

Rice RING protein OsBBI1 with E3 ligase activity confers broad-spectrum resistance against *Magnaporthe oryzae* by modifying the cell wall defence

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Emerging evidence suggests that E3 ligases play critical roles in diverse biological processes, including innate immune responses in plants. However, the mechanism of the E3 ligase involvement in plant innate immunity is unclear. We report that a rice gene, *OsBB11*, encoding a RING finger protein with E3 ligase activity, mediates broad-spectrum disease resistance. The expression of *OsBB11* was induced by rice blast fungus *Magnaporthe oryzae*, as well as chemical inducers, benzothiadiazole and salicylic acid. Biochemical analysis revealed that OsBB11 protein possesses E3 ubiquitin ligase activity *in vitro*. Genetic analysis revealed that the loss of *OsBB11* function in a *Tos17*-insertion line increased susceptibility, while the overexpression of *OsBB11* in transgenic plants conferred enhanced resistance to multiple races of *M. oryzae*. This indicates that OsBB11 modulates broad-spectrum resistance against the blast fungus. The *OsBB11*-overexpressing plants showed higher levels of H₂O₂ accumulation in cells and higher levels of phenolic compounds and cross-linking of proteins in cell walls at infection sites by *M. oryzae* compared with wild-type (WT) plants. The cell walls were thicker in the *OsBB11*-overexpressing plants and thinner in the mutant plants than in the WT plants. Our results suggest that OsBB11 modulates broad-spectrum resistance to blast fungus by modifying cell wall defence responses. The functional characterization of OsBB11 provides insight into the E3 ligase-mediated innate immunity, and a practical tool for constructing broad-spectrum resistance against the most destructive disease in rice.

Keywords: blast fungus; cell wall defence; disease resistance; E3 ligase activity; *OsBB11*; rice

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Introduction

Plants have developed a precisely regulated innate immune machinery to defend themselves against potential microbial attack. Recent studies have revealed that innate immune system in plants comprises two basic inducible

defence responses [1-5]. The first layer of innate immune response is activated on detection of conserved pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) by cell surface pattern-recognition receptors (PRRs), resulting in PAMP/MAMP-triggered immunity (PTI). The second layer of innate immune response is activated on recognition of pathogen-secreted effectors by host disease resistance (*R*) gene-encoding proteins, leading to race-specific effector-triggered immunity (ETI). Similar signalling events are involved in regulating PTI and ETI, including changes in cellular redox status and cytoplasmic Ca²⁺ levels, modification of specific proteins (e.g., phosphorylation), generation of signalling molecules (e.g., salicylic acid (SA), jasmonic acid (JA), ethylene (ET), nitric oxide, and reactive oxygen species

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(ROS)), induction of defence-related genes, and reinforcement of cell walls [1-6]. Despite their importance in defence responses, our knowledge on the molecular mechanisms of PTI and ETI is still limited.

Blast disease, caused by *Magnaporthe oryzae*, is the most serious constraint on rice production. Significant progress has been made in the last decade on understanding the molecular basis of innate immune response in rice against *M. oryzae* by applying combined molecular and genomic approaches. Nine blast *R* genes were cloned and characterized [7]. The recognition of *M. oryzae*-secreted effector protein Avr-Pita by the R protein Pita inside the rice cell could activate innate immune response [8]. Two *R* genes, *Pi9* and *Pi21*, confer broad-spectrum resistance against multiple races of *M. oryzae* [9, 10]. Rac GTPase-mediated events (e.g., generation of ROS), activation of MAP kinase, and other key components, such as RAR1, SGT1, Hsp90, and WRKY45, play important roles in regulating the immune response against *M. oryzae* [11-24]. Endogenous SA is involved in defence against *M. oryzae* [25]. A germin-like protein gene family acts as a complex quantitative trait locus conferring broad-spectrum resistance against multiple races of *M. oryzae* [26]. However, extensive studies are required to elucidate the precise molecular mechanism of immune response in rice against *M. oryzae*, which could provide tools for transgenic improvement of rice blast resistance.

In studies aimed at elucidating the molecular basis of plant immune response, one main strategy is approaches based on identifying novel proteins resulted from the induction of gene expression, in addition to map-based cloning of *R* and mutant genes. Recent studies showed that protein degradation is one of the most important biochemical events that play critical roles in regulating immune response. The ubiquitin (Ubi)/26S proteasome system constitutes a primary pathway for degrading proteins in eukaryotes. It starts with the ubiquitination of substrate proteins, which are then targeted for degradation (for review see [27]). Emerging experimental evidence indicates that protein degradation via the Ubi/26S proteasome system plays important roles in plant innate immune response (for review see [28]).

In rice, several proteins with activity of E3 Ubi ligases, a group of enzymes required for ubiquitination of substrate proteins in the Ubi/26S proteasome system, are involved in regulating innate immune response [29-31]. For example, SPL1, a U-box protein with E3 Ubi ligase activity, is a negative regulator of cell death and innate immunity against *M. oryzae* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the causal agent of bacterial leaf blight disease [30]. XB3, an E3 Ubi ligase, is necessary for full accumulation of the XA21 protein and for XA21-

mediated innate immune response against *Xoo* [31]. XA21 was recently identified as a PRR with race-specific feature against *Xoo* [32]. We characterized here a rice gene, *OsBBII*, which is induced by blast fungus and benzothiadiazole (BTH), and encodes a RING protein with E3 Ubi ligase activity *in vitro*. Functional analyses using a *Tos17*-insertion mutant and transgenic overexpression lines demonstrated that *OsBBII* mediates broad-spectrum resistance against multiple races of blast fungus by modifying cell wall defence responses. These findings provide new insights into the cellular and molecular mechanisms of broad-spectrum resistance of rice against blast disease.

Results

OsBBII is induced by BTH and *M. oryzae*

To understand the molecular basis of immune response in rice, we performed microarray gene expression profiling, and identified a group of genes upregulated by *M. oryzae* and/or *Xoo* [33]. Our analysis indicated that one gene, designated as *OsBBII* (*BLAST AND BTH-INDUCED 1*, GenBank accession number Os06g03580), was strongly induced by *M. oryzae* infection, and by SA and BTH, a functional analogue of SA. Expression of *OsBBII* was induced in *M. oryzae*-infected plants within a period of 48 h, and reached the peak at 24 h after inoculation (Figure 1A). *OsBBII* was upregulated by BTH and SA treatment and reached the peak at 12 h (BTH) or at 24 h (SA) after treatment, respectively; but was not inducible by JA and *Xoo* infection (Figure 1B, data not shown). The *OsBBII* gene is expressed in root, stem, sheath, and leaf tissues (Supplementary information, Figure S1). These data suggest that *OsBBII* may function in disease resistance to rice blast fungus.

