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Rice WRKY45 plays important roles in fungal and bacterial disease resistance

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SUMMARY

Plant 'activators', such as benzothiadiazole (BTH), protect plants from various diseases by priming the plant salicylic acid (SA) signalling pathway. We have reported previously that a transcription factor identified in rice, WRKY45 (OsWRKY45), plays a pivotal role in BTH-induced disease resistance by mediating SA signalling. Here, we report further functional characterization of WRKY45. Different plant activators vary in their action points, either downstream (BTH and tiadinil) or upstream (probenazole) of SA. Rice resistance to *Magnaporthe grisea*, induced by both types of plant activator, was markedly reduced in *WRKY45*-knockdown (*WRKY45*-kd) rice, indicating a universal role for WRKY45 in chemical-induced resistance. Fungal invasion into rice cells was blocked at most attempted invasion sites (pre-invasive defence) in *WRKY45*-overexpressing (*WRKY45*-ox) rice. Hydrogen peroxide accumulated within the cell wall underneath invading fungus appressoria or between the cell wall and the cytoplasm, implying a possible role for H_2O_2 in pre-invasive defence. Moreover, a hypersensitive reaction-like reaction was observed in rice cells, in which fungal growth was inhibited after invasion (post-invasive defence). The two levels of defence mechanism appear to correspond to Type I and II nonhost resistances. The leaf blast resistance of *WRKY45*-ox rice plants was much higher than that of other known blast-resistant varieties. *WRKY45*-ox plants also showed strong panicle blast resistance. BTH-induced resistance to *Xanthomonas oryzae* pv. *oryzae* was compromised in *WRKY45*-kd rice, whereas *WRKY45*-ox plants were highly resistant to this pathogen. However, *WRKY45*-ox plants were susceptible to *Rhizoctonia solani*. These results indicate the versatility and limitations of the application of this gene.

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INTRODUCTION

Rice is one of the most important cereal crops for human consumption worldwide. Rice diseases, such as fungal blast caused by *Magnaporthe grisea* and bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), cause serious yield losses and pose a constant threat to rice supplies. A defence signalling pathway mediated by salicylic acid (SA) plays a crucial role in systemic acquired resistance in dicots, in which endogenous SA levels rise rapidly on pathogen attack, leading to the activation of defence reactions. In rice, SA levels are high in the absence of pathogen infection and do not appear to change after infection. This implies that the defence mechanism in rice is different from that in dicots. However, some reports have suggested that an SA-mediated defence signalling pathway is also present in rice (Silverman *et al*., 1995; Yang *et al*., 2004). Moreover, several studies have shown that rice shares signalling components with the SA signalling pathway in dicots (Chern *et al*., 2005; Jiang *et al*., 2009; Shimono *et al*., 2007; Yuan *et al*., 2007).

Chemical inducers, also called plant 'activators', protect plants from a variety of diseases by acting on the SA defence signalling pathway. Plant activators are effective in rice (Iwai *et al*., 2007; Iwata *et al*., 1980; Shimono *et al*., 2007), and include benzothiadiazole (BTH) (Görlach *et al*., 1996; Lawton *et al*., 1996), probenazole (3-allyloxy-1,2-benzisothiazole-1,1-dioxide) (Iwata *et al*., 1980; Yoshioka *et al*., 2001) and tiadinil [*N*-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide] (Yasuda *et al*., 2006). Probenazole acts upstream of SA (Iwai *et al*., 2007; Yoshioka *et al*., 2001), whereas BTH and tiadinil, which are functional analogues of SA, act downstream (Görlach *et al*., 1996; Lawton *et al*., 1996; Yasuda *et al*., 2006). When plant activators are applied to plants at appropriate dosages, they entail only small costs to plant growth and development, owing to the 'priming effect' (Conrath *et al*., 2006). Plant activators also have long-lasting protective effects, broad spectra for pathogens and relatively small safety concerns. These advantages have led to their wide use in recent decades for disease control in operational agriculture.

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In *Arabidopsis*, a transcriptional cofactor, NPR1, plays a key role in the regulation of the SA signalling pathway (Cao *et al*., 1994). Some *Arabidopsis* WRKY transcription factors, including WRKY18, WRKY70 and WRKY58, are regulated downstream of NPR1 and play positive or negative roles in SA-dependent defence (Wang *et al*., 2006). Of these, WRKY70 acts at a conversion point determining the balance between SA- and jasmonic acid (JA)-dependent defence (Li *et al*., 2004). WRKY62 also acts downstream of cytosolic NPR1 and represses the JA pathway (Mao *et al*., 2007). The rice genome encodes more than 100 *WRKY* genes (Ramamoorthy *et al*., 2008; Ross *et al*., 2007). Several of these genes (e.g. OsWRKY13, OsWRKY53, OsWRKY71, OsWRKY31 and OsWRKY89) have been associated with rice resistance to *M. grisea* and/or *Xoo* by overexpression studies (Chujo *et al*., 2007; Liu *et al*., 2007; Qiu *et al*., 2007; Zhang *et al*., 2008), whereas OsWRKY62 is linked with a negative role in basal and *Xa21*-mediated resistance to *Xoo* (Peng *et al*., 2008). Of these genes, OsWRKY13 has been reported to exert its function by activating SA synthesis whilst suppressing JA signalling (Qiu *et al*., 2007, 2008).

In our previous work aimed at the elucidation of the molecular mechanisms underlying the plant-protective actions of BTH, we identified a BTH-inducible rice transcription factor, WRKY45 (Shimono *et al*., 2007). We showed that knockdown of the *WRKY45* gene (*WRKY45*-kd) compromised BTH-induced blast resistance, indicating that WRKY45 plays a pivotal role in the BTH-induced defence response in rice by acting through the SA signalling pathway (Shimono *et al*., 2007). *WRKY45*-overexpressing (*WRKY45*-ox) rice lines grown under certain conditions showed strong blast resistance with minor accompanying growth retardation, even with barely detectable levels of pathogenesis-related (*PR*) gene expression prior to pathogen infection, suggesting that *WRKY45*-ox plants are ‘primed’ for defence reactions. A rice counterpart, NH1/OsNPR1, has been implicated in SA-dependent defence in rice (Chern *et al*., 2005; Yuan *et al*., 2007). An epistasis analysis showed that the rice SA pathway branches into two, namely the WRKY45-dependent and NH1/OsNPR1-dependent pathways, unlike the *Arabidopsis* SA pathway in which NPR1 regulates most of the BTH-regulated genes (Wang *et al*., 2006). Our genome-wide transcript profiling of *NH1/OsNPR1*-regulated genes supported this model (Sugano *et al*., 2010).

In this study, we further characterized *WRKY45* loss- and gain-of-function phenotypes. We aimed to understand more about the role of *WRKY45* in SA pathway-mediated resistance to blast and leaf blight diseases, and to gain a mechanistic insight into the resistance. We found that *WRKY45* is involved in the protective effects of plant activators that have different action mechanisms. Microscopic analysis of the blast fungus infection process revealed a two-layered defence mechanism in *WRKY45*-ox rice, consisting of pre- and post-invasive resistance, and possible involvement of H_2O_2 in the former. In addition, *WRKY45* was found to play a positive role in BTH-induced blight resistance and conferred extremely strong resistance to leaf blight when overexpressed. On the basis of these results, we discuss the importance of the *WRKY45* transcription factor and the mechanism of resistance, as well as its potential applications.

