Blast resistance of CC-NB-LRR protein Pb1 is mediated by WRKY45 through protein–protein interaction

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Panicle blast 1 (Pb1) is a panicle blast resistance gene derived from the indica rice cultivar “Modan.” Pb1 encodes a coiled-coil–nucleotide-binding site–leucine-rich repeat (CC-NB-LRR) protein and confers durable, broad-spectrum resistance to Magnaporthe oryzae races. Here, we investigated the molecular mechanisms underlying Pb1-mediated blast resistance. The Pb1 protein interacted with WRKY45, a transcription factor involved in induced resistance via the salicylic acid signaling pathway that is regulated by the ubiquitin proteasome system. Pb1-mediated panicle blast resistance was largely compromised when WRKY45 was knocked down in a Pb1-containing rice cultivar. Leaf blast resistance by Pb1 overexpression (Pb1-ox) was also compromised in WRKY45 knockdown Pb1-ox rice. Blast infection induced higher accumulation of WRKY45 in Pb1-ox than in control Nipponbare rice. Overexpression of Pb1-Quad, a coiled-coil domain mutant that had weak interaction with WRKY45, resulted in significantly weaker blast resistance than that of wild-type Pb1. Overexpression of Pb1 with a nuclear export sequence failed to confer blast resistance to rice. These results suggest that the blast resistance of Pb1 depends on its interaction with WRKY45 in the nucleus. In a transient system using rice protoplasts, coexpression of Pb1 enhanced WRKY45 accumulation and increased WRKY45-dependent transactivation activity, suggesting that protection of WRKY45 from ubiquitin proteasome system degradation is possibly involved in Pb1-dependent blast resistance.

Oryza sativa | field resistance

Rice blast, which is caused by the fungus Magnaporthe oryzae, is one of the most widespread and destructive plant diseases worldwide. Panicle blast 1 (Pb1) is a blast resistance gene originally derived from the indica cultivar “Modan” (1) and was introduced into japonica rice cultivars. Rice cultivars containing Pb1 (Pb1 cultivars) show a characteristic pattern of blast resistance during plant development that is referred to as “adult resistance”: they are blast susceptible during young vegetative stages, but the resistance level increases as the plants grow, and persists even after heading (2). Pb1 has been classified as a field resistance gene because Pb1-dependent blast resistance is nonrace specific, durable, and quantitative. Pb1 encodes a coiled-coil (CC)–nucleotide-binding site (NB)–leucine-rich repeat (LRR) protein similar to Arabidopsis (Arabidopsis) R proteins for gene-for-gene disease resistance (3). Its expression in Pb1 cultivars increases during plant development, and the strength of blast resistance is correlated with the expression pattern (3). In R proteins, several motifs such as the P loop are conserved in their NB domains and required for nucleotide binding. ATP hydrolysis, or transduction of pathogen perception into R protein activation (4–6). Interestingly, Pb1 lacks the P loop and the other motifs are highly degenerated, suggesting that the action mechanisms of Pb1 are different from those of R proteins (3).

It has been reported that nuclear localization or translocation to the nucleus is required for defense activation by R proteins such as barley mildew A (MLA)10 (7), tobacco N (8) and Arabidopsis RPS4 and RPS6 (9, 10, 11). MLA10 translocates to the nucleus and interacts with Hordeum vulgare (Hv)WRKY1/2, which are suppressors of basal defense, during incompatible interaction with powdery mildew fungus (7). Rice Xanthomonas resistance (Xa)21, a pattern recognition receptor for Xanthomonas oryzae pv. oryzae (Xoo) of receptor kinase structure, has also been reported to interact with Oryza sativa (Os)WRKY62 in yeast two-hybrid (Y2H) assay (12). Both HvWRKY1/2 and OsWRKY62 belong to group Ia of the WRKY superfamily (13), which is characteristic of repressor-type transcription factors (TFs).

The salicylic-acid (SA) signaling pathway is crucial in systemic acquired resistance and a transcriptional cofactor, nonexpressor of PR1 (NPR1), has a central role in Arabidopsis (14). In rice, the SA pathway branches into OsNPR1- and WRKY45-dependent subpathways (15–17). WRKY45 is a transcriptional activator of group III and plays a major role in rice resistance to M. oryzae and Xoo induced by chemical inducers such as benzo(thio)diiazole (BTH) (15, 18). In Arabidopsis, NPR1 is regulated by the ubiquitin proteasome system (UPS) and a dual role has been proposed for this regulation: suppression of defense in the absence of pathogens and enhancement of the transcription triggered by SA signaling (19). In rice, we have shown that WRKY45, but not OsNPR1, is regulated by UPS to suppress unnecessary defense reactions in the absence of pathogens and possibly to enhance its transcriptional activity upon activation of the SA pathway (20).