Mutation in OsBBII leads to increased susceptibility to *M. oryzae*

To determine whether *OsBBII* has a function in rice innate immunity, we first examined whether *OsBBII* is required for resistance to blast fungus. A *Tos17*-insertion mutant line *osbbil* in the Nipponbare mutagenesis resource (ND7061, <http://pc7080.abr.affrc.go.jp/~miyao/pub/tos17/index.html.en>) that displays no significant morphological phenotype was identified. The *Tos17* element was inserted in the last intron of the *OsBBII* gene (Figure 2A). Reverse transcriptase-polymerase chain reaction (RT-PCR) analyses with a pair of primers amplifying the coding region of the *OsBBII* gene and a pair of primers spanning the *Tos17* insertion site showed no detectable transcript of *OsBBII* in *osbbil* plants (Figure 2B). However, the level of *OsBBII* transcript in *osbbil* plants was similar to that in wild-type (WT) plants when

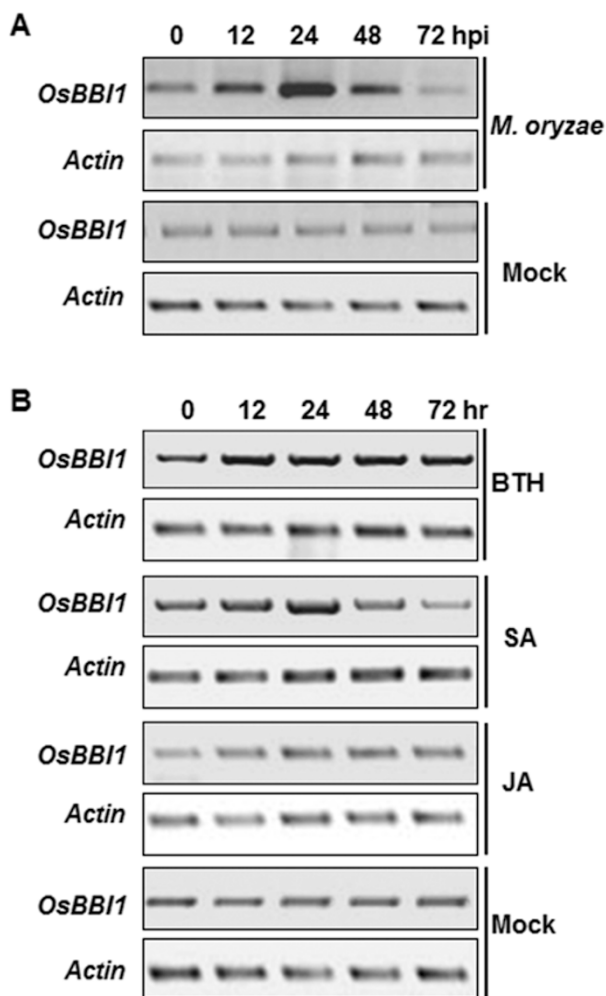


Figure 1 Induction of *OsBB11* in rice by BTH, SA and *M. oryzae*. (A) Expression of *OsBB11* induced by *M. oryzae*. (B) Expression of *OsBB11* induced by BTH and SA. Three-week-old rice seedlings were treated by foliar spraying of 300 μ M BTH, 1 mM SA, 100 μ mol/l JA, or water as a control, inoculated by foliar spraying of spore suspension of *M. oryzae* (10^5 spores/ml) or 0.02% Tween 20 in water as mock inoculation. Leaf samples were collected as indicated after treatment or inoculation and expression of *OsBB11* was analysed by RT-PCR for 32 cycles with actin gene as an internal control (26 cycles). JA did not induce *OsBB11*. Results are representative of two independent experiments with similar results.

using another pair of primers amplifying the truncated N-terminal region of the *OsBB11* transcript (Figure 2B, right). Thus, it is likely that the insertion of the *Tos17* element in the *OsBB11* gene results in a “loss-of-function” mutant.

We then analysed the disease phenotype of *osbbil* mutant plants on infection by different races of *M. oryzae*. With seedling inoculation, the *osbbil* plants showed in-

creased susceptibility to races ZD1 (strain 99-188), ZD3 (strain 98-271), and ZG1 (strain 94-10), resulting in an average of one grade higher disease index than the WT Nipponbare plants (Figure 2C and 2D). Similar results were obtained in detached leaf assays (Supplementary information, Figure S2A). Disease lesions on the *osbbil* leaves were significantly larger than on the WT leaves after inoculation with two races of *M. oryzae* (Supplementary information, Figure S2B). These results indicate that *OsBB11* is required for full immunity in rice against *M. oryzae*.

Overexpression of *OsBB11* leads to broad-spectrum resistance against *M. oryzae*

To explore the function of *OsBB11* in rice blast disease resistance, we generated independent *OsBB11*-overexpressing transgenic lines (*OsBB11*-OE) and compared the disease phenotype of these transgenic lines against *M. oryzae*. RT-PCR analysis showed that the expression levels of *OsBB11* in representative stable transgenic lines (OE-255, OE-256, and OE-259, T3-T5 generations) were higher than that in the WT TP09 plants (Supplementary information, Figure S3). We used another four different races of *M. oryzae*, ZE1 (strain 98-48-3), ZA49 (strain 89-151), ZB1 (strain 2000-2), and ZB15 (strain 09-31-1), which are known to be highly virulent on *japonica* rice. Figure 3 shows that the overall disease scores in the three *OsBB11*-OE lines were lower than those in the WT plants (Figure 3A). Disease severities on the *OsBB11*-OE plants were on average 1.1-2.5 grades lower than those on the WT TP309 plants (Figure 3B). Similar results were obtained in detached leaf assays (Supplementary information, Figure S4). We compared the disease lesion sizes on the *OsBB11*-OE and WT plants after inoculation with traces of ZB1 (strain 2000-2) and ZB15 (strain 09-31-1). As shown in Figure 3C, lesion sizes on the *OsBB11*-OE plants were significantly reduced compared with those on the WT plants, resulting in 50-80% and 45-50% reduction when infected by ZB1 and ZB15, respectively. These results confirm that *OsBB11*-mediated resistance is efficient against multiple races of blast fungus.

Increased accumulation of H_2O_2 in *OsBB11* overexpression plants

Since ROS, such as H_2O_2 , are actively involved in defence responses in rice [34, 35], we further analysed the accumulation of H_2O_2 in sheath epidemic cells at infection sites, using 3,3'-diaminobenzidine (DAB) staining [36]. The accumulation of H_2O_2 was evaluated at 24 h.p.i. and was grouped into five types (A-E) for each infection site (Figure 4A). Most cells at infection sites were not, or only slightly stained in the WT (types A and B), while

the percentages of H₂O₂-stained cells (type C and D) increased in OsBB11-OE plants (Figure 4B). At 48 h.p.i., the percentages of H₂O₂-stained epidermal cells (type C + D) were significantly higher in OsBB11-OE plants than in WT plants (Figure 4B). In contrast, the percentages of H₂O₂-stained epidermal cells in mutant *osbbi1* plants were slightly and significantly lower than those in WT plants at 24 and 48 h.p.i., respectively (Figure 4C). The results indicate that overexpression of *OsBB11* in trans-

genic plants increases the capacity to prime for accumulation of H₂O₂ in response to *M. oryzae* infection.

