Novel Bifunctional Nucleases, OmBBD and AtBBD1, Are Involved in Abscisic Acid-Mediated Callose Deposition in Arabidopsis[1][W][OA]

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Screening of the expressed sequence tag library of the wild rice species *Oryza minuta* revealed an unknown gene that was rapidly and strongly induced in response to attack by a rice fungal pathogen (*Magnaporthe oryzae*) and an insect (*Nilaparvata lugens*) and by wounding, abscisic acid (ABA), and methyl jasmonate treatments. Its recombinant protein was identified as a bifunctional nuclease with both RNase and DNase activities in vitro. This gene was designated *OmBBD* (for *O. minuta* bifunctional nuclease in basal defense response). Overexpression of *OmBBD* in an Arabidopsis (*Arabidopsis thaliana*) model system caused the constitutive expression of the *PDF1.2*, *ABA1*, and *AtSAC1* genes, which are involved in priming ABA-mediated callose deposition. This activation of defense responses led to an increased resistance against *Botrytis cinerea*. *atbbd1*, the knockout mutant of the Arabidopsis ortholog *AtBBD1*, was susceptible to attack by *B. cinerea* and had deficient callose deposition. Overexpression of either *OmBBD* or *AtBBD1* in *atbbd1* plants complemented the susceptible phenotype of *atbbd1* against *B. cinerea* as well as the deficiency of callose deposition. We suggest that OmBBD and AtBBD1 have a novel regulatory role in ABA-mediated callose deposition.

The phytohormones ethylene (ET), jasmonic acid (JA), and salicylic acid (SA) are essential for the regulation of plant defense responses against pathogen attacks. The JA/ET-mediated signaling pathways are engaged in defense responses to necrotrophic pathogens, such as *Botrytis cinerea* or *Alternaria brassicicola* (Thomma et al., 1999; Diaz et al., 2002), and commonly induce the expression of several defense genes, including *PDF1.2* and *Thi2.1* (Penninckx et al., 1996, 1998; Thomma et al., 2001). The SA-dependent defense mechanism providing the plant with protection against biotrophic pathogens (Glazebrook, 2005) activates the *pathogenesis-related* (*PR*) genes, such as *PR1*, *PR2*, and *PR5* (Thomma et al., 2001). Another phytohormone, abscisic acid (ABA), participates in various essential physiological processes, including seed development and germination (Finkelstein et al., 2002), and in plant resistance to abiotic stresses, such as drought and salinity (Yamaguchi-Shinozaki and Shinozaki, 2006). Recently, ABA-mediated resistance has been also highlighted in callose deposition and in interplay between JA, SA, and ABA against some necrotrophs (Mauch-Mani and Mauch, 2005; Adie et al., 2007; Flors et al., 2008; Ton et al., 2009).

The deposition of a linear β-1,3-glucan polymer, callose, in response to pathogen attacks/wounding stresses is a basic defense mechanism that enables the plant to arrest pathogen proliferation by reinforcing the cell wall in both monocots and dicots (Jacobs et al., 2003; Glazebrook, 2005; Hardham et al., 2007; Hao et al., 2008). In the callose synthase-deficient mutant *pmr4-1*, which shows impaired pathogen-induced callose deposition, SA-dependent defense responses are strongly induced to augment the resistance to powdery mildew (Nishimura et al., 2003), whereas JA-dependent defense responses are down-regulated, resulting in its susceptibility to *A. brassicicola* (Flors et al., 2008). These findings indicate that pathogen-induced callose deposition plays an important role in resistance to necrotroph fungus and is closely related

[1] This work was supported by the Crop Functional Genomics Center of the 21st Century Frontier Research Program (grant no. CG3112); by SIGNET, funded by the Ministry of Science and Technology (grant no. R11–2003–008–04006–0); by the BioGreen21 Program of the Rural Development Administration, Republic of Korea; and by a Korea University Grant.

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www.plantphysiol.org/cgi/doi/10.1104/pp.109.147645

to antagonistic interactions between JA-dependent responses and SA-related responses against fungal pathogens. *cob-5* mutants showing the constitutive deposition of callose were found to overproduce JA and the JA-responsive defense genes, such as *PDF1.2* (Ko et al., 2006), indicating that JA-dependent responses are positively involved in callose deposition against pathogen attacks. Callose deposition can be also induced by wounding (Hildmann et al., 1992) or during seed dormancy (Ellis and Turner, 2002), and in these cases, the synergistic actions of ABA and JA are involved. Ton and Mauch-Mani (2004) observed that β-aminobutyric acid-induced resistance (BABA-IR) against some necrotrophs, such as *A. brassicicola* and *Plectosphaerella cucumerina*, was caused by priming the enhanced callose deposition on infected sites. The ABA-deficient mutant *aba1-5* and the ABA-insensitive mutant *abi4-1* were unable to accumulate callose on infection sites and subsequently failed to develop BABA-IR against necrotrophic pathogens (Ton and Mauch-Mani, 2004; Ton et al., 2005). BABA plays an important role in sensitizing the infected tissues to ABA and subsequent augmentation of PMR4-derived callose deposition (Flors et al., 2008), indicating that ABA plays important roles in PMR4-derived callose deposition against necrotrophic fungal pathogens. Comprehensive analyses of endogenous hormonal levels, transcriptomic changes, and callose deposition in the wild type and JA-/ET-/SA-/ABA-related mutants after inoculation with a necrotrophic fungal pathogen, *Pythium irregulare*, revealed that ABA is essential to pathogen-induced callose deposition and has important functions in the activation of JA biosynthesis and the suppression of SA-dependent responses in defense responses (Adie et al., 2007). Collectively, ABA has positive roles in resistance against some necrotrophs by enhancing JA-dependent responses or reinforcing callose deposition (Mauch-Mani and Mauch, 2005; Ton et al., 2009).

More recent studies of *constitutive disease susceptibility2-1D* have also suggested that ABA plays an antagonistic role in SA-dependent defense responses and has indirect or direct positive effects on the activation of JA biosynthesis (Fan et al., 2009). The negative roles of ABA in SA-dependent responses against biotrophs have been clearly demonstrated, but the role of ABA in defense responses against necrotrophs is more obscure. Moons et al. (1997) reported that ABA and JA interact antagonistically on abiotic stress responses. Studies on an ABA-deficient *sitiens* tomato (*Solanum lycopersicum*) mutant showing a reduced tolerance to abiotic stress have also provided strong evidence that ABA has antagonistic effects on defense responses as a virulent factor against necrotrophs by suppressing SA and JA/ET signaling pathways (Audenaert et al., 2002; Anderson et al., 2004; Asselbergh et al., 2007). It has been suggested that the positive or negative involvement of ABA in defense responses to biotic stresses is regulated by the ABA-controlled global switch in response priority toward biotic or abiotic stress, based on the large and complex overlap between abiotic and biotic stress signaling pathways at multiple levels (Asselbergh et al., 2008). However, how the positive or negative role of ABA can be fine-tuned between biotic and abiotic stresses is still largely unknown.