RESULTS

Rice WRKY45 is involved in blast resistance induced by plant activators with different action points

We have shown previously that BTH-induced blast resistance is largely compromised in *WRKY45*-kd rice plants in the genetic background of a *japonica* cultivar, Nipponbare (Shimono *et al*., 2007). To further characterize the role of WRKY45, we examined the effects of the knockdown of *WRKY45* on different rice–*M. grisea* pathosystems. These included compatible and incompatible interactions with or without pretreatment with different plant activators. In *WRKY45*-kd rice lines, basal levels of *WRKY45* transcripts, in addition to their BTH-induced upregulation (Shimono *et al*., 2007), were reduced (data not shown). We also confirmed by immunoblotting that the basal level of the WRKY45 protein was reduced in *WRKY45*-kd rice (Fig. 1a). Basal resistance to a compatible blast race (007.0) without pretreatment of plant activators was not affected significantly in *WRKY45*-kd plants compared with control Nipponbare rice (Fig. 1b,c). We also tested blast races incompatible with Nipponbare (102.0 and 002.0), but observed no difference in disease symptoms between *WRKY45*-kd plants and control Nipponbare plants after inoculation of these fungal races (data not shown).

Different plant activators act at different points of the SA signalling pathway (Görlach *et al*., 1996; Iwai *et al*., 2007; Lawton *et al*., 1996; Yasuda *et al*., 2006; Yoshioka *et al*., 2001). We determined whether WRKY45 was involved in the disease resistance induced by plant activators with different action mechanisms. First, we examined the effects of *WRKY45* knockdown on blast resistance induced by tiadinil and its derivative, 4-methyl-1,2,3-thiadiazole-5-carboxylic acid (SV-03), which is an active form of tiadinil. Young seedlings of nontransformed Nipponbare and two lines of *WRKY45*-kd (T_3, homozygous) rice plants at the 3.5-leaf stage were drench treated with different amounts of tiadinil or SV-03 for 6 days, followed by spray inoculation of a compatible blast race (007.0). Examination of the disease symptoms at 5 days post-inoculation (dpi) showed that both treatments markedly decreased the number of blast lesions in nontransformed Nipponbare rice plants compared with mock-treated control plants (Fig. 1b). In *WRKY45*-kd plants, however, blast lesion numbers were increased compared with those in control Nipponbare plants treated with the same amounts of chemical inducers (Fig. 1b). Probenazole treatment also decreased blast lesion numbers in a

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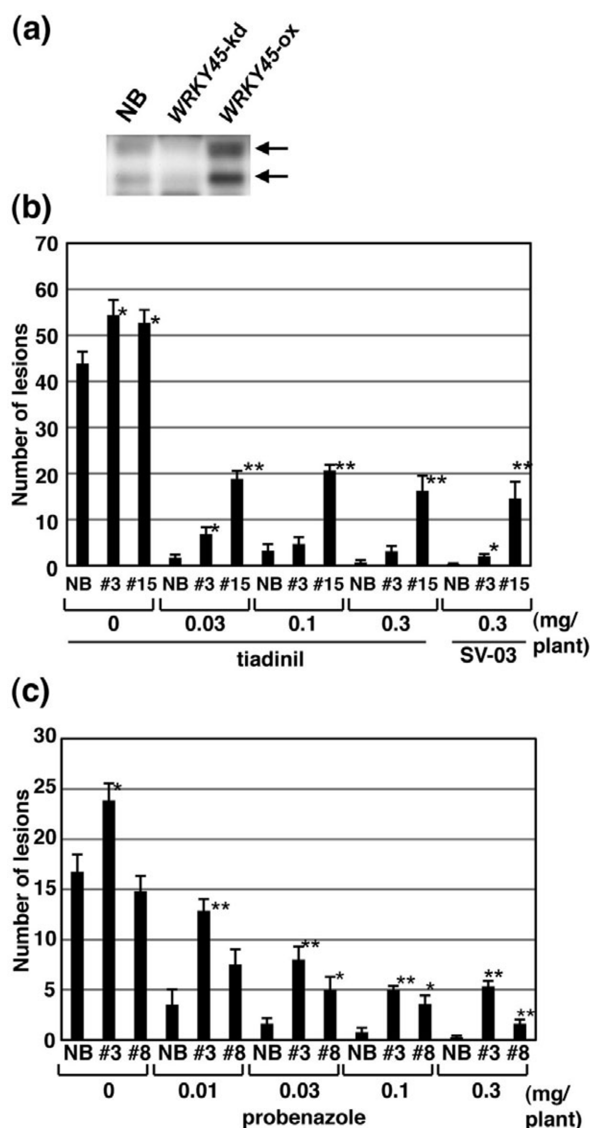

Fig. 1 Effects of *WRKY45* knockdown on tiadinil- and probenazole-induced blast resistance. (a) Expression of WRKY45 protein in rice transformants (*WRKY45*-kd and *WRKY45*-ox) and control untransformed Nipponbare (NB) rice. Arrows indicate phosphorylated (top) and unphosphorylated (bottom) forms of the WRKY45 protein. (b) Tiadinil-induced blast resistance in *WRKY45*-kd rice plants. Two independent lines of *WRKY45*-kd (lines #3 and #15, T_3) and control Nipponbare (NB) rice plants were treated for 6 days with different amounts of tiadinil and SV-03 at the 3–3.3-leaf stage. Plants were inoculated with conidia of *Magnaporthe grisea* (race 007.0) and disease symptoms were characterized at 5 days post-inoculation (dpi). (c) Probenazole-induced blast resistance in *WRKY45*-kd rice plants. Two lines of *WRKY45*-kd (lines #3 and #8, T_3) and Nipponbare (NB) rice plants grown in a glasshouse were treated with different amounts of probenazole for 6 days at the 3.5-leaf stage. Plants were inoculated with *M. grisea* (race 007.0) and disease symptoms were characterized at 5 dpi. Values are the mean numbers (±standard error of the mean, SEM) of susceptible-type lesions in the 10-cm central region of the fourth leaves (n = 7–10). Asterisks indicate significant difference from the control (NB) using a t-test: $^*P < 0.05$ and $^{**}P < 0.01$.

dose-dependent manner, and the effects were partially negated by *WRKY45* knockdown (Fig. 1c). These results indicate that the defence-inductive actions of these chemical inducers are at least partially dependent on *WRKY45*. RNA interference (RNAi)-mediated gene knockdown, which is based on post-transcriptional mRNA degradation, is usually incomplete and can become even less complete when the transcription of the RNAi target gene is strongly induced (by the chemical inducers in this case); therefore, it is difficult to evaluate the extent of WRKY45 dependence for each chemical inducer.