Enhancement of cell wall defence response in *OsBB11* overexpression plants

The cell wall is the first layer of defence against pathogen infection, and cell wall enhancement during defence is associated with ROS accumulation. Our observations that blast fungus penetrated less into and higher H₂O₂ accumulation in the OsBB11-OE cells suggested that cell wall defence might be enhanced in OsBB11-OE plants, resulting in enhanced blast resistance. To explore this possibility, we analysed the levels of phenolic compounds and cross-linking of wall-associated proteins in sheath epidermal cells at infection sites of *M. oryzae* by autofluorescence and Coomassie Brilliant Blue staining. Strong autofluorescence and cross-linking of wall-associated proteins were observed at infection sites and adjacent cells of the OsBB11-OE plants, compared with relatively weak results in WT cells at 24 and 48 h.p.i. (Figure 5A and 5C). The percentages of autofluorescent cell walls and cross-linking increased by ~10% in the OsBB11-OE plants compared with that in the WT plants at 24 h.p.i. This increase was much more significant at 48 h.p.i. (Figure 5B and 5D). In contrast, the percentages of autofluorescent cell walls and cross-linking of wall-associated proteins at infection sites were lower in the *osbbi1* plants than those in the WT plants at 24 and 48 h.p.i. (Figure 5E and 5F). We further measured the thickness

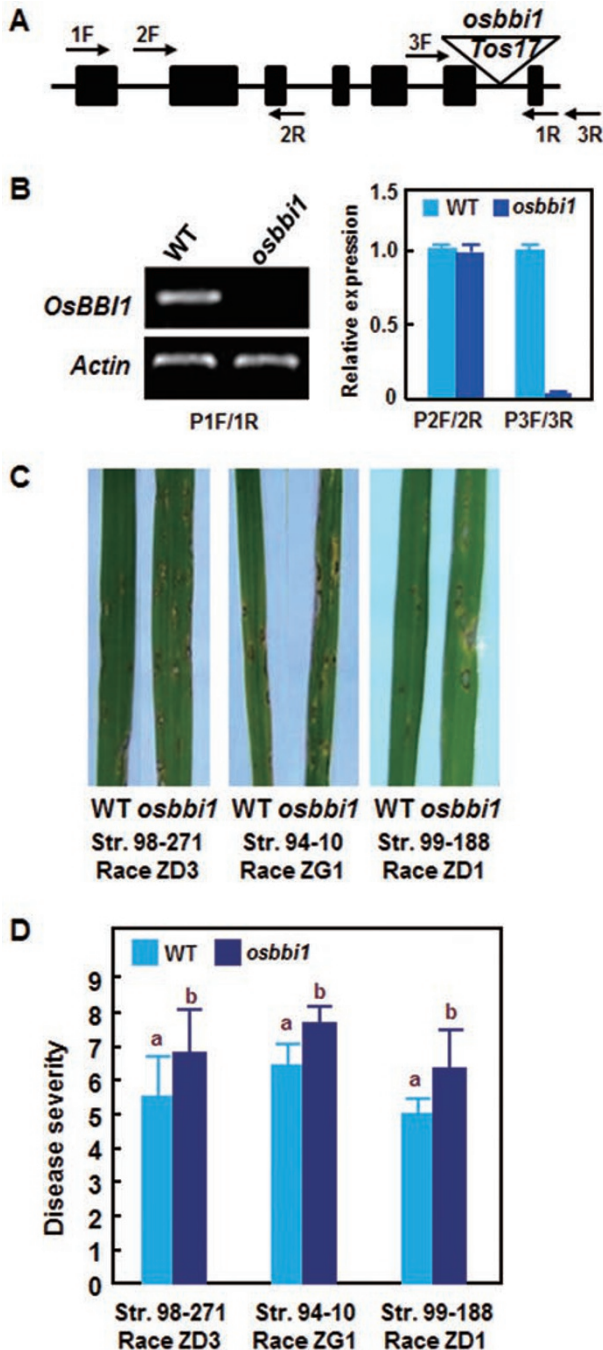


Figure 2 Mutation in *OsBB11* led to enhanced susceptibility to *M. oryzae*. **(A)** Genomic structure of the *OsBB11* gene and location of the *Tos17* element. Filled boxes indicate exons while black lines indicate introns. The primers are indicated to detect the *OsBB11* transcripts. **(B)** Detection of the *OsBB11* transcript in *osbbi1* mutant. Left, semi-quantitative RT-PCR was performed to detect the full *OsBB11* transcript in *osbbi1* and WT plants using a pair of primers (1F/1R) as indicated in **A**. The actin gene was used as an internal control. Right, quantitative real-time PCR was performed to detect the *OsBB11* transcripts using two pairs of primers (2F/2R and 3F/3R) as indicated in **A**, respectively. Note that the primers 2F and 2R also detected same levels of the *OsBB11* coding region in *osbbi1*. **(C)** Representative disease symptoms on leaves of the *osbbi1* and WT plants to different strains (str.), races are indicated. **(D)** Disease severity on leaves of the *osbbi1* and WT plants. Three-week-old plants at the four-leaf stage were inoculated by foliar spraying spore suspension (5×10^5 spores per ml) prepared from different races of *M. oryzae* and photos were taken at 5 d.p.i. At least 40 plants in each experiment were evaluated for disease scores using an international nine-scale standard. Data presented are the means \pm standard errors from three independent experiments. Different letters indicate significant difference at $P = 0.05$.