To adjust to extracellular stimuli, plants have evolved sophisticated regulatory systems that are functionally categorized as transcriptional, posttranscriptional, and posttranslational. Posttranscriptional regulation includes the alternative splicing of expressed genes, small interfering RNA- or microRNA-derived mRNA degradation, and alternative maturation of mRNA stability (Glisovic et al., 2008). This form of an mRNA decay system controls the transcript level of specific genes and enables organisms to rapidly and flexibly adjust their transcriptomes in response to extracellular stimuli (Tourriere et al., 2002). Nucleases involved in gene regulation have been evolutionarily conserved (Wilusz et al., 2001; Wilusz and Wilusz, 2004), but the cellular functions of only a few nucleases have been identified in terms of their regulation of physiological stress responses or pathogenesis. Tobacco (*Nicotiana tabacum*) S-like RNase (Galiana et al., 1997; Hugot et al., 2002) and wheat (*Triticum aestivum*) PR protein of class 4 (Caporale et al., 2004) are induced by pathogen attacks, and their nuclease activities are necessary for inhibiting pathogen proliferation through slicing RNAs originating from fungal pathogens. RNS1 (S-RNase family) in Arabidopsis (*Arabidopsis thaliana*; LeBrasseur et al., 2002) and LE (RNase T2 family) in tomato (Gross et al., 2004) have been observed to be induced locally and systemically by wounding stress. Furthermore, several bifunctional nucleases having both RNase and DNase activities, such as BFN1 (Nuclease I family) in Arabidopsis (Perez-Amador et al., 2000), Bnuc1 (Nuclease I family) in barley (*Hordeum vulgare*; Muramoto et al., 1999), and PhNUC1 in *Petunia× hybrida* corollas (Langston et al., 2005), have been reported to be induced during leaf senescence. However, the molecular and cellular action mechanisms involved in the regulation of these responses of plant bifunctional nucleases (BFNs) remain largely unknown.

The wild rice species *Oryza minuta* (2n = 48; the genome type BBCC; accession no. 101141) is known to be resistant to various rice pathogens (Vaughan, 1994) and to contain useful resistance genes against serious insects and pathogens, such as brown planthopper (BPH; *Nilaparvata lugens*) and rice blast (*Magnaporthe oryzae*; Brar and Khush, 1997). Rice blast is a hemibiotrophic fungal pathogen that initially penetrates into host plant cells by mechanical wounding and subsequent enzymatic dissolution of host cell wall polymers. It then feeds on the initial infected sites as a biotroph, ultimately killing the infected host cell for proliferation and feeding on the cell remnants as a necrotroph (Howard and Valent, 1996; Ribot et al., 2008). On the other hand, the BPH causes mechanical wounding by chewing, but it does not kill the host cells as a biotrophic insect (Sogawa, 1976; Kim and Sohn, 2005). In an earlier study, we screened *O. minuta*

transcriptomes from wound-, BPH-, or rice blast-treated leaves and normal conditioned leaves in a high-throughput system to identify useful defense-related genes from *O. minuta* (Cho et al., 2004a, 2004b, 2005; Shim et al., 2004). In the study reported here, we identified a novel gene induced by wound-, BPH-, and *M. oryzae* in *O. minuta*. In vitro biochemical assays revealed that its recombinant protein has a novel BFN with both RNase and DNase activities. We have designated this BFN as OmBBD (for *O. minuta* bifunctional nuclease related to basal defense responses). Its Arabidopsis ortholog, AtBBD1, which contains high sequence conservation, was also identified. Here, we report the gene expression profiles, protein subcellular localization, and functions of OmBBD and AtBBD1 in ABA-mediated callose deposition during a defense response to attack by the necrotrophic pathogen *B. cinerea*.

RESULTS

Identification and Expression Analysis of the *OmBBD* Gene in *O. minuta*

We screened *O. minuta* ESTs involved in rice pathogen defense (Cho et al., 2004b). The gene expression patterns were closely monitored by northern analyses following the experimental treatments of rice leaves with wounding, BPH attack, and inoculation with rice blast. For further study, we selected EST 442, as its transcript was strongly induced 6 h after the BPH attack and wounding treatment. The expression of this transcript showed a biphasic induction pattern following inoculation with *M. oryzae*, with distinct increased expression 6 and 72 h post inoculation but drastically decreased expression 24 h post inoculation (Fig. 1A). It has been suggested that the hemibiotic nature of *M. oryzae* may be the cause of this biphasic induction pattern (Howard and Valent, 1996; Ribot et al., 2008). The full-length cDNA obtained by 5′-RACE-PCR and an end-to-end PCR was 1,337 nucleotides long and consisted of an open reading frame (ORF) of 996 nucleotides encoding 331 amino acids (deposited in GenBank under accession no. ABI79452).

Changes in *OmBBD* gene expression in response to important plant defense factors were studied by treating the leaves of four-leaf-stage *O. minuta* plantlets with methyl jasmonate (MeJA; 100 μM), ethephon (ETP; 5 mM), SA (200 μM), and ABA (100 μM; Fig. 1B). *OmBBD* transcription was induced in the leaf tissues of *O. minuta*, which is a wild rice species resistant to BPH and *M. oryzae*, upon treatment with wounding, BPH, and *M. oryzae* and also by JA, ET, SA, and ABA, suggesting its possible roles in defense against pathogens or mechanical wounding (Fig. 1).

The Predicted OmBBD and Its Homologs Contain Highly Conserved DUF151, UVR, and HCR Domains

The predicted amino acid sequences of OmBBD shared 99%, 59%, 56%, 57%, and 59% amino acid identities, respectively, with its homologs BAG89510 and BAG97425 from *Oryza sativa*, NP_849890 and NP_564093 from Arabidopsis, and BAA95791 from tobacco. The mRNA expression of the rice and Arabidopsis homologs, BAG89510 and NP_849890, was also turned on at a basal level in healthy leaves and then rapidly induced by MeJA, ETP, SA, and ABA treatments (Supplemental Fig. S1); these expression patterns were similar to those of the *OmBBD* gene. We designated the *O. sativa* and Arabidopsis homologs of *OmBBD*, BAG89510 and NP_849890, as *OsBBD1* and *AtBBD1*, respectively.

Putative amino acid sequence alignment of OmBBD and other plant homologs using the ClustalW algorithm (LeBrasseur et al., 2002) revealed a highly conserved domain of unknown function 151 (DUF151) and UVR (a dimerization domain between UvrB and UvrC) domains as well as a highly conserved region adjacent to the variable N-terminal region (designated as a highly conserved region [HCR]; Fig. 2). *OmBBD* homologs in various EST databases of monocots and dicots (e.g. for barley, http://www.tigr.org; for sweet potato [*Ipomoea batatas*] and poplar [*Populus* species], http://mycor.nancy.inra.fr) have been found to be widely conserved, with high sequence similarities in the DUF151 domain, even though their functional roles have not been elucidated.