These results, in conjunction with our previous work using BTH (Shimono *et al*., 2007), indicate that WRKY45 is commonly involved in the action of plant activators that act on the SA signalling pathway, regardless of whether their action points are upstream or downstream of SA. The partial influence of *WRKY45* knockdown in reducing induced blast resistance could be because of the incompleteness of *WRKY45* expression shutdown in the *WRKY45*-kd lines (Shimono *et al*., 2007). However, it is possible that probenazole and tiadinil induce blast resistance in rice through a WRKY45-independent pathway in addition to the WRKY45-dependent pathway.

Microscopic analysis of the *M. grisea* infection process in *WRKY45*-ox rice

We have shown previously that *WRKY45* overexpression confers blast resistance in rice (Shimono *et al*., 2007). To gain a mechanistic insight into the blast resistance induced in *WRKY45*-ox rice, we characterized microscopically the infection process of the fungus. Sheaths of fifth leaves from soil-grown rice plants at the six-leaf stage were laid in a humidified plastic box, and a fungal suspension was poured into the cavities of the leaf sheaths. At 2 and 4 dpi, we examined the leaf sheaths under a light microscope and counted the number of appressoria that had or had not developed invading hyphae in rice cells. In leaf sheaths of control Nipponbare plants, about 50% and 70% of fungal appressoria developed invading hyphae in rice cells at 2 and 4 dpi, respectively (Fig. 2a). In contrast, only a small percentage of appressoria developed invading hyphae at both 2 and 4 dpi in *WRKY45*-ox leaf sheath cells (Fig. 2a). We used plasmolysis as an indicator of viability by observing infection sites after immersion of inoculated leaf sheaths in 0.8 M sucrose solution, and found that secondary invading hyphae extended into plasmolysed cells in Nipponbare (Fig. 2b). The development of invading hyphae was also seen by transmission electron microscopy (TEM) of a Nipponbare epidermal cell (Fig. 3a), in which compartmentalization was observed. In *WRKY45*-ox rice, 24 h after inoculation, no invading hyphae were seen around the majority of appressoria, and most of the epidermal cells under attack showed plasmolysis (Fig. 2c). Most of the fungi ceased producing hyphae just before invading cells (Fig. 3b–d), but some succeeded in penetrating the cell wall without

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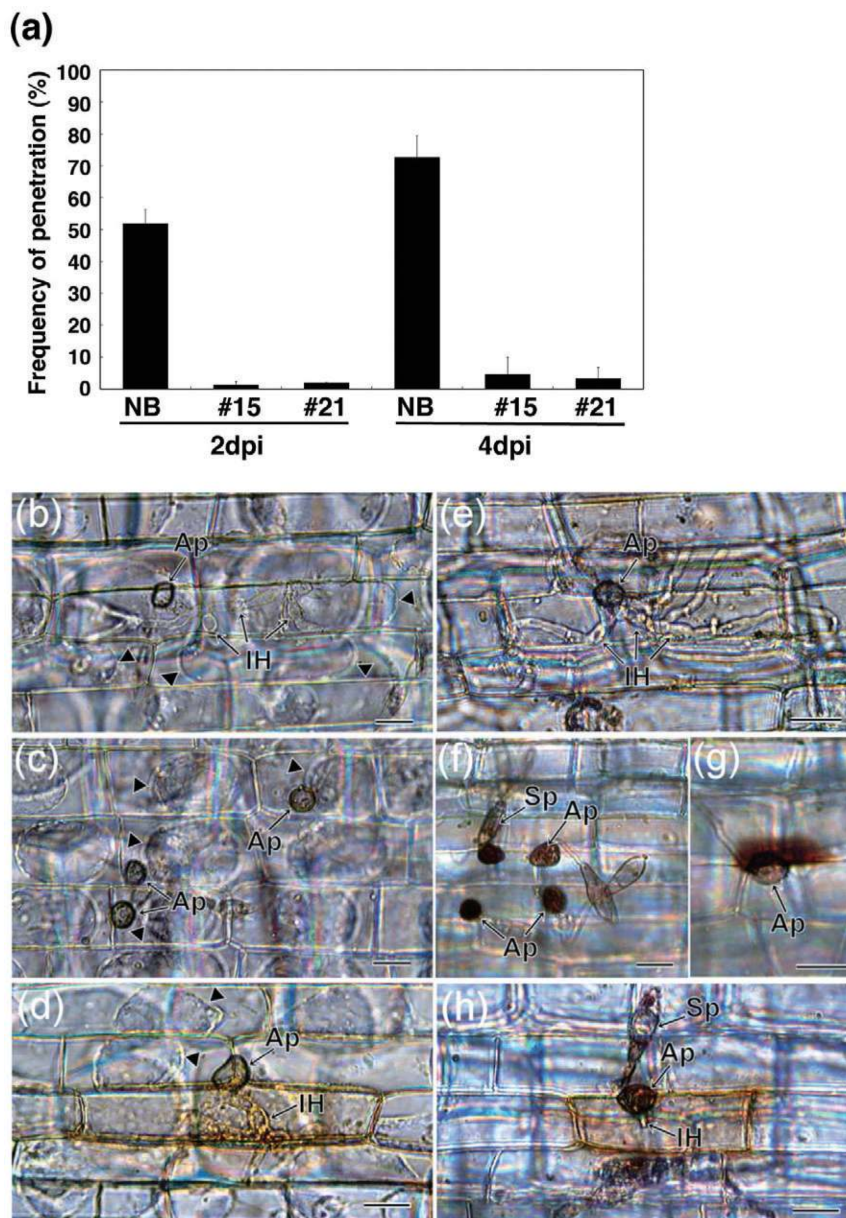

Fig. 2 Microscopic analysis of the *Magnaporthe grisea* infection process in *WRKY45*-ox plants. (a) Frequency of fungal invasion into rice cells. Nipponbare (NB) and *WRKY45*-ox (line #15, T_3 and line #21, T_4) rice plants were inoculated with *M. grisea* by intact leaf sheath inoculation, and examined for hyphal invasion into rice cells under a light microscope at 2 and 4 days post-inoculation (dpi). Values are the mean percentages (±standard deviation; $n = 3$ experiments) of 50 appressoria with extended invading hyphae for each infection. (b–h) Light micrographs of infection sites 48 h after inoculation. Leaf sheaths were treated with 0.8 M sucrose solution for 20 min before observation (b–d) and stained with diaminobenzidine (DAB) to detect H_2O_2 accumulation at the infection sites (e–h). In Nipponbare, well-developed invading hyphae with their tips expanding into rice cells were observed; the cells were plasmolysed (b) and were rarely stained with DAB (e). In *WRKY45*-ox leaves, most appressoria did not extend invading hyphae into rice cells or provoke host reactions (c). In uninvaded *WRKY45*-ox cells, DAB staining was not observed (f), or was observed in a limited area of the cell wall under an appressorium (g). Fungus-invaded *WRKY45*-ox cells often exhibited cytoplasmic granulations (d) and DAB staining in the whole cell (h). Ap, appressorium; IH, invading hypha; Sp, spore. Arrow heads indicate plasma membranes of plasmolysed epidermal cells. Bars, 10 μm.