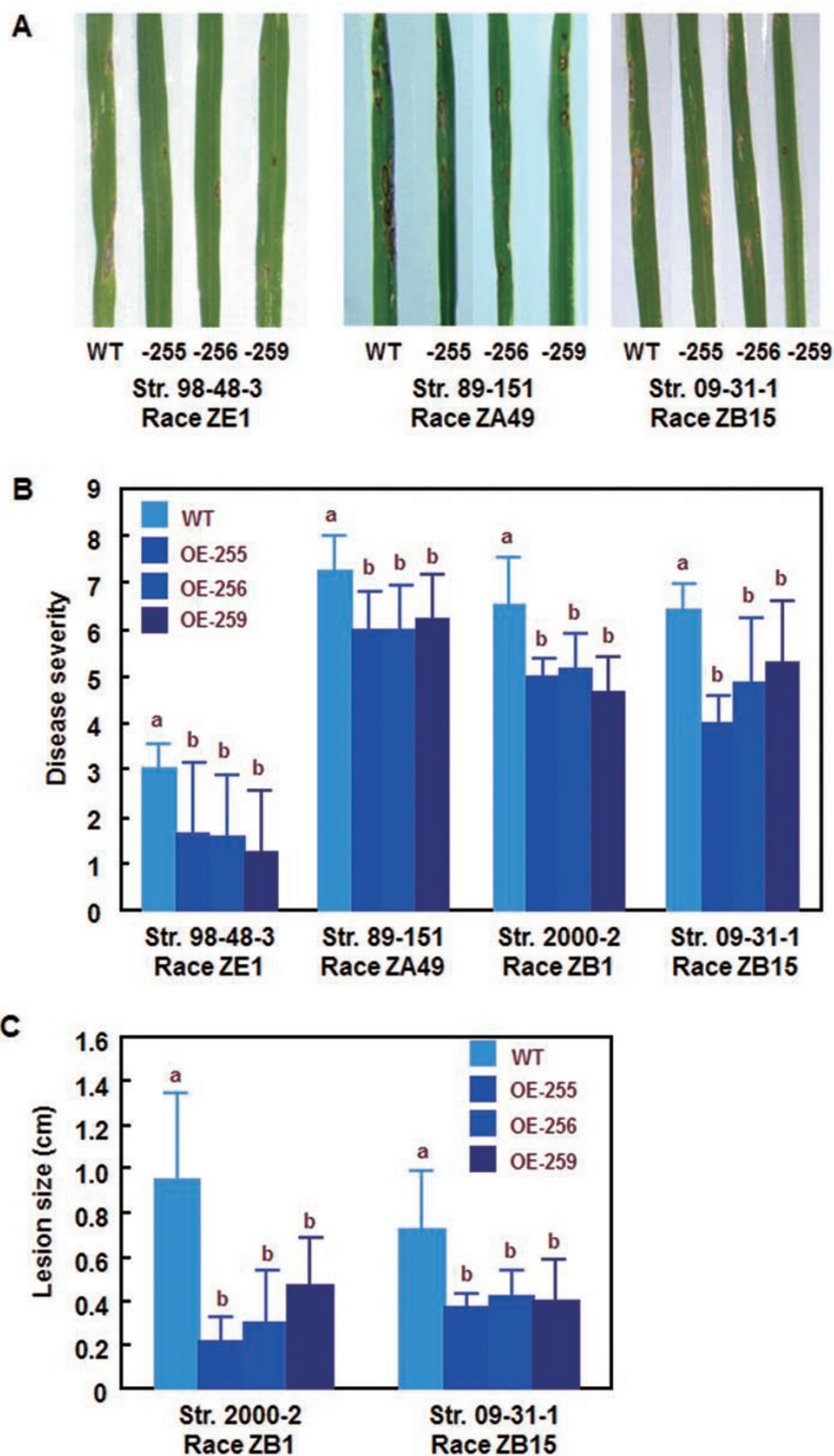


Figure 3 Overexpression of *OsBB11* in transgenic rice confers broad-spectrum resistance against multiple races of *M. oryzae*. **(A)** Representative disease symptoms on leaves of the *OsBB11*-OE and WT plants. Three-week-old plants were inoculated with different races of *M. oryzae* and photos were taken at 5 d.p.i. **(B)** Disease severity on leaves of the *OsBB11*-OE and WT plants. At least 40 plants in each experiment were evaluated for disease scores. **(C)** Lesion sizes on leaves of the *OsBB11*-OE and WT plants. At least 100 lesions from 20 representative diseased leaves of 20 plants were measured. Data presented are the means \pm standard errors from three independent experiments. Different letters indicate significant difference at $P = 0.05$ (**B**, **C**).

of cell walls in sheath tissues. The cell walls were 25-30% thicker in OsBB11-OE cells than in WT cells (Figure 6A and 6B). In contrast, the thickness of cell walls in the *osbbi1* cells showed a 35% decrease compared with that in the WT cells (Figure 6C and 6D). These data indicated that OsBB11 mediates defence response by reinforcing cell walls, associated with the increase in ROS accumulation and cross-linking of wall-associated proteins.

OsBB11 encodes a RING protein with E3 ligase activity

The *OsBB11* gene encodes a 261-aa protein, which contains a conserved RING domain at the C-terminal end. Seven types of RING domains have been identified, including two canonical RING types, RING-HC (C3HC4) and RING-H2 (C3H2C3), and five modified RING do-

main types, RING-v, RING-C2, RING-D, RING-S/T, and RING-G [37]. The RING domain in OsBB11 contains the highly conserved C-X2-C-X14-C-X1-H-X2-H-X2-C-X10-C-X2-C structure (Figure 7A), which is commonly present in the RING-H2 type of RING proteins [37]. Therefore, we predicted that OsBB11 belongs to the RING-H2 type of the RING family (Supplementary information, Figure S5). Of these, *Arabidopsis* BIG BROTHER, SDIR1, SIS3, RING1, NLA, SINAT5, and XBAT32, and rice EL5 and GW2 possess E3 ligase activity *in vitro* [38-46]. NLA, SIS3, SDIR1, and RING1 play roles in biotic and abiotic stress responses [40-43]. Furthermore, the RING domain and some well-conserved residues within this domain are critical to E3 ligase activity for most RING proteins [37]. To explore

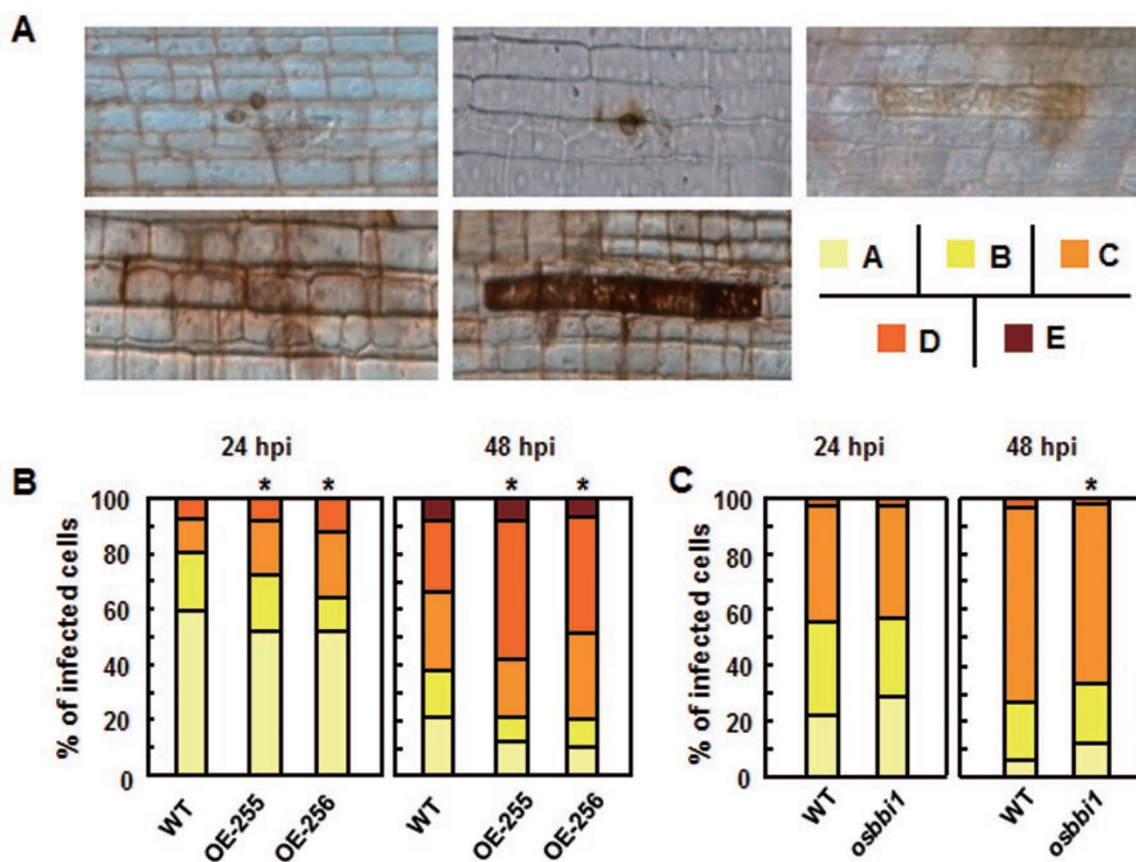


Figure 4 Accumulation of H₂O₂ in sheath epidermal cells at infection sites by *M. oryzae*. Leaf sheaths from 4-week-old plants were inoculated by injection with spore suspension of *M. oryzae*. (A) Micrographs of the cells with distinct H₂O₂ accumulation levels in inoculated leaf sheaths stained with DAB at 24 h.p.i. Leaf sheaths from 2-month-old plants were used for experiments. Type A, successful fungal colonization of cells with no DAB staining visible; type B, DAB staining in appressoria site; type C, DAB staining in a primary epidermal cell following fungal invasion; type D, DAB staining a primary epidermal cell and adjacent cells at infection sites; type E, deep DAB staining in a primary epidermal cell and adjacent cells. (B, C) Frequency distribution of the DAB staining cell types at 24 and 48 h.p.i. in sheath epidermal cells of the OsBB11-OE and WT plants (B) and of the *osbbi1* and WT plants (C). Data presented are the mean from three independent experiments with leaf sheaths from five individual plants in each experiment (B, C). At least 100 single-cell infection sites were examined in each experiment. Asterisks above the bars indicate significant difference at *P* = 0.05.