In this study, we investigated the molecular mechanisms underlying Pb1-dependent defense signaling. Our results showed that the Pb1 protein interacted with WRKY45 and the blast resistance by Pb1 depended on WRKY45. In addition, WRKY45 was protected from degradation by coexpressed Pb1. Based on these results, we propose a mechanism of Pb1-mediated blast resistance through direct interaction with WRKY45.

Results

Pb1 Interacts with WRKY45 Through Its CC Domain. To explore the possibility that defense signaling by Pb1 involves direct interaction with WRKY TFs, similarly to MLA10 and Xa21, we performed targeted Y2H analysis between Pb1 and rice WRKY TFs. The N-terminal region of Pb1 encoded a putative CC domain (amino acids 1–44), which was predicted by Marcoil 1.0 (http://bcf.isb-sib.ch/webmarcoil/webmarcoilC1.html) (21) based on the periodic occurrence of hydrophobic amino acids at

Author contributions: H.I. and H.T. designed research; H.I., N.H., A.M., L.X., A.N., S.S., and C.-I.I. performed research; and H.I. and H.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1221551110/-/DCSupplemental.
positions 16 (I), 23 (L), 30 (V), and 37 (M). The putative Pb1-CC was fused to the GAL4 DNA-binding domain and used as bait. Several rice WRKY TFs (WRKY19, 45, 47, 66, 62, 68, 76), which were chosen arbitrarily from different classes (13, 22), were fused to the GAL4 activation domain and used as prey. Because the expression of some of their full-length forms inhibited yeast growth, we used their N-terminal regions to generate the fusion proteins. The proteins were expressed in yeast cells (Fig. S1) and tested for protein–protein interaction. The results showed that Pb1-CC interacted with only WRKY45, but not with other WRKY proteins including WRKY47, another group III WRKY protein, in yeast cells (Fig. L4). The interaction was also observed between wild-type Pb1-CC (Pb1-CC1-44 and Pb1-CC1-51) and full-length WRKY45 in a GST-pulldown assay (Fig. 1B). Then, we examined the effects of mutation of the amino acids, which served as the basis for the prediction of CC structure in Pb1. Whereas mutants of Pb1-CC that had mutations in the two hydrophobic amino acids (Pb1-CC1-51A6A23A and Pb1-CC1-51A30A37A) interacted with WRKY45 similarly to wild-type Pb1-CC1-51, Pb1-16A23A30A37A (Quad), in which the four hydrophobic amino acids were mutated, showed markedly decreased interaction with WRKY45 in comparison with its corresponding wild-type (Pb1-CC1-44 and Pb1-CC1-51) and full-length Pb1-CC in a GST-pulldown assay (Fig. 1A). A coimmunoprecipitation assay showed that full-length Pb1 interacted with full-length WRKY45, but Pb1-Quad (full-length) interacted very weakly (Fig. 1C). This Pb1–WRKY45 interaction and the reduction of the interaction by CC mutation were also observed in a split LUC system (23, 24) by using N-terminal and C-terminal luciferases fused with Pb1 and WRKY45, respectively, in rice leaf sheath cells (Fig. 1D).

**Pb1-Mediated Blast Resistance Depends on WRKY45.** When compatible races of *M. oryzae* were inoculated onto Nipponbare rice, WRKY45 proteins accumulated with a peak at 2–3 d after inoculation (dpi). WRKY45 accumulation reached higher levels and lasted longer in *Pb1* overexpression (*Pb1-ox*) plants than in Nipponbare (Fig. 2A). Transcript levels of WRKY45 were higher in *Pb1-ox* than in Nipponbare (Fig. 2B). Those of the WRKY45-regulated genes WRKY62 and RDX20 (20) were also higher in *Pb1-ox* (Fig. 2B) than in Nipponbare. The elevation of WRKY45 transcript levels was also observed in rice cultivars containing natural allele of *Pb1* (see below) in adult stages before and shortly after blast fungus infection (Fig. S2).