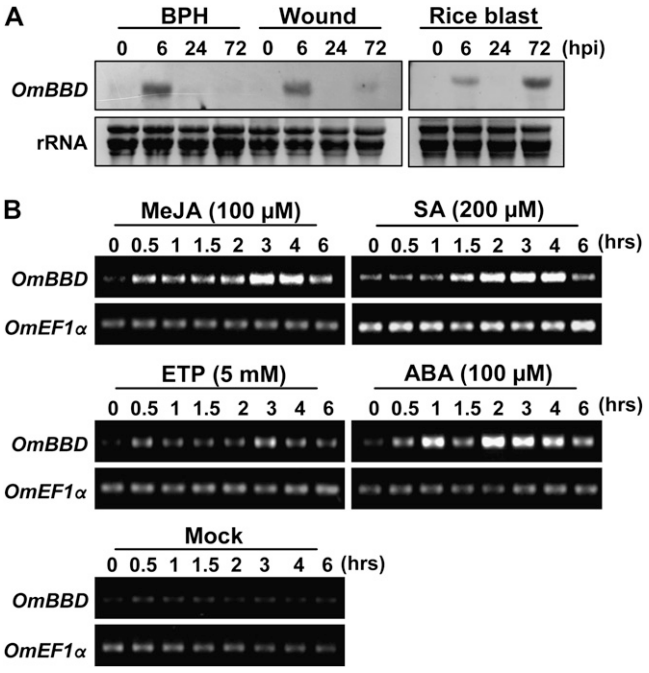

Figure 1. Expression profiles of the *OmBBD* gene after plant hormone, pathogen, and wounding treatments. A, Northern analysis using total RNAs isolated from four-leaf-stage *O. minuta* leaves treated by wounding, BPH, and rice blast. hpi, Hours post inoculation. The ethidium bromide-stained rRNA is shown as a loading control. B, RT-PCR analysis using total RNAs isolated from four-leaf-stage *O. minuta* leaves treated with various defense-related elicitors. The housekeeping gene *OmEF1α* was used as a loading control.

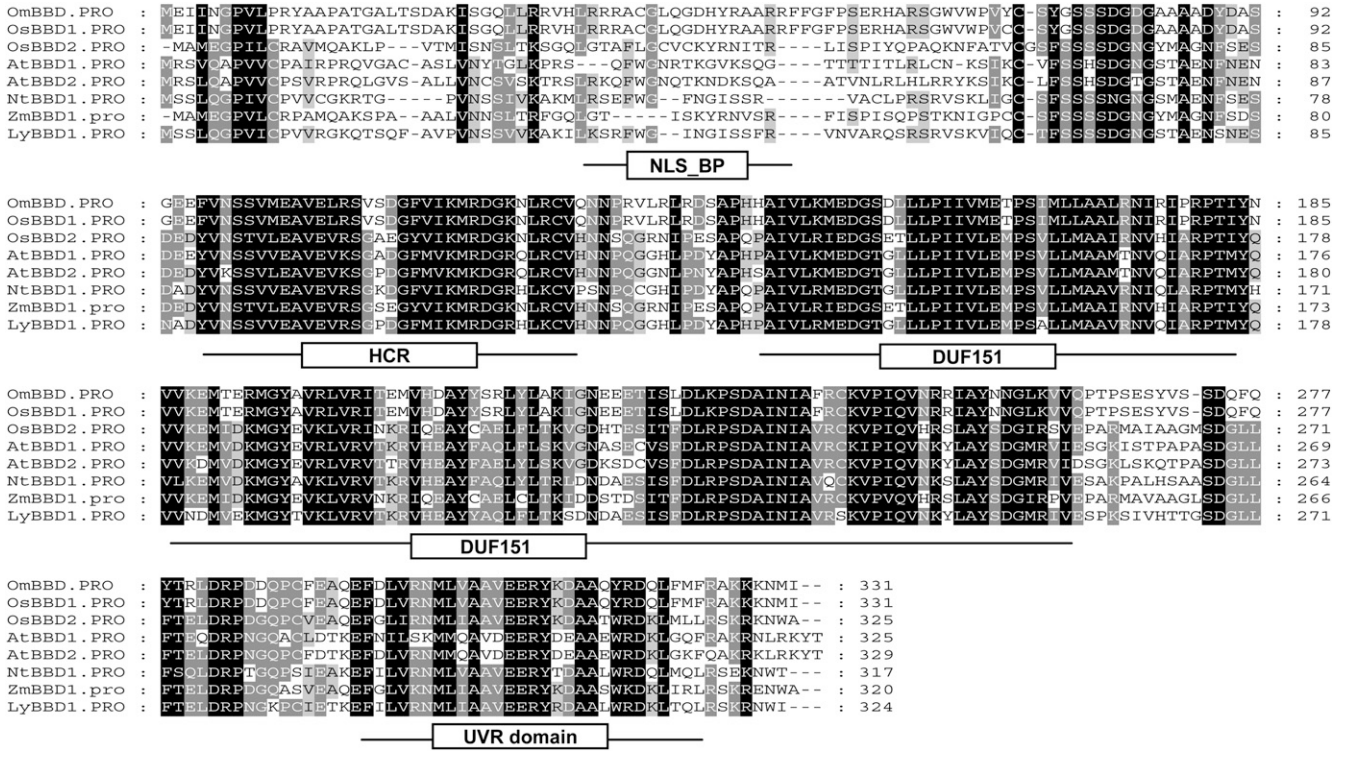

Figure 2. Alignment of the deduced amino acid sequences of OmBBD with other plant homologs using the ClustalW algorithm. Conserved amino acids are highlighted in black, and similar amino acids are highlighted in gray. NLS_BP, Bipartite nuclear localization signal.

OmBBD Is a Novel BFN with RNase and DNase Activities and Is Localized in the Nucleus of Onion Cells

The deduced amino acid sequence of OmBBD has a 60% probability of a nuclear localization (http://www.cbs.dtu.dk/services/TargetP/) and contains a putative bipartite nuclear localization signal in the N-terminal region (http://myhits.isb-sib.ch/cgi-bin/motif_scan; Fig. 2). To experimentally determine OmBBD localization, we generated constructs of *35S*:*smGFP* and *35S*:*OmBBD*::*smGFP*, introduced these into onion (*Allium cepa*) epidermal cells by particle bombardment, and then stained the cells with 4′,6-diamidino-2-phenylindole (DAPI). The nuclei were detected as bluish-white spots (Fig. 3B) using a fluorescence microscope; the corresponding GFP signals were detected by a GFP filter and merged with the DAPI-stained image by means of digital superimposition (Fig. 3B). While the control soluble-modified (sm) GFP proteins were uniformly distributed throughout the onion epidermal cell (Fig. 3A), the merged image showed that the OmBSD-smGFP fusion proteins were localized in the nuclei of the onion cells (Fig. 3B).