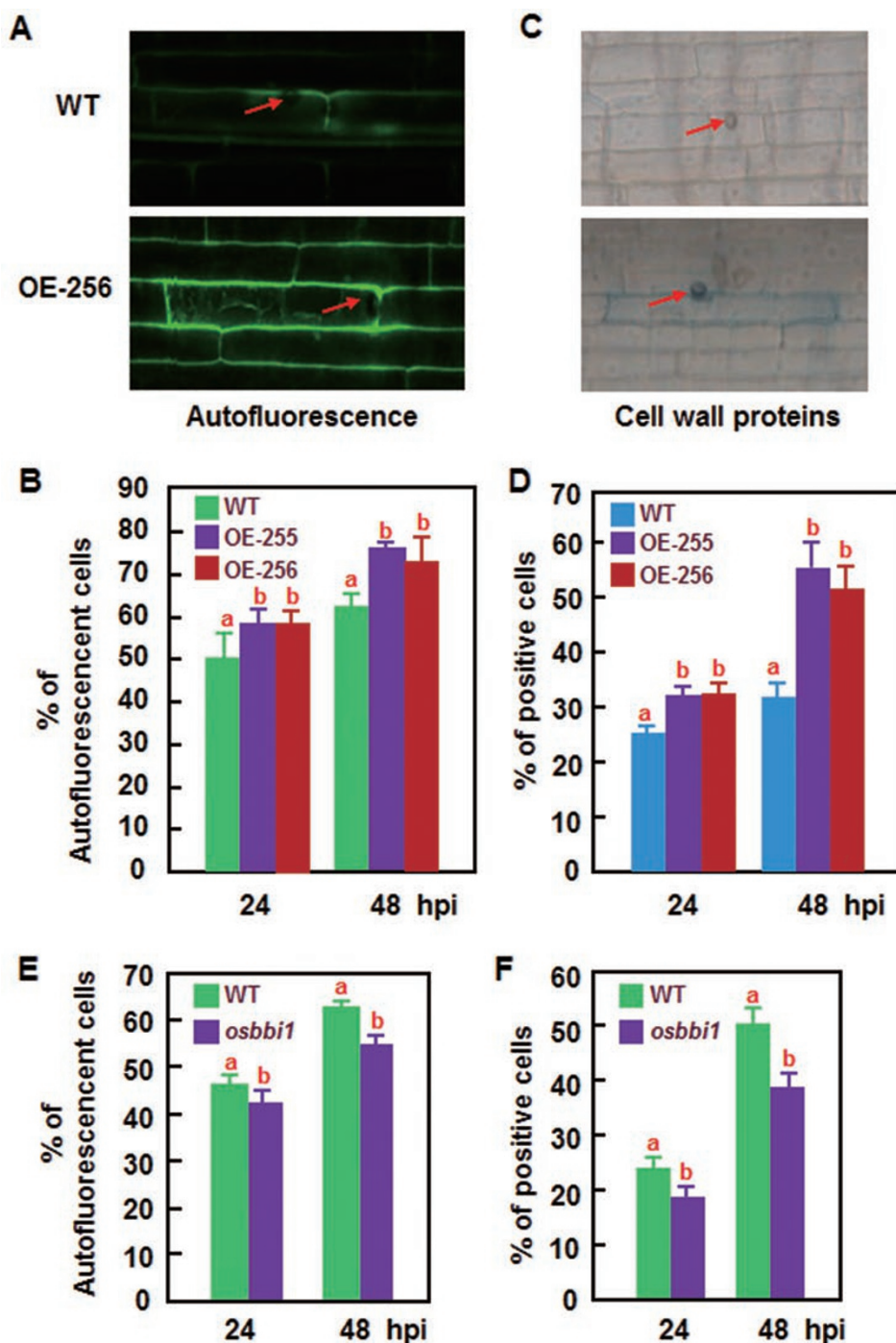


Figure 5 Accumulation of phenolic compounds and cross-linking of proteins in cell walls of rice sheath epidermal cells at infection sites. Leaf sheaths from 4-week-old plants were inoculated by injection with spore suspension of *M. oryzae*. **(A)** Representative autofluorescence images of sheath epidermal cells at infection sites in OsBB11-OE and WT TP09 plants. **(B, E)** Deposition of autofluorescent phenolics in sheath epidermal cells of the OsBB11-OE and WT plants **(B)** and of the *osbbi1* and WT Nipponbare plants **(E)**. **(C)** Representative micrographs of cross-linking of wall-associated proteins stained with Coomassie Brilliant Blue dye in sheath epidermal cells at infection sites in the OsBB11-OE and WT plants. **(D, F)** Cross-linking of proteins in sheath epidermal cell walls of the OsBB11-OE and WT plants **(D)** and of the *osbbi1* and WT plants **(F)**. Arrows in **A** and **C** indicate fungal appressoria at infection sites. Data presented in **B, D, E,** and **F** are the means \pm standard errors from three independent experiments with leaf sheaths from five individual plants in each experiment. At least 100 single-cell infection sites were examined in each experiment. Different letters indicate significant difference at $P = 0.05$.

whether the OsBB1 protein has E3 Ubi ligase activity, we expressed full-length OsBB1 as a six His-tagged fusion protein (His-OsBB1) in *Escherichia coli* and purified the recombinant protein for enzyme activity assay (Figure 7). To examine the importance of the RING domain in E3 Ubi ligase activity, a truncated mutant of OsBB1, OsBB1^{ARING}, in which the RING domain was deleted, was also generated as a His-tagged fusion protein, and assayed for E3 ligase activity (Figure 7). In the time-course experiments, clear protein ubiquitination was observed at 1 h after incubating His-OsBB1 with Ubi, E1, and E2 enzymes in the reactions. The level of protein ubiquitination increased with incubation time, indicating the E3 ligase activity of His-OsBB1 (Figure 7B). In the presence of Ubi, E1, and E2 enzymes, His-OsBB1 could carry out self-ubiquitination, while no clear protein ubiquitination was detected in the absence of E1, E2, or His-OsBB1 (Figure 7C). The truncated mutant, OsBB1^{ARING}, did not show E3 ligase activity in the reactions (Figure 7C). These results show that OsBB1 is a functional E3

ligase, and the RING domain in OsBB1 is essential to its E3 Ubi ligase activity.