To examine whether the panicle blast resistance by Pb1 depends on WRKY45, we introduced a *WRKY45* knockdown (*WRKY45-kd*) construct (15) into Aichi-Koshihikari-SBL (a rice cultivar in which the *Pb1* locus was introgressed) and its blast susceptible near-isogenic cultivar without *Pb1* (Koshihikari), generating the rice lines *Pb1*-/*WRKY45-kd and *Pb1*/WRKY45-kd, respectively. We previously showed that WRKY45-kd largely compromised BTH-induced resistance to blast diseases (15), suggesting the absence of major functional redundancy for WRKY45. The expression levels of *Pb1* in *Pb1*/WRKY45-kd lines were similar to those in the parental *Pb1* line (Fig. S3), indicating that the WRKY45 knockdown did not affect *Pb1* expression in these transformants. A panicle blast resistance test showed that the resistance was compromised in *Pb1*/*WRKY45-kd (lines 4 and 11), in which *WRKY45* transcripts in panicles were reduced, to the levels of *Pb1* (Koshihikari) lines (Fig. 3A and Fig. S3). The *Pb1*/WRKY45-kd line 6, in which the *WRKY45* transcript level was somewhat higher than those in the other two lines (4 and 11), exhibited weaker panicle resistance, showing a correlation between the residual levels of *WRKY45* transcripts and the strength of panicle blast resistance (Fig. 3A and Fig. S3). The levels of panicle resistance in *Pb1*/WRKY45-kd plants were similar to those in the *Pb1* line (Koshihikari) line, indicating that *WRKY45* knockdown did not affect the basal levels of blast resistance in the panicle (Fig. 3A).

Constitutive *Pb1-ox* confers leaf blast resistance (3). To examine the effect of *WRKY45* knockdown on the leaf blast resistance induced by *Pb1* overexpression, we generated rice transformants that overexpressed *Pb1* in a *WRKY45-kd* Nipponbare background (*Pb1-ox/WRKY45-kd*). We selected several *Pb1-ox/WRKY45-kd* lines in which *Pb1* was overexpressed at levels similar to some *Pb1*-ox lines (Fig. S4A) and *WRKY45* was knocked down (Fig. S4B). Leaf blast resistance tests showed that the resistance by *Pb1* overexpression was largely compromised by *WRKY45* knockdown (Fig. 3B). In rice transformants overexpressing *Pb1-Quad* in Nipponbare (*Pb1-Quad-ox*) (Fig. S5), leaf blast resistance was weaker than in *Pb1*-ox plants expressing wild-type *Pb1* at comparable levels (Fig. 3C). Collectively, these results indicated that *WRKY45* is required for *Pb1*-mediated blast resistance both in the panicle and the leaf and suggested that direct interaction with *WRKY45* through the CC domain is required for the resistance.

The *WRKY45*-ox rice plants showed strong preinvasive defense against rice blast (18). When *Pb1*-ox rice plants were inoculated with *M. oryzae* in leaf sheaths (25), fungal invasion into rice cells was observed from only 15–20% of fungal conidia, whereas the invasion rate was 80% in Nipponbare. The invasion rate into *Pb1-ox*/WRKY45-kd rice was comparable to Nipponbare (Fig. 3D). These results were also consistent with *WRKY45*-dependent blast resistance by *Pb1*.

**Nuclear Localization of Pb1 Is Essential for Blast Resistance.** Intracellular localization of *Pb1* and *WRKY45* were examined by expressing GFP-*Pb1* and GFP-WRKY45 fusion proteins in rice protoplasts. GFP-*Pb1* was detected in both the cytoplasm and the nucleus (Fig. 4A), whereas GFP-WRKY45 was localized to
The blast resistance by NahG lines (Fig. S7). The rice lines overexpressing ox/Pb1 partially depends on the SA signaling pathway. lines were more resistant than the NahG expression: The Pb1-ox/NahG lines were more resistant than the NahG lines but less resistant than Nipponbare. These results indicated that blast resistance by Pb1 partially depends on the SA signaling pathway.

Pb1 Prevents Ubiquitin-Proteasome Degradation of WRKY45. When we expressed WRKY45 proteins in wheat germ extracts, the addition of the proteasome inhibitor MG132 resulted in the accumulation of WRKY45 proteins (Fig. S4), suggesting that WRKY45 was degraded by UPS in the extract, as in rice plants (20). Interestingly, coexpression of Pb1, instead of MG132 addition, stabilized WRKY45 proteins in the extract (Fig. 5A). Coexpression of Pb1-Quad resulted in smaller amounts of WRKY45 proteins (Fig. 5B).