To determine the biochemical function of nuclear OmBBD, we first needed to investigate the functions of the highly conserved DUF151 and UVR domains in OmBBD. A search of the Pfam databank of a public database (http://pfam.sanger.ac.uk) for other proteins containing a DUF151 domain identified 93 DUF151 domain-containing proteins whose functions are still totally unknown. The fact that these were collected only from prokaryote and eukaryote databases and that no DUF151 domain-containing protein had been found in animals, yeast, and fungi indicated that the DUF151 family is specific to bacteria and plant organisms. Phylogenetic analysis of selected representative bacterial (33) and plant (nine) genes using the ClustalW method in the MegAlign program of Lasergene software (DNASTAR; http://www.dnastar.com) revealed that putative amino acid sequences of these 42 genes were clustered into four major groups (Supplemental Fig. S2), with group A consisting of only the DUF151 domain, group B consisting of a DUF151 domain plus a UVR domain, group C consisting of a DUF151 plus a UVR and HCR domain, and group D consisting of variable regions within the HCR, DUF151, and UVR domains.

The constructed phylogenetic tree suggests that the DUF151 family is derived from a common ancestor and developed conservatively from bacteria to plant and that it may be crucial for the biological function of this DUF151 domain-containing gene family. An x-ray crystal analysis of the TM0160 protein (181 amino acids) suggested that this protein possesses a putative active site with novel catalytic activity, but it failed to identify any exact biochemical function (Spraggon et al., 2004). The DUF151 domain (137 amino acids) is composed entirely of the TM0160 protein (181 amino

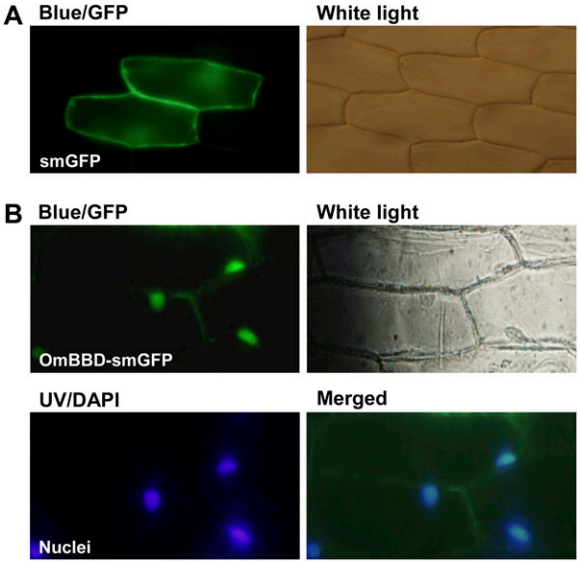

Figure 3. Localization of OmBBD-smGFP fusion proteins in onion epidermal cells. A, Localization of control smGFP proteins in onion cells. The smGFP signals were detected by a GFP filter, and onion cells were viewed under white light. B, Localization of OmBBD-smGFP fusion proteins in onion cells. The onion cells were stained with DAPI and viewed under UV light. The bluish white spots indicate stained nuclei of onion cells. The image obtained through the GFP light filter was merged with the one obtained through a UV light filter using digital superimpositions.

acids), leading to the suggestion that this domain is closely related to the putative catalytic activity of this protein. The phylogenetic tree analysis also showed that the UVR domain may be important for the biological function of group C and D proteins. In support of this hypothesis, a UVR domain has been reported to be crucial in protein-protein interactions between UvrB and UvrC in the UVR protein complex (Moolenaar et al., 1995; Sohi et al., 2000).

The predictive catalytic activity of a DUF151 domain and the nucleus localization of OmBBD led to the conjecture that OmBBD is a nuclear protein with nuclease activity. RNA and DNA degradation assays using a recombinant OmBBD protein fused with a glutathione *S*-transferase tag (GST-OmBBD) were carried out as previously described (Bantignies et al., 2000) to investigate this possibility. For the RNA degradation assay, total RNAs extracted from *O. minuta* were incubated with GST-OmBBD. The reaction mixtures (including positive and negative controls; Fig. 4) were loaded and run on a 1.2% formaldehyde agarose gel. The ribosomal RNA bands, but not those of the GST tag protein, which was the negative control, were degraded and smeared by the GST-OmBBD protein (Fig. 4A). The GST-OmBBD protein retained its nuclease activity after boiling, indicating that the RNase activity of OmBBD is thermostable (Fig. 4A). Torula yeast RNAs on a polyacrylamide gel were consistently digested by GST-OmBBD, based on the presence of bands stained negatively with toluidine blue O (Yen and Green, 1991; Fig. 4B, left panel). Western-blot analysis using a GST antibody verified the presence of the corresponding GST-OmBBD protein in the ribonuclease activity spot on the gel (Fig. 4B, right panel). Based on these results, therefore, we concluded that OmBBD confers ribonuclease activity.

For the DNA degradation assay, the genomic DNAs extracted from *O. minuta*, salmon sperm DNA, circular plasmids, and digested linear plasmids were incubated with the GST-OmBBD protein (Fig. 4C) and subsequently analyzed on a 0.8% agarose gel. The GST-OmBBD protein showed a DNase activity based on the appearance of smeared DNA running patterns on the gel. Confirmation that the degradation of DNAs was derived from the DNase activity of OmBBD was obtained by running an in-gel DNase assay with a polyacrylamide gel containing salmon sperm DNA as a substrate. Reaction buffers were supplemented with $MnCl_2$, $MgCl_2$, $ZnCl_2$, or $CaCl_2$ as cofactor for DNase activity. At 3 h of incubation, a negatively stained band was detected only in the buffer supplemented with $MnCl_2$ (Fig. 4D). Weak bands were detected in the buffers with other cofactors only after more extended incubation times (data not shown). Thus, the DNase activity of OmBBD may not be cofactor dependent but only enhanced in the presence of $MnCl_2$. In contrast to its RNase activity, the GST-OmBBD protein failed to digest the DNAs after boiling (Fig. 4D), indicating that the DNase activity of OmBBD is thermounstable.

Based on these results, we suggest that the OmBBD protein is a bifunctional nuclease with both RNase and DNase activities. Alignment of the amino acid sequences of the plant OmBBD homologs with those of known BFN homologs did not reveal any significant conservative residue. We were also unable to find the two conserved His residues that characterize the catalytic activity of the S1/P1 nuclease family with both RNase and DNase activities (Maekawa et al., 1991). These results show that the OmBBD homologs are novel BFN proteins that differ from the previously reported BFN family.