Discussion

Blast disease is the most economically devastating disease in rice, and blast resistance is a prerequisite for commercial variety registration and release in China. However, blast resistance can be rapidly broken down due to the race diversity of *M. oryzae* [47]. Thus, breeding rice for broad-spectrum blast resistance has been a long-term task for rice breeders, with limited success due to the availability of resistance genes and our limited knowledge of resistance mechanisms. This study shows that a blast fungus- and BTH-induced gene *OsBB1*, encoding a RING protein with E3 ligase activity, plays a role in basal resistance to blast fungus. Loss of *OsBB1* function resulted in increased susceptibility, whereas overexpression of *OsBB1* in transgenic plants led to enhanced resistance against multiple races of *M. oryzae*

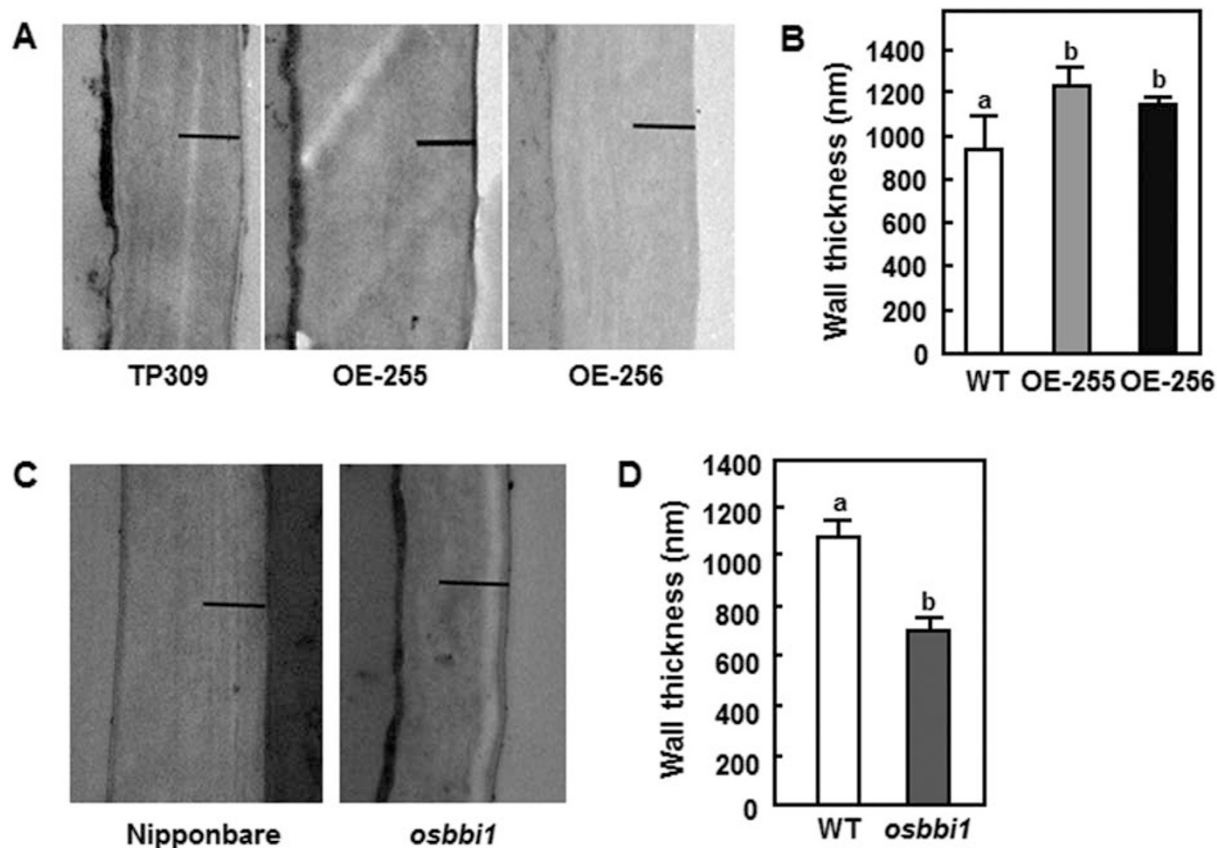


Figure 6 Comparison of cell wall thickness in OsBB1-OE, *osbbi1* mutant and WT plants. (A, B) Cell walls in OsBB1-OE and WT Nipponbare plants. (C, D) Cell walls in *osbbi1* and WT TP309 plants. Sheath sections from five individual plants were used for observation and at least 20 measurements were carried out for each sheath section. Data presented in B and D are the means \pm standard errors. Different letters indicate significant difference at $P = 0.05$. Bar = 500 nm.

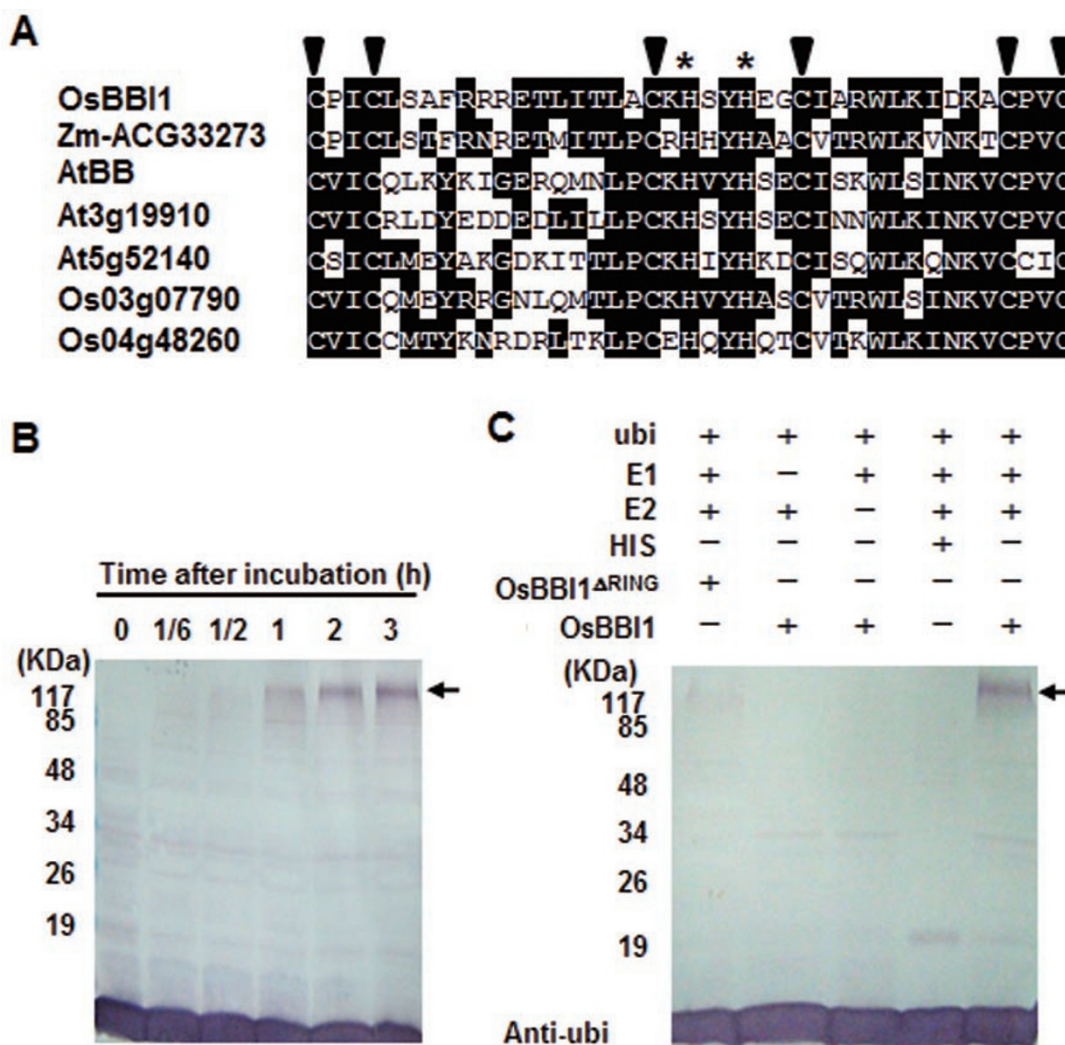


Figure 7 OsBBI1 is a RING-H2 protein with E3 ligase activity *in vitro*. **(A)** Amino acid alignments of the OsBBI1 RING domain with RING domains of other proteins from *Arabidopsis* (At), rice (Os) and maize (Zm). Filled triangles indicate the conserved cysteine (C) residues, while asterisks indicate conserved histidine (H) residues. **(B, C)** E3 ligase activity of OsBBI1 *in vitro*. His-tagged OsBBI1 or OsBBI1^{ΔRING} fusion protein was assayed for E3 ubiquitin ligase activity in the presence of E1, E2, and Ubi. Ubiquitinated proteins were detected by protein blot analysis using an antibody to Ubi, with time-course increase in ubiquitinated proteins shown in **B**. Arrow indicates the ubiquitinated proteins.