To investigate this phenomenon, myc-WRKY45 was transiently expressed in rice protoplasts and the effect of Pb1 coexpression on the WRKY45 protein levels was tested. As shown in Fig. 5C, myc-WRKY45 was accumulated by coexpression of Pb1 and, to a lesser extent, by coexpression of Pb1-Quad or Pb1-NES. These results suggested that Pb1 protected WRKY45 proteins from UPS-dependent degradation through a protein–protein interaction.

The effects of Pb1 coexpression on transcriptional activities by WRKY45 were examined in a transient transactivation assay by using rice sheath cells. A reporter construct that contained W-box sequences fused upstream of the luciferase gene (15) was delivered to rice leaf sheath cells by particle bombardment together with effector constructs for WRKY45 and/or Pb1. Coexpression of Pb1 enhanced the luciferase activity due to WRKY45 by approximately fourfold (Fig. 5D). In contrast, coexpression of Pb1-Quad or Pb1-NES enhanced the activity by only twofold (Fig. 5D). No significant activity was detected with Pb1 only (Fig. S8), indicating that the luciferase activity depended on introduced WRKY45. These results are consistent with Pb1-stabilizing WRKY45 proteins without inhibiting their transcriptional activity. Blast fungus

![Fig. 2. Expression of WRKY45 proteins and downstream genes in Pb1-ox rice after blast inoculation. (A) Temporal expression patterns of WRKY45 proteins after rice blast infection. Nipponbare and Pb1-ox plants were spray inoculated with blast fungal conidia (6 × 10^6 mL^-1). Total proteins were extracted from the leaves over time, and WRKY45 proteins were detected by Western blot using anti-WRKY45 antibody. The experiments were performed four times by using different lines with similar results. (B) Transcript levels of the WRKY45 gene and WRKY45-regulated genes after rice blast inoculation. WRKY45, WRKY62 (AK067834), and RDX (AK104089) were analyzed by quantitative RT-PCR in Nipponbare and Pb1-ox plants. Means of two technical repeats are shown with SD. We obtained similar results in two independent experiments.

the nucleus (Fig. 4A). In subcellular fractionation of leaf cells from Pb1-ox plants, ~1/20th of total Pb1 proteins were detected in nuclei-enriched fractions and the remainder in nuclei-depleted fractions (Fig. 4C). To examine whether the nuclear localization of Pb1 is essential for Pb1-dependent blast resistance, we overexpressed Pb1 fused with a nuclear exclusion sequence (NES), which were largely excluded from nuclei when expressed in rice protoplasts (Fig. S6), in rice transformants (Pb1-NES-ox). A blast resistance test showed that Pb1-NES-ox lines were markedly less blast resistant than Pb1-ox lines (Fig. 4B). Fractionation of cell extracts from blast-infected leaves of Pb1-ox rice plants showed that the subcellular distribution of Pb1 did not change after blast fungus infection.

Pb1-Mediated Blast Resistance Partially Depends on SA. WRKY45 is a key transcription factor of the SA pathway in rice (15). To examine whether the depletion of endogenous SA influences the blast resistance by Pb1, we overexpressed NahG, the gene encoding a SA-degrading protein, in a Pb1-ox background (Pb1-ox/NahG) and tested for blast resistance along with Pb1-ox and NahG lines (Fig. 5). The rice lines overexpressing NahG only (NahG) were more blast susceptible than control Nipponbare, as reported (26). The blast resistance by Pb1 overexpression was largely compromised by NahG expression: The Pb1-ox/NahG lines were more resistant than the NahG lines but less resistant than Nipponbare. These results indicated that blast resistance by Pb1 partially depends on the SA signaling pathway.