The *atbbd1* Mutant Shows Susceptible Phenotypes against *B. cinerea*, and the Ectopic Expression of the *OmBBD* Gene Complements the Phenotypes of *atbbd1*

OmBBD expression in wild rice was induced by wounding and at a late infection phase of *M. oryzae*. Using the Arabidopsis model system to investigate the biological functions of OmBBD, we assessed the expression patterns of the *AtBBD1* gene in response to attack by a necrotrophic pathogen, *B. cinerea* (Fig. 5A). *AtBBD1* expression was found to increase concurrently with the development of the disease symptoms caused by *B. cinerea* infection, suggesting a possible function of *OmBBD* in the defense or pathogenesis response. The biological function of the *OmBBD* or *AtBBD1* gene in plant fungal defense was also analyzed by introducing the *OmBBD* and *AtBBD1* genes into an *atbbd1* knockout mutant line, in which a T-DNA had been inserted in the third exon of the *AtBBD1* gene (courtesy of the Salk

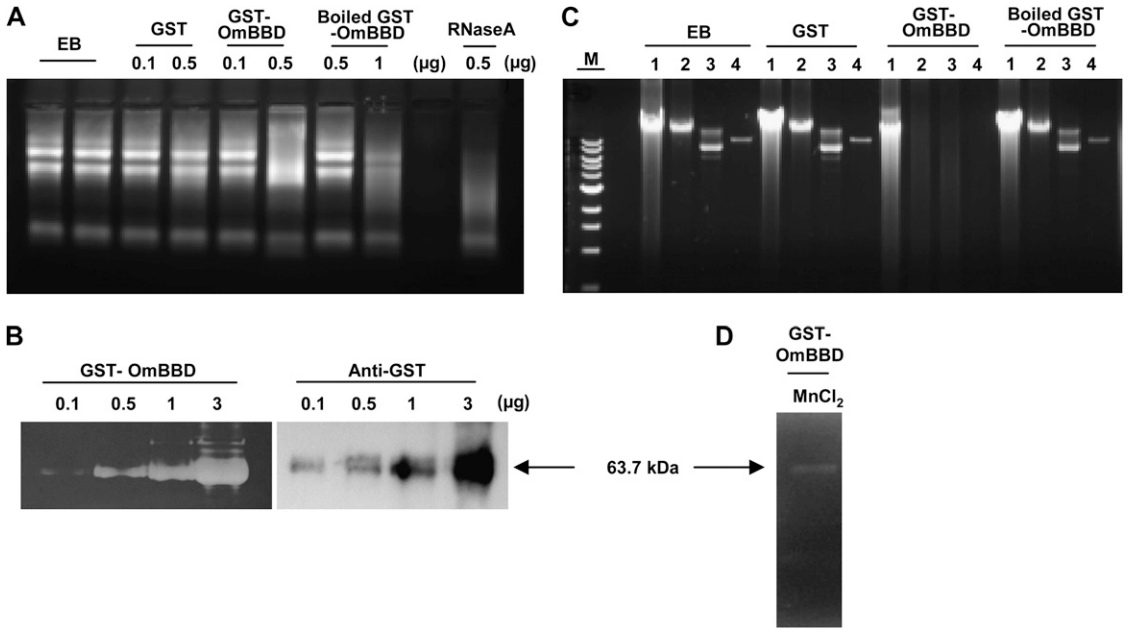

Figure 4. Bifunctional nuclease activity of a recombinant GST-OmBBD fusion protein. A, RNase activity of the GST-OmBBD fusion protein was assayed using total RNA prepared from *O. minuta* leaves for 2 h at 50°C. The degraded RNA products were loaded onto a 1.2% formaldehyde agarose gel. EB, Elution buffer (30.8 mg mL^{-1} reduced glutathione in 50 mM Tris-HCl, pH 8.0); GST, GST protein only. RNase A was used as a positive control, and elution buffer and GST were used as negative controls. B, RNase activity was confirmed on a polyacrylamide gel containing 2.4 mg mL^{-1} yeast RNA for 0.1, 0.5, 1, and 3 μg of GST-OmBBD fusion proteins. Boiled proteins and native proteins were loaded onto an SDS-PAGE system and electrophoresed; the SDS-PAGE gel was then stained with a toluidine blue O solution. The negative bands on the stained gel were confirmed to be from GST-OmBBD fusion proteins by western-blot analysis with GST antibody (right panel). C, DNase activity of GST-OmBBD was tested using various kinds of DNAs for 2 h at 50°C. Lane M, 1-kb marker; lane 1, 5 μg of salmon sperm DNA; lane 2, 3 μg of *O. minuta* genomic DNA; lane 3, 2 μg of circular DNA; lane 4, 2 μg of digested linear plasmid. The degraded DNA products were loaded onto a 1.0% agarose gel. D, DNase activity was tested on a native PAGE gel containing 0.4 mg mL^{-1} salmon sperm DNA as a substrate using an incubation buffer supplemented with 50 μM $MnCl_2$ as cofactor for DNase activity. All results were obtained in three independent experiments.

Institute). The results confirmed the ectopic expression of each of these transcripts in the *atbbd1* mutant line (Fig. 5B). An examination of fungal (*B. cinerea*) and bacterial (*Pseudomonas syringae* pv *tomato* DC3000 [*Pst*DC3000]) pathogen responses in the *atbbd1* mutant revealed that relative to the wild type (Columbia ecotype [Col-0]), this mutant showed increased susceptibility only against the fungal pathogen (Fig. 5C; Supplemental Fig. S4A). Necroses following fungal infection were observed 5 d after inoculation, with the average size of the necrotized area of *atbbd1* leaf tissues being approximately 2-fold larger than that of the wild type (Fig. 5C). The fungal susceptibility of *atbbd1* was rescued by complementation with ectopic *OmBBD* or *AtBBD1* expression under the control of the 35S promoter, suggesting that both AtBBD1 and OmBBD are conserved in the defense response to *B. cinerea* (Fig. 5C).

OmBBD and AtBBD1 Have a Role in the Induction of the PDF1.2-Related Defense Response in Arabidopsis against *B. cinerea* Attacks

To investigate whether OmBBD acts in defense responses against *B. cinerea* in Arabidopsis, we ectopically overexpressed the *OmBBD* gene in Arabidopsis (Col-0). Five individual T3 homozygous lines were originally selected. Of these, three were further selected based on transgene expression levels: OM-OE1 (*OmBBD*-overexpressed line 1), OM-OE3, and OM-OE2, with the highest, medium, and basal levels of transgene expression, respectively (Fig. 6B).

Leaves of wild-type (Col-0) and *35S:OmBBD* (OM-OE1, OM-OE2, and OM-OE3) plants and of *atbbd1* mutant plants were inoculated with the fungal pathogen *B. cinerea*, and the size of the necrotic regions was measured 5 d after inoculation. All three transgenic lines were more resistant to *B. cinerea* than the wild-type and *atbbd1* plants, with the resistance levels of *35S:OmBBD* plants being dependent on the expression level of *OmBBD*. In contrast, plants of the *atbbd1* mutant line were highly susceptible (Fig. 6A). These results are also consistent with those of the pathogen assay in *atbbd1* and transgenic *atbbd1* plants expressing *OmBBD* (Fig. 5C).

