependence of the BTH-Responsive Genes on *WRKY45*.

**Supplemental Table 1.** BTH-Responsive Genes in Microarray Experiments.

**Supplemental Table 2.** Primers for PCR Used in This Study.

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