![Fig. 3. Pb1-mediated panicle blast resistance is WRKY45 dependent. (A) Effects of WRKY45 knockdown on panicle blast resistance. The plants were spray inoculated with blast fungus conidia (1–3 × 10^6 mL^-1) at full-heading stage and evaluated for disease symptoms by percent diseased grains. Averages of 20 plants each are shown with SEs. The experiments were performed four times with similar results. (B) Effects of WRKY45 knockdown on leaf-blast resistance by Pb1 overexpression. The plants were spray inoculated with blast fungal conidia (6 × 10^6 mL^-1) and evaluated for diseased leaf area at 11 dpi. Average lesion areas (%) in 20 plants are shown with SE. The experiments were performed four times with similar results. NB, Nipponbare. (C) Pb1–WRKY45 interaction is required for blast resistance. Leaf blast resistance tests were performed using transformants that overexpressed HA-St-tagged Pb1 or Pb1-Quad at comparable levels (Fig. 5). The experiments were performed three times with similar results. (D) Pb1-dependent blast resistance is mainly due to preinvasive defense. The plants were inoculated with rice blast fungus by leaf sheath inoculation and examined for the rate of invasion into rice at 2 dpi. Means are shown with SD. Inoue et al.}
inoculation elevated the accumulation of WRKY45 proteins in Pb1-ox rice to higher levels than in Nipponbare (Fig. 2), which suggests that the inhibition of WRKY45 degradation contributes to blast resistance by Pb1.

Discussion

We showed in this report that the CC-NB-LRR protein Pb1 interacts with WRKY45, a key transcription factor of the SA pathway, and that the blast resistance by Pb1 depends on WRKY45. Data suggested that protection of WRKY45 from UPS degradation is involved in the blast resistance by Pb1.

Defense signaling through protein–protein interaction between pathogen sensors and WRKY TFs has been reported in barley and rice. In barley, MLA10, a CC-NB-LRR-type R protein, binds to HvWRKY1/2 in the nucleus to induce effector-triggered immunity (ETI) against a specific race of *Blumeria graminis* f. sp. *hordei* (*Bgh*) (7). HvWRKY1/2 are transcriptional repressors that presumably suppress defense activation in the absence of pathogens, and transcription becomes derepressed upon *Bgh* infection triggered by binding of MLA10, leading to defense activation (7). In rice, Xa21, a pattern recognition receptor for *Xoo* resistance, interacts with rice WRKY62 (12). WRKY62 is also a transcriptional repressor and suppresses *Xoo* resistance when overexpressed (12). The Xa21-mediated *Xoo* resistance through interaction with WRKY62 is also thought to be based on derepression of defense (12, 27). Whereas HvWRKY1/2 and WRKY45 are transcriptional repressors, rice WRKY45 is a transcriptional activator; therefore, the mechanisms of defense activation by Pb1-WRKY45 interaction must be different from the two cases mentioned above. Our data suggest that Pb1 prevents the UPS-dependent degradation of WRKY45, which serves to suppress untimely defense activation in the absence of pathogens (20). The UPS-dependent WRKY45 degradation leads to defense repression; therefore, its prevention is also a derepression of defense, although the molecular mechanisms involved are different.

Nuclear localization of R proteins is essential for ETI in various pathosystems (7–9). barley MLA10, triggered by the binding of an effector, avrA10, translocates to the nucleus where it interacts with HvWRKY1/2 (7). A nuclear accumulation requirement for triggering defense responses has also been reported for RPS4, an *Arabidopsis* R protein (9). Our results showed that the addition of a nuclear exclusion signal to Pb1 reduced its ability to induce blast resistance; thus, nuclear localization of Pb1 is required for inducing blast resistance. However, Pb1 proteins overexpressed in rice cells were mainly detected in nuclei-depleted fractions, with only a minor portion localized to the nucleus, and this distribution pattern did not change by blast inoculation, unlike MLA10 (Fig. 4C). Thus, constitutive nuclear localization of Pb1, rather than its nuclear translocation after pathogen infection, appears to be required for Pb1-mediated blast resistance, although we cannot
exclude nuclear translocation of part of the Pb1 proteins after infection. Some sequence motifs highly conserved in NB-LRR-type R proteins, such as the ATP-binding motif, have been shown to be important for the activation of defense signaling. The activation of defense signaling by nuclear translocation of MLA10 also involves its activation through the NB domain (28). In Pb1, these motifs are missing (P loop) or highly degenerated, suggesting that the NB domain of Pb1 is functionally different from those of R proteins (3). These characteristic sequence features could be a key to elucidating the activation mechanism of Pb1-dependent defense signaling.