provoking appreciable host cell responses (Fig. 3e). The latter presumably invaded cells and developed a short invading hypha 48 h after inoculation, but provoked a hypersensitive reaction (HR)-like reaction in the host cells, causing granulation of the cytoplasm (Figs 2d, h and 3f). This presumably restricted further extension of the invading hyphae. In these cells, the accumulation of H_2O_2 was detected by diaminobenzidine (DAB) staining (Fig. 2h), whereas DAB staining was barely observed in Nipponbare cells that

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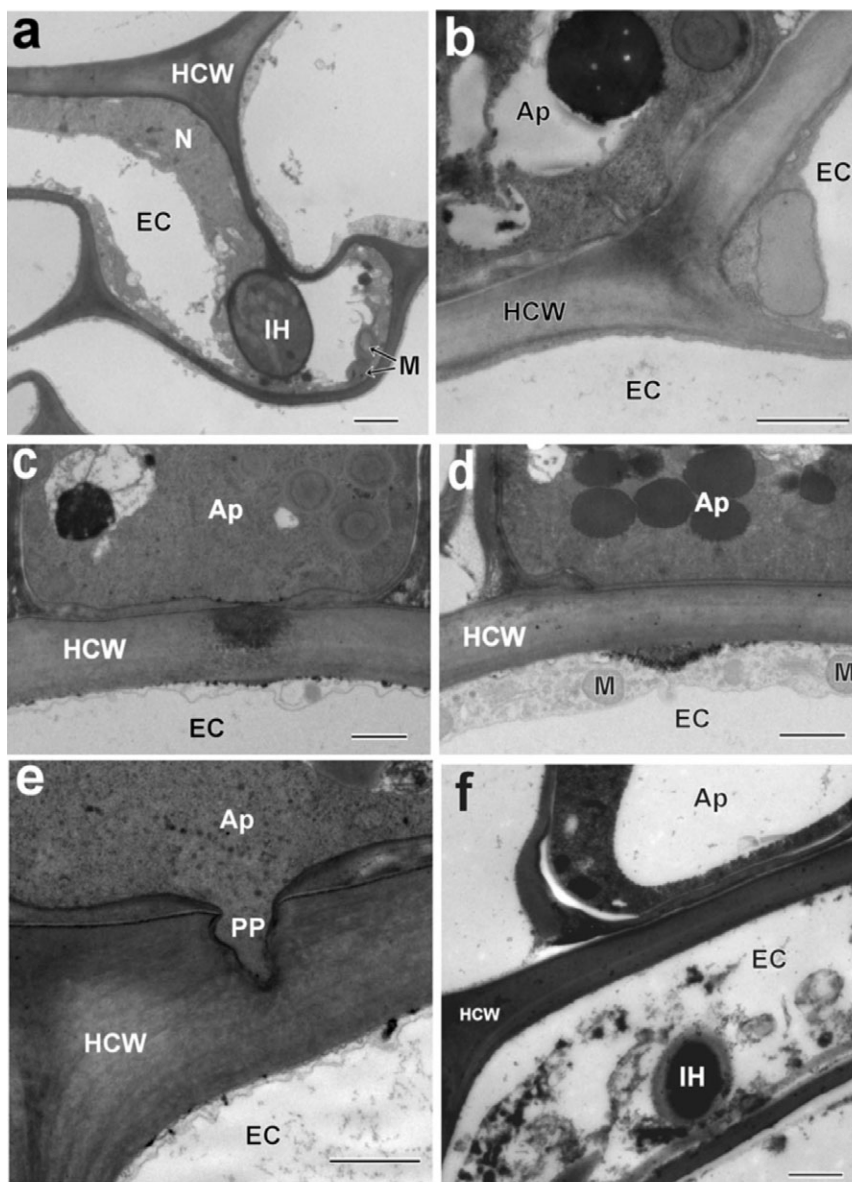

Fig. 3 Ultrastructural localization of H_2O_2 in epidermal cells of *WRKY45*-ox rice leaf sheaths attacked by appressoria of *Magnaporthe grisea*. (a) Development of an invading hypha of *M. grisea* in an epidermal cell of Nipponbare 30 h after inoculation. Nucleus and mitochondria are clearly observed in the invaded epidermal cell, indicating compartmentalization of the cell. (b–e) Four types of host cell reaction in *WRKY45*-ox rice in response to fungal attack 24 h after inoculation. Epidermal cells appear to be compartmentalized in all the types. (b) No penetration, with no host reaction. (c) No penetration, with H_2O_2 accumulation (fine black spots) in the cell wall just beneath an appressorium. (d) No penetration, with H_2O_2 accumulation between the cell wall and cytoplasm in the epidermal cell. (e) Penetration peg invading into the epidermal cell wall. Slight H_2O_2 accumulation is observable around the penetration peg. (f) Development of an invading hypha in the epidermal cell 48 h after inoculation. Note that the epidermal cell no longer shows compartmentalization and the fragmented cytoplasm and the invading hypha are electron dense as a result of H_2O_2 accumulation. Ap, appressorium; EC, epidermal cell; HCW, host cell wall; IH, invading hypha; M, mitochondrion; N, nucleus; PP, penetration peg. Bars, 1.0 μm.

allowed fungus invasion (Fig. 2e). Thus, *WRKY45*-ox rice plants exhibited two layers of defence mechanism against blast fungus: a pre-invasive defence that blocked fungal invasion into cells, and a post-invasive defence accompanying HR cell death that confined fungal growth to primarily invaded cells.

In *WRKY45*-ox rice, DAB staining was not observed in many cells under attack from appressoria (Fig. 2f). However, partial staining was observed in part of the cell wall underneath an appressorium in a small portion of cells (Fig. 2g). In the epidermis of *WRKY45*-ox rice, 24 h after inoculation, H_2O_2 accumulation was

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not detected in the cells at the majority of infection sites (Fig. 3b). However, in some infection sites, precipitation of $Ce(OH)_2OOH$ (as a result of reaction of H_2O_2 with $CeCl_3$) was detected in the cell wall, just underneath an appressorium (Fig. 3c) or between the cell wall and the plasma membrane (Fig. 3d). This TEM observation was consistent with those made using light microscopy (Fig. 2g). Minor H_2O_2 accumulation was also seen around the penetration peg invading the plant cell wall (Fig. 3e). We did not observe H_2O_2 accumulation in *M. grisea*-infected cells of Nipponbare rice. Therefore, H_2O_2 accumulation is likely to be one of the host reactions of *WRKY45*-ox rice that is elicited on attack of fungal appressoria.

Microscopic analysis of the *M. grisea* infection process in probenazole-treated rice

We also characterized microscopically the *M. grisea* infection process in probenazole-treated rice and categorized infection sites into three types (no penetration, restricted and developed), as described previously (Fig. 4; Tanaka *et al*., 2007). Probenazole (0.25 mM) treatment markedly increased the frequency of the 'restricted' type and decreased that of the 'developed' type, indicating the occurrence of post-invasive defence. However, the frequency of the 'no penetration' type did not change significantly. We also tested a higher dosage (1 mM) of probenazole with similar results (data not shown). Thus, probenazole treatment did not induce pre-invasive defence in our experimental conditions. The post-invasive defence was obviously dependent on WRKY45 (Fig. 4), consistent with the results in Fig. 1c.