(Figures 2 and 3), indicating that *OsBBII* confers broad-spectrum resistance in rice against blast disease.

Several E3 ligases in rice have been reported to be involved in innate immunity [29-31]. However, OsBBI1 is distinct from the E3 ligases previously reported to be involved in rice innate immunity in terms of structural characteristics and biological functions. Structurally, OsBBI1 belongs to the RING-H2 type of RING proteins, and only contains a conserved RING domain at its C-terminus. SPL1, a U-box protein with E3 Ubi ligase activity, contains both a U-box domain and an armadillo repeat domain, while XB3, an XA21-binding protein

with E3 Ubi ligase activity, has an ankyrin repeat domain in its N-terminus in addition to a RING domain at its C-terminus [30, 31]. Functionally, experimental data presented in this study demonstrate that OsBBI1 positively regulates rice defence response against *M. oryzae*, but not against *Xoo* (data not shown). SPL1 negatively regulates cell death and innate immunity against both *M. oryzae* and *Xoo* [30], while XB3 seems to have a specific function in XA21-mediated innate immune response against *Xoo* [31]. Therefore, this study adds OsBBI1, representing a novel E3 Ubi ligase, to the list of proteins with E3 ligase activity involved in rice innate immunity.

Broad-spectrum resistance is generally referred to as resistance to majority of geographically different isolates of the same pathogen and/or resistance to two or more unrelated pathogens. We did not observe the involvement of *OsBBII* in disease resistance against bacterial blight, indicating that *OsBBII*-mediated resistance is likely specific to defence against blast fungus, consistent with its induction by BTH and *M. oryzae* but not by *Xoo*. In rice, several *R* genes/loci such as *Pi5*, *Pi9*, *Pi21*, *Pi33*, *Pi39(t)*, and *Pi40(t)*, have been identified to confer broad-spectrum blast resistance [9, 10, 48-51]. However, molecular mechanisms by which these *R* genes provide broad-spectrum resistance remain elusive. The characterization of *OsBBII* as a novel component in rice immunity against *M. oryzae* may provide an additional approach to dissect the molecular and biochemical mechanisms downstream of pathogen recognition. Furthermore, *OsBBII* may provide a practical tool for engineering broad-spectrum blast resistance in rice breeding.

R gene-mediated resistance often provides full race-specific protection. In contrast, resistance controlled by quantitative trait loci (QTLs) is non-race-specific but partial (for review see [7]). Compared with *R* gene-mediated high level of disease resistance, overexpression of *OsBBII* only conferred a relatively low level of resistance and mutation in the *OsBBII* gene resulted in partial loss of resistance against multiple races of *M. oryzae* (Figures 2 and 3). This indicates that OsBBII may mediate non-race-specific basal resistance against *M. oryzae* in rice, and is probably involved in an unrecognized PTI network. Some blast resistance QTLs have been identified in rice and some have been characterized [7, 10, 26]. The *OsBBII* gene (Os06g03580) is located in a region of chromosome 6, in which a number of blast resistance QTLs have been identified [52-55]. The features of OsBBII-mediated basal and partial resistance suggest that *OsBBII* might be a QTL against *M. oryzae*, such as other rice defence genes [56].

Extensive studies have indicated that ROS is an important component of plant defence responses associated with cell wall reinforcement [57, 58]. The invasion of *M. oryzae* hyphae often stimulates rapid production of H₂O₂ in rice cells at infection sites as an early defence response [59]. Small GTPase Rac complex-mediated accumulation of ROS generated through NADPH oxidases is required for innate immune response against *M. oryzae* [11-15]. Most recently, Chi *et al.* [60] reported that virulent *M. oryzae* strains have developed ROS-scavenging ability through the DES1 protein, thus suppressing ROS-mediated innate immune response in host cells. OsBBII-OE plants accumulated higher, while *osbbil* mutant plants generated lower levels of H₂O₂ than WT plants in

cells at the sites of *M. oryzae* infection (Figure 4). This correlates with increased levels of autofluorescence and cross-linking of proteins in cell walls in the OsBBII-OE plants. However, overexpression of *OsBBII* does not result in constitutive accumulation of H₂O₂, autofluorescent materials, and cross-linking of cell wall proteins because the levels of H₂O₂, autofluorescence, and cross-linking of proteins in the walls of cells beyond the infection sites were similar with OsBBII-OE and WT plants. Thus, the OsBBII-mediated ROS generation and cell wall defences can only be activated in OsBBII-OE plants on infection of *M. oryzae*. This can be explained by an unrecognized mechanism, in which OsBBII may target the degradation of negative regulator(s) of defence responses, which is probably induced after pathogen infection. OsBBII could also target the degradation of suppressor of host defence responses, which is secreted by *M. oryzae* during infection.

Recent studies demonstrated the importance of the cell wall in immune responses against diverse pathogens [61-65]. Thickness or reinforcement of the cell wall plays important roles in counteracting the penetration of fungal pathogens [66, 67]. Consistent with ROS accumulation and cross-linking of cell wall proteins, OsBBII-OE plants develop thicker cell walls, while the *osbbil* mutant generates thinner walls compared with corresponding WT plants (Figure 6). This indicates that functions of OsBBII in cell wall defence responses are likely executed via regulating ROS production and/or the cell wall synthesis pathway. Further identification of the OsBBII target (s) will provide deeper insights into the biochemical and molecular basis of OsBBII-mediated immunity in rice.

Materials and Methods

Growth of rice plants

Rice (*Oryza sativa* L.) subsp. *indica* cv. Yuanfengzao, *japonica* cv. Taipei 309 (TP309), and Nipponbare were used in this study. Transgenic plants were selected by GUS staining and PCR detection. All rice seedlings were grown in a growth room at 25 °C with 14 h light/10 h darkness. Three-week-old seedlings were used for blast disease assays and other experiments.

Generation and characterization of overexpression and *osbbil* mutant plants

A *Tos17* insertion line of the *OsBBII* gene, ND7061, was obtained from the *Tos17* insertion resource (<http://pc7080.abr.affrc.go.jp/~miyao/pub/tos17/index.html.en>). PCR-based genotyping was performed to screen for homozygous plants. Genotyping was performed using *OsBBII*-specific primers, OsBBII-g-1F (5'-CTC-CTCACACGCTAGGAAGG-3') and OsBBII-g-1R (5'-GTC-TATCCGCTTTCAGACGC-3'), along with a *Tos17*-specific primer *Tos17*-1R (5'-ATTGTTAGGTTGCAAGTTAGTTAAGA-3'). Homozygous plants were used for studies. The plasmid for over-

expression was generated by cloning the full-length coding region (1.3 kb) of *OsBB11* into the rice expression vector 35S-C1301, and was transformed into cv. TP309 (*japonica*) to generate more than 20 independent lines. Stable lines were assayed for disease resistance with the continuous generations (T1 to T3).