JA signaling in Arabidopsis has been reported to be essential in the defense responses against necrotrophic pathogens. In addition, the plant defensin gene

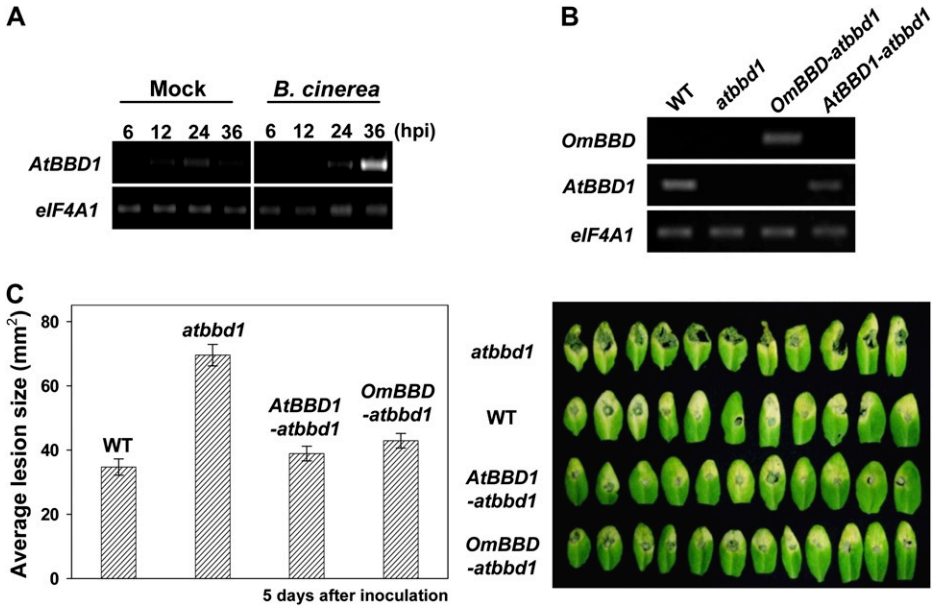

Figure 5. The susceptible phenotypes of *atbbd1* against *B. cinerea* and the rescue of susceptibility by ectopic expression of either *OmBBD* or *AtBBD1* in *atbbd1* plants. A, Expression patterns of the *AtBBD1* gene after *B. cinerea* infection. *B. cinerea* (8×10^5 spores mL^{-1}) in 0.001% Triton X-100 was sprayed onto four leaves of each 4-week-old plant. The 0.001% Triton X-100 solution was treated as a mock control. The housekeeping gene *eIF4A1* was used as a loading control. hpi, Hours post inoculation. B, RT-PCR analysis of *AtBBD1* and *OmBBD* in 2-week-old seedlings of *atbbd1* and rescue lines. *OmBBD-atbbd1* and *AtBBD1-atbbd1* represent the rescue lines of *AtBBD1* and *OmBBD*, respectively. WT, Wild type. C, Degree of disease development (mm^2) 5 d after the inoculation of *B. cinerea* (left). Photographs were taken 5 d after inoculation (right). A 10-μL suspension of *B. cinerea* (8×10^4 spores mL^{-1}) was dropped onto four leaves of each 4-week-old plant; a total of 36 plants were used for each line. Average lesion sizes were determined 5 d after inoculation. Values presented are means ± SE (n = 100) obtained from three independent experiments.

PDF1.2, a downstream gene of the JA/ET signaling pathway, is known to be closely related to the resistance against *B. cinerea* (Penninckx et al., 1998; Glazebrook, 2005). To determine whether OmBBD and AtBBD1 function in the JA/ET signaling pathway, we identified the transcript levels of *PDF1.2* against *B. cinerea* in wild-type, OM-OE1, and *atbbd1* plants. *PDF1.2* was induced to a basal level in OM-OE1 plants in the absence of fungal inoculation when compared with the responses of the wild-type and *atbbd1* plants. Upon *B. cinerea* inoculation, *PDF1.2* expression increased relatively slower in *atbbd1* plants than in wild-type plants but relatively faster and more sensitively in OM-OE1 plants (Fig. 6C). In addition, differential expression patterns of *PDF1.2* in *atbbd1* and OM-OE1 plants were observed after the MeJA or ETP treatment (Supplemental Fig. S3). These results show that both OmBBD and AtBBD1 play important roles in the defense-related signaling pathway by influencing the induction of the *PDF1.2* gene.

OmBBD and AtBBD1 Function in ABA-Related Callose Deposition in Response to Attack by *B. cinerea*

The finding that both the *OmBBD* and *AtBBD1* genes were also strongly induced by ABA suggested the possibility that the gene products are involved in ABA-induced defense. A test of *RD29a* expression revealed that the transcript level of *RD29a* was reduced in ABA-treated *atbbd1* mutant plants compared with wild-type plants (Supplemental Fig. S3). In addition, *35S:OmBBD* plants consistently showed a reduced rate of seed germination in comparison with wild-type and *atbbd1* plants upon exogenous ABA treatment (Fig. 7A). These results indicate that OmBBD and AtBBD1 have a potential role in ABA-related responses in Arabidopsis.

ABA possibly has important functions in the defense response by regulating the rapid induction of callose deposition (Ton and Mauch-Mani, 2004; Mauch-Mani and Mauch, 2005; Ton et al., 2005). Ton et al. (2005) reported that both the *AtSAC1b* (i.e. phosphoinositide phosphatase) and *ABA1* genes appeared to function in the augmented callose deposition process during the expression of BABA-IR against another fungal pathogen, *Hyaloperonospora arabidopsidis*. Our analysis of the expression levels of those genes in 4-week-old leaves of wild-type, *35S:OmBBD*, and *atbbd1* plants by reverse-transcription (RT)-PCR revealed that *AtSAC1b* and *ABA1* were constitutively expressed in OM-OE1, OM-OE2, and OM-OE3 plants (Fig. 7B) and that this expression reflected that of *OmBBD* in Arabidopsis. These results imply that OmBBD and AtBBD1 function in cell wall reinforcement through the ABA-derived callose deposition that is induced following infection by a necrotrophic path-