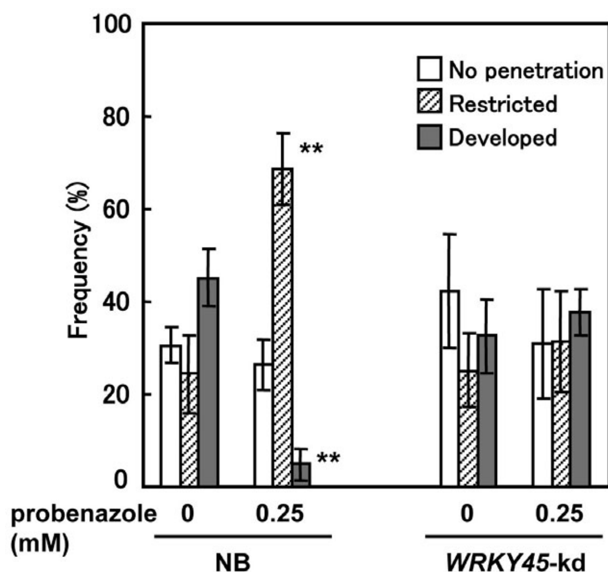

Fig. 4 Microscopic analysis of *Magnaporthe grisea* infectious processes in probenazole-treated rice plants. *WRKY45*-kd and control Nipponbare (NB) rice plants were treated with 0 or 0.25 mM probenazole for 1–2 weeks (Watanabe *et al*., 1977) and inoculated with *M. grisea* (race 007.0) at the six-leaf stage using the leaf sheath inoculation method. Approximately 100 appressoria were observed microscopically at 48 h after inoculation and classified into three categories as follows: no penetration, appressoria formed but failed to penetrate epidermal cells; restricted, primary invading hyphae were restricted within primarily invaded dead host cells; developed, invading primary hyphae grew extensively in living host tissues. Values are the means ± standard deviation from four independent experiments. Asterisks indicate significant difference from the control (0 mM probenazole) using a *t*-test: $^{**}P < 0.01$.

WRKY45 overexpression confers extremely strong leaf and panicle blast resistance

The strength of blast resistance conferred by *WRKY45* overexpression was compared with that in five *japonica* rice cultivars (Sensho, Chubu 32, Koganenishiki, Mutsuhikari and Nipponbare), which exhibit different degrees of blast resistance (Fukuoka and Okuno, 2001; Sugiura *et al*., 2002). Spray inoculation of compatible blast fungus (race 007.0) at the seven-leaf stage demonstrated that both *WRKY45*-ox lines were more resistant than any of the other rice cultivars (Table 1). Among the cultivars used as references, Sensho is known for its high blast resistance, which is comparable with gene-for-gene-based blast resistance, owing to four quantitative trait loci that include *pi21* (Fukuoka and Okuno, 2001; Fukuoka *et al*., 2009). The percentage lesion area in the most susceptible cultivar, Nipponbare, was approximately 70%, whereas that in Sensho was only approximately 0.3%. Under the same conditions, the lesion areas in *WRKY45*-ox plants were even smaller. Therefore, the constitutive expression of *WRKY45* induces extremely strong leaf blast resistance in rice.

Panicle blast infection affects directly the yield and grain quality of rice, thereby reducing crop production more severely than does leaf blast. Generally, plant activators are not as effective against panicle blast as they are against leaf blast, although the reason for this is unclear. We tested the panicle blast resistance of

Table 1 Comparative evaluation of leaf blast resistance in *WRKY45*-ox rice.

	Lesion area (%)	SEM
WRKY45-ox #24	0.0416	0.042
WRKY45-ox #21	0.00416	0.004
Sensho	0.292	0.086
Chubu 32	8.29	1.00
Koganenishiki	26.4	5.60
Mutsuhikari	38.4	5.70
Nipponbare	67.7	5.70

Two lines of *WRKY45*-ox rice (#21 and #24, T_3) and five *japonica* cultivars (Sensho, Chubu 32, Koganenishiki, Mutsuhikari and Nipponbare) were grown in a glasshouse during September–October and inoculated with *Magnaporthe grisea* (race 007.0, 2×10^5 conidia/mL) at the five- to six-leaf stage. Disease symptoms were characterized by lesion areas (%) at 10 days post-inoculation (dpi). Values are the means and standard error of the mean (SEM; $n = 12$). *P* values for the significance of differences between *WRKY45*-ox lines and Sensho were 0.016 (*WRKY45*-ox #24 vs. Sensho) and 0.003 (*WRKY45*-ox #21 vs. Sensho) using Student's *t*-test.

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Fig. 5 Panicle blast resistance of *WRKY45*-ox plants. Control Nipponbare (NB) and *WRKY45*-ox (#15, T3) plants grown in a glasshouse were inoculated with *Magnaporthe grisea* (race 007.0) at the panicle neck. Disease development was characterized at 14 days post-inoculation (dpi).

WRKY45-ox plants by inoculating the panicle neck with fungal conidia and evaluating the disease symptoms at 14 dpi. Severe disease symptoms were observed in the panicle of all but one of 11 Nipponbare plants, resulting in whitish dead panicles (Fig. 5, left). In contrast, none of five (line #5) or 11 (line #21) *WRKY45*-ox plants exhibited disease symptoms (Fig. 5, right). These results show that *WRKY45*-ox plants are more resistant to panicle blast than are Nipponbare plants, with P values of 1.37×10^{-3} (line #5) and 3.40×10^{-5} (line #21), respectively, according to Fisher's exact test (Cochran, 1954). Thus, constitutive overexpression of *WRKY45* also induces strong blast resistance in rice panicles, which is an agronomically important trait.

WRKY45 is also required for rice resistance to leaf blight disease but not sheath blight disease

Plant activators are known for their effects on a broad spectrum of rice diseases, including leaf blight caused by the bacterial pathogen *Xoo*. This prompted us to examine whether WRKY45 plays a role in *Xoo* resistance in rice. The youngest fully expanded leaves at the seven-leaf stage of control Nipponbare and *WRKY45*-kd rice plants were drench treated with BTH and inoculated with bacteria by the scissors-dip method after 4 days (Kauffman *et al*., 1973). At 14 dpi, *Xoo* lesion lengths in mock-treated *WRKY45*-kd plants were slightly longer than those in mock-treated Nipponbare plants (Fig. 6a). This indicates that WRKY45 plays some minor role in basal *Xoo* resistance in the absence of pretreatment by plant activators. Treatment with BTH markedly reduced the lesion lengths in Nipponbare plants compared with mock-treated plants. BTH-induced *Xoo* resistance was partially, but clearly, negated by *WRKY45* knockdown (Fig. 6a), indicating that *WRKY45* has a positive role in BTH-inducible *Xoo* resistance. We then tested *Xoo* resistance in *WRKY45*-ox rice. T_1 segregants of *WRKY45*-ox rice lines, containing zero, one or two copies of the transgene at a single locus, were inoculated with *Xoo* at the eight- to nine-leaf stage. In *WRKY45*-ox lines, disease symptoms in terms of average lesion lengths were markedly suppressed in a manner clearly dependent on transgene number (Fig. 6b, top). Disease severity cosegregated with transgene copy number in individual plants (Fig. 6b, bottom). Transcript levels of *WRKY45* in individual plants correlated well with transgene number and degree of leaf blight resistance (Fig. 6c). Increased expression of WRKY45 proteins of normal size was confirmed in the *WRKY45*-ox line by immunoblotting (Fig. 1a). Taken together, these results show that WRKY45 plays a positive role in *Xoo* resistance.