Treatment of chemical inducers

Seedlings of cv. Yuanfengzao (*indica*) were sprayed with BTH (0.3 mM), SA (1.5 mM, pH 6.5), and JA (0.1 mM) solutions, and with distilled water containing 0.05% Tween 20 as a control. Leaf samples were collected within a period and stored at -80°C until use.

Inoculation and disease assays

Different strains of *M. oryzae*, belonging to races of ZA49 (strain 89-151), ZD3 (strain 98-271), ZG1 (strain 94-10), ZE1 (strain 98-48-3), ZD1 (strain 99-188), ZB1 (strain 2000-2), and ZB15 (strain 09-31-1) were used for blast resistance assay. Three-week-old seedlings were spray-inoculated with spores (5×10^5 per ml with 0.05% Tween-20 in water) as described [68]. Disease severity was evaluated using a standard international 0-9 scale (0 = resistant and 9 = susceptible) at 6 d.p.i. Leaf blast spot inoculation was performed as described [16]. To observe cellular defence, a sheath inoculation method was used as described [69]. For microscope observation, sheath sections were sampled at 24 and 48 h.p.i. At least five sections were observed for each sample.

Visualization of cytological responses

To detect H_2O_2 accumulation at infection sites, the sheath sections were vacuum infiltrated in DAB-HCl solution (1 mg/ml, pH 3.8) [36] for 30 min and incubated in the growth chamber for 8 h. Trimmed sheath segments were mounted in 50% glycerol and examined for the formation of brown-red precipitate under bright-field with a Leica CTR5000 microscopy (Leica Microsystems, Hong Kong, China). To detect phenolic compounds, sheath segments were stained for 30 min in a solution containing 0.01% (w/v) aniline blue and 0.15 M K_2HPO_4 . They were visualized as autofluorescence under blue light epifluorescence with a Leica TCS SP5 fluorescent microscopy (GPF filter set; excitation at 405 nm, dichroic beamsplitter of 500-550 nm; Leica Microsystems, Hong Kong, China) according to the method described previously [59]. To detect protein cross-linking, sheath segments were submerged in 1% sodium dodecyl sulphate (SDS) for 24 h at 80°C and stained in 0.1% Coomassie Brilliant Blue in a solution of 40% ethanol and 10% acetic acid for 15 min. They were subsequently rinsed in a solution of 40% ethanol and 10% acetic acid, followed by mounting in 50% glycerol and examination using a Leica CTR5000 microscopy (Leica Microsystems, Hong Kong, China).

Transmission electron microscopy (TEM)

Sheath segments from 2-month-old rice plants were fixed with 2.5% glutaraldehyde in phosphate buffer (100 mmol/l, pH7.0) for at least 4 h, washed three times with the same phosphate buffer for 15 min each, post fixed with 1% osmium tetroxide in the phosphate buffer for 1 h, and washed three times with the phosphate buffer. The sheath segments were then embedded in Epon 812, and ultra-thin sections were stained by uranyl acetate and alkaline lead citrate for 15 min, respectively, and observed in TEM of Model H-7650 (Hitachi, Tokyo, Japan).

E3 Ubi ligase activity assay

The coding sequence of the *OsBB11* gene was cloned into pET-32 vector at *Bam*HI and *Hind*III sites, and the recombinant plasmid was introduced into the *E. coli* strain (DE3). To generate the truncated mutant *OsBB11*^{ARING}, the coding sequence was amplified using a pair of primers, *OsBB11*-dRING-1F (5'-GCGGGATC-CATGGCCACCGTGGGGCAGCCT-3' and *OsBB11*-dRING-1R (5'-GCGAAGCTTCTAATCATGGTTTGCTTCCTTG-3', and cloned into pET-32 vector at *Bam*HI and *Hind*III sites. Expression of *OsBB11* and *OsBB11*^{ARING} fusion proteins in *E. coli* cells was induced by 1 mmol/l isopropyl-D-thiogalactoside at 37°C for 4 h. The His-tagged *OsBB11* fusion protein was purified using a His-Bind Kit following the manufacturer's protocols (NovaGen, Madison, WI, USA). Assays for *in vitro* ubiquitination were carried out as described previously, with slight modifications [38]. In brief, 0.1 μg human E1 (Merk BioSciences, Nottingham, UK), 0.22 μg human E2 (UbcH5b) (Merk BioSciences, Nottingham, UK), 10 μg Ubi, and 1 μg purified His-tagged *OsBB11* or *OsBB11*^{ARING} fusion protein were incubated in a 30 μl reaction mix (50 mM Tris-HCl, 5 mM MgCl_2 , 2 mM ATP and 0.5 mM DTT, pH 7.5) at 30°C for 0-3 h. The reaction was stopped with $1 \times$ SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer and boiled in water for 5 min. *In vitro* ubiquitination of the samples were analysed by SDS-PAGE and polyubiquitinated proteins were detected by protein blotting using an anti-Ubi antibody (Merk BioSciences, Nottingham, UK).

Analysis of gene expression

Total RNA was prepared from the samples using the TRIZOL reagent according to the manufacturer's procedure (Invitrogen, Shanghai, China) and treated with DNase RQ1 (Promega, Madison, WI, USA). First-strand cDNA was synthesized from 600 ng of total RNA using superscript III RT (Invitrogen, Shanghai, China). Semi-quantitative RT-PCR was performed for 28-32 cycles based on the abundance of transcripts of the genes analysed. Primers used in this study are *OsBB11*-rt-1F (5'-AGGCAACAACGAA-GAGGCTGC-3') and *OsBB11*-rt-1R (5'-TAGTTGGTCAGC-CCTGTTGATC-3') for *OsBB11* expression and detection of *OsBB11* transcript in *osbb11* mutant, and *OsActin*-1F (5'-TATG-GTCAAGGCTGGGTTTCG-3') and *OsActin*-1R (5'-CCATGCTC-GATGGGGTACTT-3') for *actin* expression as an internal control. Quantitative real-time PCR was carried out using SYBR premix Ex Taq (TaKaRa, Dalian, China) in a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) with 100 ng cDNA and 7.5 pmol of each gene-specific primer. Data were collected from two independent biological samples in triplicate. Expression ratios were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method with the elongation factor 1 α as reference gene. Relative expression levels were compared with the expression levels before treatment as 1.0. Gene-specific primers used are as follows: *OsBB11*-rt-2F, 5'-GAAACACTGCTCTTGC-CCAATCTG-3'; *OsBB11*-rt-2R, 5'-TGCCTAGCGAACACCA-AGTCATAC-3'; *OsBB11*-rt-3F, 5'-AGACGCCGAGAAACACT-CATTACA-3'; *OsBB11*-rt-3R, 5'-CAGCCTTGTGTATCGCCTC-CTC-3'; *OsEF1a*-rt-1F, 5'-GTCATTGGCCACGTCTCGACTC-3'; *OsEF1a*-1R, 5'-TGTTTCATCTCAGCGGCTTCC-3'.

Statistical analysis

All experiments were repeated independently at least three times. Data collected were statistically analysed by one-way analy-

sis of variance followed by least significant difference test at $P = 0.05$ using DPS software (<http://www.chinadps.net/index.htm>).

Accession Number

Sequence data from this article can be found in GenBank under the following accession number: *OSBB11* genomic DNA and proteins (Os06g03580).

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(**Supplementary Information** is linked to the online version of the paper on the *Cell Research* website.)