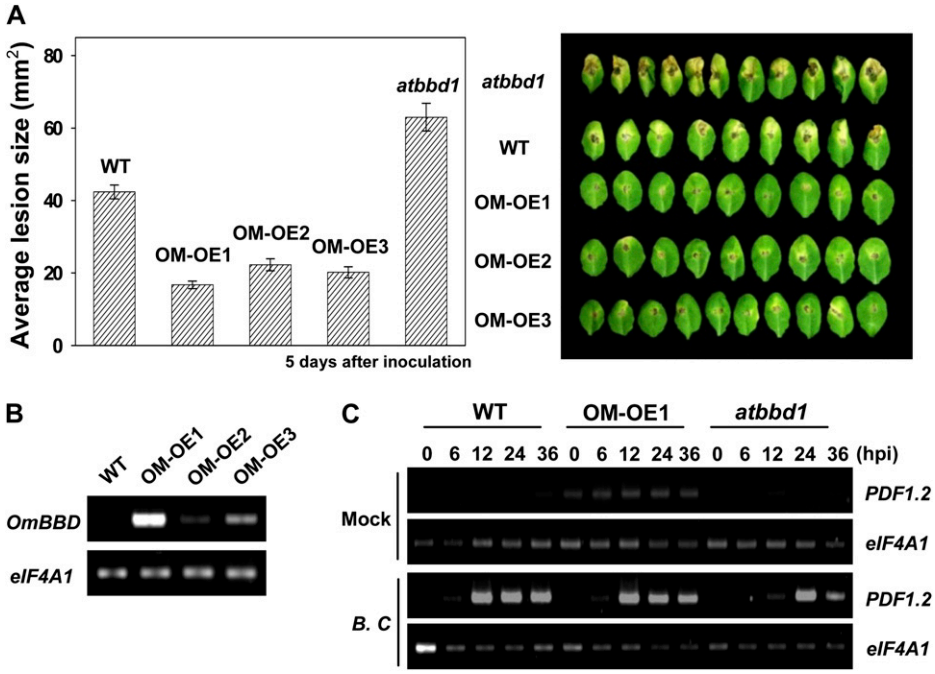

Figure 6. Disease development in wild-type (WT), *35S:OmBBD*, and *atbbd1* plants following inoculation with *B. cinerea*. A, Degree of disease development (mm^2) 5 d after inoculation of *B. cinerea* (left). Photographs were taken 5 d after inoculation (right). A 10-μL suspension of *B. cinerea* (8×10^4 spore mL^{-1}) was dropped onto the four leaves of each 4-week-old plant; a total of 36 plants were used for each line. Average lesion sizes were determined 5 d after inoculation. Values presented are means ± SE of the mean ($n = 100$) obtained from three independent experiments. B, Expression levels of *OmBBD* in leaves of 2-week-old wild-type and three independent *35S:OmBBD* plants (OM-OE1, OM-OE2, and OM-OE3). C, Expression analysis of *PDF1.2* in wild-type, OM-OE1, and *atbbd1* mutant plants at different time periods after *B. cinerea* (B. C) inoculation. Four-week-old plants were sprayed with the *B. cinerea* suspension (5×10^5 spore mL^{-1}) in 0.001% Triton X-100 or 0.001% Triton X-100 (mock control). hpi, Hours post inoculation.

ogen. To verify this hypothesis, we examined the accumulation of callose in wild-type, OM-OE1, and *atbbd1* leaves that had been sprayed with *Botrytis* spores (Fig. 8). The inoculated leaves were stained with aniline blue. Callose deposition was identified as bluish-white spots under UV illumination, and fungal hyphae were observed under white light (Fig. 8A). The presence of bluish-white spots on the inoculated leaves of wild-type plants showed that normal callose deposition occurred at the penetration sites of these plants. Callose was much more strongly and more rapidly deposited from the early stage of fungal penetration (12 h post inoculation) in the infected leaves of OM-OE1 plants than in those of wild-type and *atbbd1* plants (Fig. 8B). In contrast, callose deposition was reduced at the infected sites of *atbbd1* plants (Fig. 8). This "deficiency" in callose deposition in *atbbd1* plants was rescued by complementation with ectopic *OmBBD* or *AtBBD1* expression under the control of the 35S promoter (Fig. 8C). These results show that OmBBD and AtBBD1 participate in callose deposition following infection by *B. cinerea*.

DISCUSSION

We have demonstrated the potential role of OmBBD and AtBBD1 in a host defense system against a necrotrophic pathogen, *B. cinerea*, via JA- and ABA-dependent plant regulatory pathways and callose deposition.

Based on our results, we identified OmBBD as a novel BFN with both RNase and DNase activities. However, we were unable to determine whether or not the nuclease activity of OmBBD is essential for the defense responses in Arabidopsis. This led to the interesting question of just how OmBBD, the BFN protein shown to have nonspecific substrates (Fig. 4), can be involved in the regulation of specific defense responses, such as callose deposition. Consequently, special attention was given to the fact that the UVR domain plays a role in the protein-protein interaction between UvrB and UvrC in the UVR protein complex (Moolenaar et al., 1995; Sohi et al., 2000). We first screened a yeast two-hybrid library constructed from four-leaf-stage *O. minuta* leaves treated with rice blast, subsequently identifying four nonredundant clones from the primary screening and β-galactosidase assay. Carrying out a BLASTX search, we obtained two potential candidate clones from these four nonredundant clones that were homologs of *O. sativa* BAG98749 and BAG87290, respectively. The partial amino acid sequence of a BAG87290 homolog possessed the C3HC4-type zinc finger (RING finger) domain that is

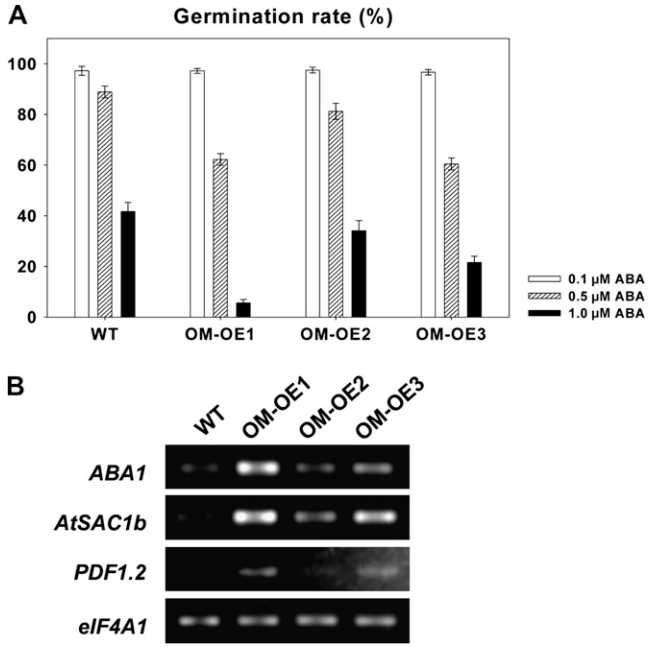

Figure 7. The test of germination inhibition by ABA and the constitutively expressed genes in *35S:OmBBD* Arabidopsis plants. A, Seeds were germinated on MS media containing three different concentrations of ABA (0.1, 0.5, and 1 μM). Germination rates were determined 9 d after seeding on 10 MS plates of each set (150 seeds for each ABA concentration). Values presented are means ± SE ($n = 5$) obtained from three independent experiments. B, RT-PCR analysis of *ABA1*, *AtSAC1b*, and *PDF1.2* in 2-week-old wild-type (WT) and three independent *35S:OmBBD* Arabidopsis plants (OM-OE1, OM-OE2, and OM-OE3).

intrinsic to protein-protein interaction in the ubiquitination pathway (Lorick et al., 1999; Supplemental Fig. S5). These results raise the possibility that the OmBBD protein may interact with any counterpart protein to control the defense responses.