To further explore the effect of *WRKY45* overexpression on different rice diseases, we examined the resistance of *WRKY45*-ox rice to *Rhizoctonia solani*, a necrotic pathogen that causes rice sheath blight disease (MacNish and Neate, 1996). However, we did not observe any resistance to this pathogen under our experimental conditions (data not shown).

DISCUSSION

WRKY45 is involved in the action of different plant activators

WRKY45 is specifically induced by BTH and SA, but barely induced by a variety of signalling-related molecules, i.e. methyl jasmonate, 1-aminocyclopropane 1-carboxylic acid, indole-3-acetic acid, gibberellin G3, brassinolide and kinetin (Shimono *et al*., 2007). BTH-induced resistance to blast (Shimono *et al*., 2007) and leaf blight (Fig. 5) in rice is negated by *WRKY45* knockdown. Taken together, these observations indicate that SA-mediated defence signalling is at least partly dependent on WRKY45. Different plant activators act at different points in the SA signalling pathway, either upstream or downstream of SA. In this study, we showed that WRKY45 is required for the action of both types of plant activator (Fig. 1). This is in agreement with our model of WRKY45 acting downstream of SA (Shimono *et al*., 2007), and further corroborates the importance of WRKY45 in the SA signalling pathway.

We did not observe any effect of *WRKY45* knockdown on the resistance to compatible *M. grisea* without pretreatment by plant activators. *WRKY45* expression is upregulated 2–3 days after *M. grisea* inoculation (Jiang *et al*., 2010), whereas it is induced within 24 h of BTH treatment (Shimono *et al*., 2007). The lack of WRKY45-dependent blast resistance in the absence of chemical pretreatment is likely to be because of this time lag. Presumably, *WRKY45* must be expressed prior to, or in the early phase of, fungal infection to exert effective defence functions. Resistance to

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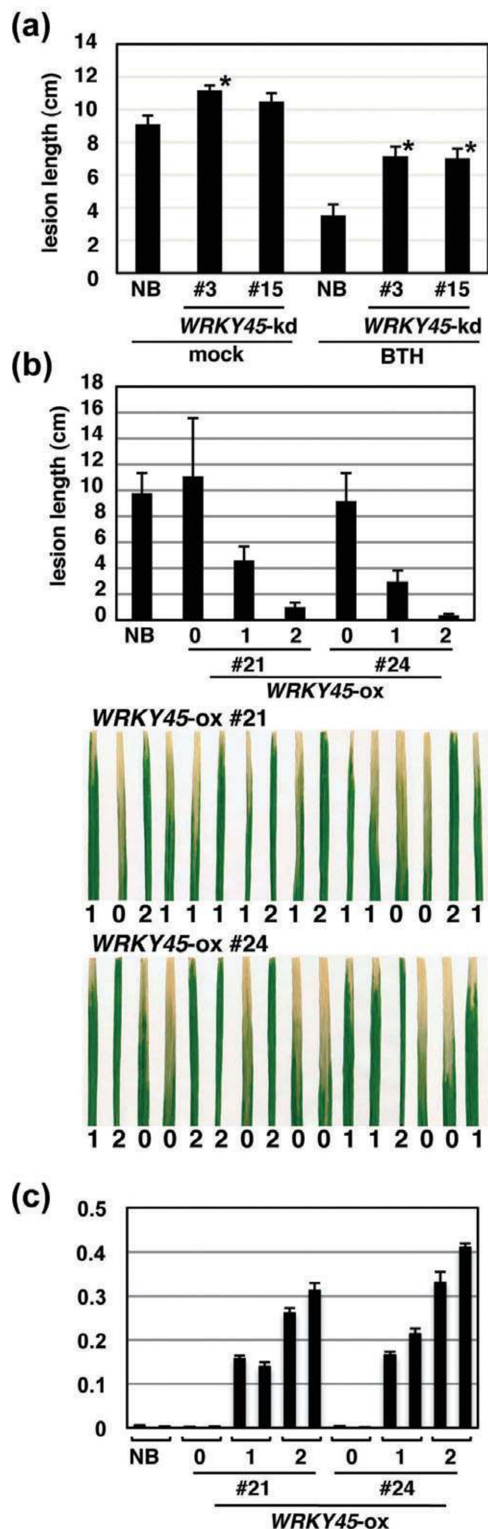

Fig. 6 Bacterial *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) resistance in *WRKY45*-kd and *WRKY45*-ox rice plants. (a) Benzothiadiazole (BTH)-induced *Xoo* resistance in *WRKY45*-kd plants. *WRKY45*-kd (T_3) and Nipponbare (NB) rice plants were grown in a glasshouse, treated with 0.5 mM BTH at the seven-leaf stage and inoculated with *Xoo* 4 days later. Disease symptoms were characterized at 14 days post-inoculation (dpi). Values are the means ± standard error of the mean (SEM) (n = 8–10 plants). Asterisks indicate a significant difference between *WRKY45*-kd and control NB plants at $P < 0.01$. The P value for untreated NB and *WRKY45*-kd (line #15) was 0.09. (b) Disease symptoms in *WRKY45*-ox plants. T_1 seeds of *WRKY45*-ox lines (#21 and #24) with a single transgene insertion were grown in a glasshouse together with Nipponbare (NB) plants. Plants were inoculated with *Xoo* at the eight- to nine-leaf stage and disease symptoms were observed at 14 dpi. Genotypes of individual plants with respect to the *WRKY45*-ox transgene copy number (0, 1 or 2) were determined by real-time polymerase chain reaction (PCR) using genomic DNAs as templates. Values of the lesion number are means ± SEM. Sample numbers (n) were 40 (NB), six (#21, 0 copy), 18 (#21, 1 copy), eight (#21, 2 copy), 18 (#24, 0 copy), 12 (#24, 1 copy) and 10 (#24, 2 copy). Photographs of inoculated leaves in individual plants are shown with the transgene copy numbers. (c) Levels of *WRKY45* transcripts in individual *WRKY45*-ox plants. *WRKY45* transcript levels were determined by real-time reverse transcription PCR in representative individual segregants carrying different transgene numbers.

incompatible blast races was not affected by *WRKY45* knockdown. We have reported previously that the timing of the onset of *WRKY45* upregulation in Nipponbare rice during incompatible interactions (with races 102.0 and 002.0) is similar to that during compatible interactions, whereas the magnitude and duration of the former are higher and longer, respectively, than those of the latter (Jiang *et al*., 2010). The link between the SA signalling pathway and gene-for-gene resistance is unclear (Katagiri *et al*., 2002). In this particular case, rapid and strong WRKY45-independent defence reactions during the incompatible *M. grisea*–rice interaction, including hypersensitive cell death, could have masked WRKY45-dependent defences.