Overexpression of NahG weakened rice blast resistance due to Pb1 overexpression (Fig. S7). These results indicate that Pb1-mediated blast resistance partially depends on the SA signaling pathway. Two possible mechanisms can account for the partial SA dependence of Pb1-mediated blast resistance. One is that Pb1 activates the SA pathway upstream of SA as many R proteins do (29), leading to WRKY45 degradation by Pb1. In the latter scenario, activation of Pb1 by pathogen recognition is not necessarily required in Pb1-dependent blast resistance because induction of WRKY45 through the SA pathway (or products) and subsequent enhancement of WRKY45 protein levels by nuclear localized Pb1 can account for the Pb1-dependent blast resistance. WRKY45 proteins, and transcripts of WRKY45 and its downstream genes, increased more highly in Pb1-ox rice compared with Nipponbare after blast inoculation (Fig. 2). It is likely that protection of WRKY45 by Pb1 plays a role in this up-regulation because WRKY45 autoregulates its own gene (20); however, it is difficult to evaluate the extent of its contribution.

Pb1 is unique in its durability of resistance and apparent absence of race specificity despite its NB-LRR structure. However, the molecular mechanism of defense induction through direct interaction with WRKY45 proposed in this study does not by itself account for the durability of Pb1-dependent blast resistance. Function-structure analysis of Pb1 protein, with respect to the defense signaling through direct interaction with WRKY45, will be required to advance our understanding of the mechanisms underlying the characteristics of Pb1-dependent rice blast resistance.

Materials and Methods

Y2H. A bait construct was generated by inserting a DNA fragment encoding the Pb1-CC domain (bp 1–132), which was PCR amplified by using Pb1-1CCFW-EcoRI/Pb1-1CCRV-PstI primers, into the pGBKTK7 vector (Clontech) using EcoRI and PstI (pGBK7-Pb1-1C). Pre-constructs were generated by inserting DNA fragments encoding the N-terminal regions of WRKY13 (1–162), 19 (1–162), 45 (1–174), 47 (1–178), 62 (1–198), 66 (1–293), 68 (1–275), and 76 (1–293) into the pGAD7 vector using an In-fusion kit (Takara Bio, www.takara-bio.co.jp).

Y2H assays were performed according to the manufacturer's protocol (Clontech, www.clontech.com). The primers used in plasmid construction are listed in Table S1.

GST Pulldown. To generate constructs for GST-pulldown assays, the Pb1-CC sequence (base pairs 1–132) was inserted into the pGEX-6-1 vector (GE Healthcare, www.gehealthcare.com). Mutations were introduced into the Pb1-CC by recombinant PCR to generate Pb1-16A23A, Pb1-30A37A, and Pb1-Quad fragments. The DNA fragments (base pairs 1–153) containing the mutations were inserted into pGEX-6-1 by using EcoRI and Xhol.

For GST-pulldown assays, GST and Pb1-CC fusion proteins were induced in Escherichia coli BL21 (DE3) strain (Novagen, www.merckmillipore.jp) at 30 °C for 4 h by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 mM. Proteins were extracted from the E. coli cells by using a Bug Buster HT kit (Takara Bio), loaded onto glutathione-sepharose resin GST accept (Nacalai, www.nacalai.co.jp), eluted with a buffer (50 mM Tris-HCl at pH 8.0 and 1 mM EDTA) containing 10 mM glutathione, and separated on a 15% (wt/vol) SDS-polyacrylamide gel. Proteins were detected with a Zinc staining kit (Bio-Rad, www.biorad.com).

WRKY45 HA protein was detected by Western blotting using anti-HA antibody.

Generation of Rice Transformants. Constructs for overexpressing Pb1 derivatives in rice were generated by modifying the pZ11- Pb1-HASt (hemagglutinin-streptavidin) construct (33). pZ11–Pb1-Quad-HAST was generated by replacing the wild-type Pb1-CC sequence in pZ11–Pb1-HASt with the Pb1-Quad sequence using Sall and Aef site. pZ11–Pb1-HASt-NESt was generated by inserting a DNA sequence for NESt (NELAKLAGLDINK), which was amplified by using Pb1-NESt rv and Pb1-3000 Fw primers, into pZ11–Pb1-HASt. A Pb1 overexpression construct was generated by PCR amplifying the bialaphos resistance (bar) maker gene (30) using Ubq-bar Fw/Ubq-bar Rv primers with pUC-BAR plasmid as a template, and inserted into pZ11–Pb1-HASt at an Ascl site, yielding pZ11–Pb1-HASt-BAR.