Collectively, the plant protective function of WRKY45 is clearly manifested only when the SA pathway is activated by plant activators. From an evolutionary point of view, the fungus could have developed an unknown mechanism to dampen the rice SA pathway during the history of its arms race with rice.

WRKY45 overexpression induces pre- and post-invasive defence reactions to blast fungi

Microscopic observation of the blast fungus infection process revealed that a two-layered defence system was induced in *WRKY45*-ox rice. Most fungal appressoria were not able to extend infecting hyphae to penetrate epidermal cells in leaf sheaths (pre-invasive defence). Fungal entry into rice cells occurred only infrequently; on these occasions, the second-layer defence mechanism accompanying HR-like cell death prevented further fungal growth (post-invasive defence). The two layers of defence phenotype seem to correspond to the Type I and II nonhost resistances proposed by Mysore and Ryu (2004).

Pre-invasive defence (Type I) has been well characterized in the nonhost resistance of *Arabidopsis* against powdery mildew fungi

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(Collins *et al*., 2003; Lipka *et al*., 2005). Studies on three *pen* mutants (*pen1*, *pen2* and *pen3*), each of which permits efficient fungus entry, identified components of a pre-invasive defence mechanism. *PEN1* encodes a syntaxin-like protein that plays a role in vesicle trafficking in eukaryotic cells (Collins *et al*., 2003). The *PEN2*-encoded protein metabolizes glucosinolates into antimicrobial substances (Bednarek *et al*., 2009). *PEN3* encodes an ABC transporter that is presumably involved in the secretion of antimicrobial substances (Lipka *et al*., 2005; Stein *et al*., 2006). The pre-invasive defence of *Arabidopsis* against powdery mildew fungi depends on these proteins, which synthesize and secrete antimicrobial proteins and metabolites. Although the components of the pre-invasive defence against blast fungi induced in *WRKY45*-ox rice are yet to be identified, similar proteins encoded by *WRKY45*-regulated genes could be involved in its mechanism. In our TEM analysis, H_2O_2 accumulation was detected in the cell wall of *WRKY45*-ox leaf epidermal cells beneath fungal appressoria, as well as between the plant cell wall and plasma membrane, where fungal invasion appeared to be blocked. This suggests that certain enzymes or metabolites that are directly or indirectly involved in H_2O_2 production are secreted in the cell walls of *WRKY45*-ox cells. The attacks of fungal appressoria then presumably elicit H_2O_2 production, either by activating the enzymes or supplying substrates to them. In addition to direct antimicrobial effects, reactive oxygen species produced in response to pathogens are known to play a critical role in defence mechanisms, such as lignin production, lipid peroxidation and phytoalexin production (Baker and Orlandi, 1995). Hydrogen peroxide is also known to drive oxidative cross-linking of proline-rich cell wall structural proteins (Bradley *et al*., 1992). Therefore, it is possible that the H_2O_2 accumulation observed contributes to the pre-invasive defence in *WRKY45*-ox rice.

Post-invasive (Type II) defence accompanying HR-like cell death was observed when *Arabidopsis pen* mutants were infected with the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Collins *et al*., 2003; Lipka *et al*., 2005), similar to *WRKY45*-ox rice plants that permitted fungus entry (Fig. 2d,h). Pre-invasive defence appears to delay hyphal invasion, if any, in both cases. It seems that this delay allows the host cells to prepare for fungal invasion by activating post-invasive defences. Alternatively, in the case of *WRKY45*-ox rice, genes potentially involved in the post-invasive defence mechanism may be constitutively expressed, with fungal invasion triggering their post-transcriptional activation. Transcriptomic analysis of *WRKY45*-ox and/or *WRKY45*-kd plants should provide answers to these questions.

In probenazole-treated rice, only post-invasive defence, and not pre-invasive defence, was observed under our experimental conditions. This is inconsistent with the defence reactions in *WRKY45*-ox rice against *M. grisea*. One possible reason for this inconsistency could be quantitative differences in the defence responses induced in the two systems. In *WRKY45*-ox rice plants, defence compounds are most probably constantly produced; consequently, larger amounts of defence compounds might be accumulated in these plants compared with probenazole-treated rice plants. in which the defence response is induced transiently. However, we cannot totally rule out the possibility that *WRKY45* overexpression induces defence responses that are qualitatively different from those induced by probenazole.

Multidisease resistance by WRKY45 overexpression

Tao *et al*. (2009) reported that two *WRKY45* alleles in *japonica* and *indica* rice have differential functions with respect to leaf blight disease. The *japonica*-derived *WRKY45* (Os*WRKY45-1*) genomic sequence (under the control of the maize *ubiquitin* promoter) was introduced into *japonica* rice cv. Mudanjiang 8, resulting in slightly increased growth of *X. oryzae* in comparison with control untransformed rice plants. The introduction of *indica*-derived *WRKY45* (*WRKY45-2*) rendered the transformants more resistant to the pathogen (Tao *et al*., 2009). In contrast, our results show that overexpression of a cDNA for Nipponbare-derived *WRKY45* (Os*WRKY45-1*), driven by the maize *ubiquitin* promoter, results in very strong *Xoo* resistance. This indicates a positive role for this gene in *Xoo* resistance, and is also in line with the partial negation of BTH-induced *Xoo* resistance in *WRKY45*-kd Nipponbare rice. We employed a standard method of overexpression using full-length cDNA, whereas Tao *et al*. (2009) used a genomic sequence that included introns and a region upstream from transcriptional start sites. We have confirmed that WRKY45 proteins of the same size as those detected in nontransformed Nipponbare rice are produced in our *WRKY45*-ox plants (Fig. 1a). The analysis of WRKY45 proteins produced in the transformants of Tao *et al*. (2009) may help to address this contradiction.

Xoo is a biotrophic pathogen, whereas *M. grisea* is a hemibiotrophic pathogen that initially feeds on living host tissue but later causes the death of plant cells (Talbot and Foster, 2001). It invades living plant cells using intracellular invasive hyphae that grow from one cell to the next; successive cell invasions are biotrophic, but each invaded cell dies when the fungus invades adjacent cells (Kankanala *et al*., 2007). In dicots, SA-dependent systemic acquired resistance plays an important role in plant resistance to biotrophic and hemibiotrophic pathogens (Glazebrook, 2005). In rice, plant activators that act on the SA pathway are effective against the above biotrophic and hemibiotrophic pathogens (Babu *et al*., 2003; Watanabe *et al*., 1977). Glazebrook (2005) have also shown that necrotrophic pathogens, which benefit from plant cell death, are not limited by the SA-dependent defence that usually accompanies HR cell death. Our results showed that *WRKY45*-ox rice plants were resistant to the two hemibiotrophic pathogens, but not to a necrotic pathogen, *R. solani*. This is consistent with the concept that overexpression of *WRKY45* activates SA-dependent defence reactions in the transformants, and hence

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protects them from hemibiotrophic pathogens. In dicots, JA-mediated defence mechanisms are generally effective against necrotrophic pathogens, and the activation of SA signalling suppresses JA signalling, rendering plants more susceptible to necrotic pathogens (Glazebrook, 2005; Thomma *et al*., 1998). In our microarray analysis in *WRKY45*-ox rice plants (A. Akagi *et al*., unpublished data), however, the expression of 13 JA-responsive genes (Yoshii *et al*., 2010) did not appear to be altered, which does not support this hypothesis.