A WRKY45-kd construct (15) was introduced into rice cv. Koshikihari-Alchi-SBL (Pb1') and its near isogenic cv. Koshikihari (Pb1) by Agrobacterium tumefaciens transformation (31). To generate Pb1-ox/WRKY45-kd plants, pZ11–Pb1-HASt-BAR was introduced into WRKY45-kd Nipponbare lines (15) and selected by the resistance to Bialaphos (Wako Chemical, www.wako-chem.co.jp). pZ11–Pb1-NESt-HAST and pZ11–Quad-HAST constructs were introduced into Nipponbare as described (32).

Nuclear Fractionation. Fresh rice leaves were ground into powder in liquid nitrogen by using a mortar and pestle and thawed in extraction buffer 1 (10 mM Tris-HCl at pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 340 mM sucrose, 10% glycerol, and 100 μM MG132). The homogenates were filtered through a nylon mesh of 48-μm pores and incubated on ice for 10 min. Then, the filtrates were centrifuged at 1,000 x g for 20 min in 30% Percoll in buffer 1 (Sigma-Aldrich, www.sigmaaldrich.com). The supernatants were used as nuclei-depleted fractions. The pellets were resuspended in extraction buffer 2 (50 mM Tris-HCl at pH 7.5, 1 mM MgCl2, 1 μM Benzamidine, 50 μM NaCl, and 100 μM MG132), incubated for 10 min on ice, centrifuged briefly, and the supernatants were used as nuclei-enriched fractions. Fractionation of rice protoplasts was performed as described above except that the extraction buffer 1 contained 0.6% Triton X-100.

Transactivation Assay. Inner leaf sheaths of rice were placed on 0.5% agar plates containing 0.4 M Mannitol. Gold particles coated with 0.5 μg of effector, 0.3 μg of reporter, and 0.1 μg of reference plasmids (33) were introduced into the rice leaf sheaths by using a PDS-1000/He Biolistic particle delivery system (Bio-Rad). After incubation at 28 °C overnight, samples were collected and ground in liquid nitrogen. Luciferase activities were assayed with the DualGlo Luciferase Reporter Assay System and the NanoLuc Luciferase technology (Promega, www.promega.com).

Split Luciferase Assay. The sequence of the maize ubiquitin promoter was PCR amplified with pZ11–Pb1-HASt as a template and inserted into the pSAt4 vector (34) by using AgeI and SacI, yielding pZ11–Pb1-HASt-BAR. The sequence for the 5′ untranslated region of rice alcohol dehydrogenase (ADH) (35), the HA tag, and the N-terminal region of Renilla luciferase (NRLuc, base pairs 1–687) were amplified by PCR with rice genomic DNA, HA synthetic nucleotides, and pGL4.70 plasmid (Promega), respectively, as templates, using the primer pairs listed in Table S1. The three fragments were then mixed and subjected to second-round PCR. The ADH-HA-NRLuc fragment obtained was inserted downstream of the ubiquitin promoter, yielding pZ11–Pb1-HASt-ADH-NRLuc (C-term Renilla Luciferase) was constructed in a similar manner except that 3×myc tag amplified with Pubi/myc/WRKY45 as a template was inserted upstream of CRLuc. The fragments for Pb1 derivatives and WRKY45 were inserted downstream of NRLuc and CRLuc in the two corresponding vectors. These plasmids were delivered into rice leaf suspensions together with a reference plasmid (PUBi-Luc) as described above. RLuc activities were normalized to the reference Luc activity.

WRKY45 Protection Assay in Protoplasts. The ADH and 3×HA sequences were amplified by PCR with the rice genome and 3×HA synthetic DNA as templates, respectively. The two fragments were mixed and subjected to second-round PCR. The ADH-HA fragment was inserted into pSAt4-Pubi by using SacI and XbaI, and then Pb1, Pb1-Quad, and Pb1-NESt fragments were inserted downstream of the 3×HA tag by using Sall and NotI. These plasmids were cotransfected with Pubi/myc/WRKY45 (20) to protoplasts of rice Oc cells by electroporation as described (36). After incubation overnight, HA-tagged Pb1 derivatives and myc-WRKY45 were detected by Western blot.
ACKNOWLEDGMENTS. This work was supported by Grant-in-Aid for Young Scientists 2270048 and Japanese Ministry of Agriculture, Forestry and Fisheries Genomics for Agricultural Innovation Grants GMA0001 and PM10008.