High-level overexpression of *WRKY45* induced extremely strong resistance to the fungal pathogen *M. grisea* and bacterial pathogen *Xoo*. *WRKY45* overexpression was also effective against panicle blast, for which plant activators are not as effective. *WRKY45* overexpression caused relatively minor growth retardation, which was condition dependent (Shimono *et al*., 2007). Considering these agronomically important traits, *WRKY45* seems to be a promising candidate for the development of multidisease-resistant crops, including rice and possibly other grass crops. The elucidation of the molecular mechanisms underlying the effective defence and minor effects on plant growth are among our next challenges.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

WRKY45-ox and *WRKY45*-kd rice plants have the genetic background of *Oryza sativa* L. cv. Nipponbare (Shimono *et al*., 2007). Rice cultivars Sensho, Chubu 32, Koganenishiki and Mutsuhikari were used to compare blast resistance (Fukuoka and Okuno, 2001; Sugiura *et al*., 2002). Plants were grown in a glasshouse in soil (Bonsol no. 2; Sumitomo Chemical Corporation, Tokyo, Japan) at 28/23 °C (day/night) and 70% humidity.

Chemical treatments of rice plants

Rice seedlings were grown in soil in a plastic seedling case (5 cm $\times$ 5 cm $\times$ 5 cm partitions). For chemical treatments, 12 mL each of BTH [Wako, Tokyo, Japan; in 0.5% (v/v) acetone + 0.05% (v/v) Tween 20], probenazole [Meiji Seika, Tokyo, Japan; in 0.5% (v/v) acetone + 0.01% (v/v) Tween 20] and tiadinil [Nihon Noyaku, Tokyo, Japan; in 0.5% (v/v) acetone + 0.01% (v/v) Tween 20] were poured onto the soil surface of each partition in the seedling case. The solvents employed for the respective chemicals were used as procedural controls.

Pathogen inoculation and disease evaluation

To evaluate leaf blast resistance, conidia of the blast fungus (*Magnaporthe grisea* Cavara) of races compatible (007.0) and incompatible (102.0 and 002.0) with Nipponbare were suspended in 0.01% Tween 20 at a density of (1–2) $\times$ 10^5 conidia/mL and sprayed onto rice plants; these were then incubated in a dew chamber for 20 h at 25 °C and grown in a glasshouse for 7 days before disease symptoms were examined, as described by Watanabe *et al*. (1977). Disease symptoms were evaluated as the number of blast lesions in the 10-cm central section of the leaves. Panicle blast assays were performed according to Koizumi and Tani (1998) and Koga *et al*. (2007) with slight modification. Kimwipe S-200 papers (Kimberly Clark Corporation, Irving, TX, USA), immersed in suspensions of blast fungi conidia at a density of 10^6 conidia/mL, were attached to the panicle neck and sealed with adhesive tapes. The plants were grown in a glasshouse for 14 days and disease development was evaluated by the proportion of diseased panicles.

The leaf blight bacterial pathogen *Xoo* strain T7174 was used to inoculate rice. The bacteria were suspended in water [optical density at 600 nm (OD_{600}) = 0.03] and inoculated to the youngest fully expanded rice leaves using the scissors-dip method (Kauffman *et al*., 1973). Lesion lengths were measured at 10–14 dpi.

The sheath blight pathogen *R. solani* (strains MAFF237699, MAFF305231 and SB2-1) was inoculated using the procedure of Sato *et al*. (2004). The mycelium grown on potato sucrose agar medium was homogenized using a food processor, and aliquots (100 μL) were injected inside a leaf sheath (two leaves below the flag leaf) using a plastic syringe at the heading date. Nine plants each were used for inoculation. The lesion area relative to the total area of the leaf sheath just below the flag leaf was evaluated at 28 dpi.

Microscopic analysis of the *M. grisea* infection process

Leaf sheaths of the fifth leaves were peeled off with leaves and roots from soil-grown rice plants at the six-leaf stage, laid horizontally in a plastic case humidified with wet filter papers and inoculated with *M. grisea* according to the procedure of Koga *et al*. (2004b). Hollow spaces enclosed by leaf sheaths above the mid-vein were filled with a suspension of fungal conidia at a density of 2 $\times$ 10^5 conidia/mL. The inoculated leaf sheaths were incubated for 2 or 4 days at 23 °C and observed under a DMR microscope (Leica Microscopy Systems, Wetzlar, Germany).

For observations of plasmolysis to assess the viability of host rice cells, leaf sheaths were immersed in 0.8 M sucrose solution for 20 min before microscopic examination (Koga *et al*., 2004a).

DAB staining

Hydrogen peroxide was detected by DAB staining according to Thordal-Christensen *et al*. (1997) with modifications. Pieces of leaf sheath (10 mm) were immersed in 1 mg/mL DAB (Nacalai Tesque, Kyoto, Japan) and incubated for 8 h at room temperature in the dark.

TEM

A modified cytochemical method for the detection of H_2O_2 was used (Bestwick *et al*., 1997). Leaf pieces (2 mm $\times$ 3 mm) were vacuum infiltrated in 5 mM $CeCl_3$ solution [buffered with 50 mM 3-(*N*-morpholino)propane sulphonic acid (MOPS), pH 7.2] at room temperature for 1 min, and then incubated at room temperature for 1 h. Some pieces were infiltrated *in vacuo* in MOPS buffer without $CeCl_3$ as a control. The leaf pieces were prefixed in 2.5% glutaraldehyde (buffered with 0.1 M cacodylate, pH 7.2) at 4 °C overnight, and then post-fixed in 1% buffered osmium tetroxide at 4 °C for 1 h. The specimens were rinsed with distilled

water for 10 min three times, dehydrated in ethanol, and embedded in Quetol 651 resin mixture (Nissin EM, Tokyo, Japan) according to Shinogi *et al*. (2003). Sections of 90–120 nm in thickness were cut from resin blocks with an ultramicrotome (EM UC6; Leica, Vienna, Austria) using a diamond knife (Diatome, Binene, Switzerland) and observed under a Hitachi 7650 electron microscope (Hitachi, Hitachinaka, Japan).

Immunoblotting

Total protein was extracted from the fourth or fifth leaf blades using protein extraction buffer [40 mM Tris-HCl pH 6.8, 5% sodium dodecylsulphate (SDS), 8 M urea and 0.1 mM ethylenediaminetetraacetic acid (EDTA)]. The extracts were separated on 10% SDS–polyacrylamide gels. Immunoblotting was performed using a standard procedure. Briefly, proteins were transferred to an Immun-Blot poly(vinylidene difluoride) (PVDF) membrane (Bio-Rad, Hercules, CA, USA) and blocked. To detect WRKY45 proteins, the blots were reacted with affinity-purified rabbit polyclonal antibody against the C-terminal peptide sequence of WRKY45, followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody. Bands were visualized with a chemiluminescence kit (ECL Plus; GE Healthcare, Little chalfont, UK).

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