Several nucleases have been reported to function as efficient and rapid regulators of gene expression in posttranscriptional regulation by affecting the stability of the target mRNAs (van Hoof et al., 2002). AtPARN [for poly(A)-specific ribonuclease] participates in the fine-tuning of the correct cellular responses against ABA, SA, and stress signaling by deadenylating the target mRNAs, thereby changing them into a more easily degradable molecule (Nishimura et al., 2005). In their report, Nishimura et al. (2005) suggest that the cellular functions of AtPARN in the stress response are regulated by a number of other components, including microRNA or RNA-binding proteins. In an animal system, CUG-BP, an RNA-binding protein, has been reported to bind to RNA substrates and recruit PARN deadenylase, thereby showing the possibility that some counterpart proteins play a key role in the regulatory functions of PARN deadenylase (Moraes et al., 2006). A review of the *S-RNase* gene, which is located at a single, highly polymorphic genetic locus called the *S*-locus, suggests that this gene plays a key role in the self-incompatibility interaction through rejecting self-pollen (Cruz-Garcia et al., 2003; Wang et al., 2003). The RNase activity of S-RNases is essential to the proper functioning of S-RNases in the self-incompatibility response (Huang et al., 1994), but it remains unclear just how the S-RNases can specifically affect the rejection of self-pollen. It has been reported recently that several *S*-locus F-box proteins control the functions of S-RNases through the ubiquitin/26S proteasome pathway of protein degradation (Qiao et al., 2004; Hua et al., 2007; Newbigin et al., 2008). These reports provide additional support to our hypothesis of the existence of a counterpart protein whose function is to control the regulatory roles of OmBBD in defense responses. Consequently, in further study, it should be determined whether or not the nuclease activity of OmBBD is essential for the defense responses in plants by identifying essential residues of OmBBD on its nuclease activity.

Callose deposition arrests the proliferation of fungal or bacterial pathogens by reinforcing the cell walls around the infected regions. As such, this process is a crucial basal defense response in both dicot (Glazebrook, 2005; Hardham et al., 2007) and monocot (Yang et al., 2004; Hao et al., 2008) plants. Studies on the callose deficient mutant *pmr4-1* (Flors et al., 2008), the ABA-deficient mutant *aba2-12* (Adie et al., 2007), and the *cob-5* mutant overproducing JA and callose (Ko et al., 2006) have revealed that ABA and/or JA synergistically play essential roles in the callose deposition process against necrotrophic fungal pathogens and that the pathogen-induced callose deposition is primed or augmented by ABA and causes enhancement of JA-dependent defense responses to suppress SA-dependent responses. Our results are in agreement with this working model. In the *35S:OmBBD* lines, the expression of both the *PDF1.2* and *ABA1* genes was constitutively up-regulated (Fig. 7B) relative to that of wild-type plants, but the expression of the *PDF1.2* and *RD29a* genes in *atbbd1* was reduced (Supplemental Fig. S3). Callose deposition in response to *B. cinerea* was less in the *atbbd1* plants, and this deficiency was rescued by complementation with overexpression of either *OmBBD* or *AtBBD1* (Fig. 8). Following SA treatment, the expression of the *PR1* gene increased more slowly in OM-OE1 plants and much more quickly in *atbbd1* plants relative to wild-type plants (Supplemental Fig. S3). These results are consistent with previous observations that ABA-derived callose deposition suppresses SA-dependent responses and increases the JA-dependent defense responses indirectly (Flors et al., 2008). Interestingly, we also observed similar callose deposition patterns in wild-type and *atbbd1* plants in response to treatment with a virulent biotrophic bacterial pathogen, *Pst*DC3000 (Supplemental Fig. S4B). However, we failed to detect any change in resistance against *Pst*DC3000 (Supplemental Fig. S4A). Despite the lack of callose deposition in the *pmr4-1* mutant, it is resistant to attacks by fungal pathogens, powdery mildew, and the bacterial pathogen *Pst*DC3000 (Nishimura et al., 2003). One possible

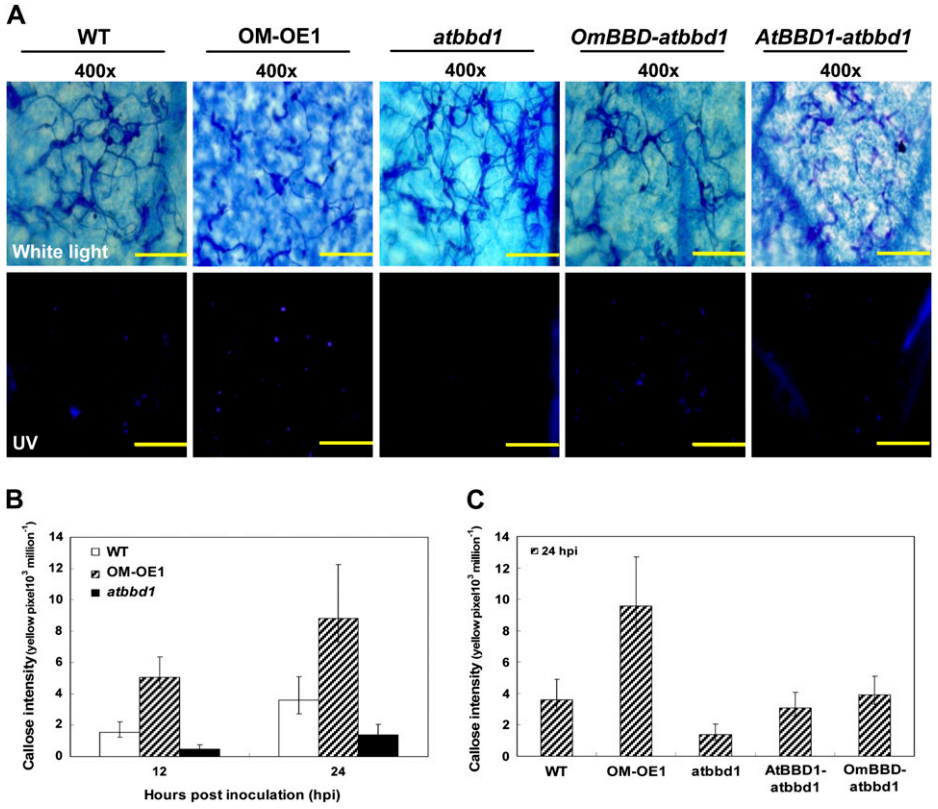

Figure 8. Comparison of callose accumulation in wild-type (WT), OM-OE1, *atbbd1*, and complementation lines during fungal penetration of the necrotrophic pathogen *B. cinerea*. A, Callose and fungal hyphae on infected sites in wild-type, OM-OE1, *atbbd1*, and complementation lines. Four-week-old plants were sprayed with the *B. cinerea* suspension (5×10^5 spores mL^{-1}). Leaves were harvested after 24 h, stained with aniline blue solution, and photographed under UV light to detect callose and under a bright channel (white) to detect fungal hyphae. Bars = 100 μm. B and C, Comparison of callose accumulation in wild-type, OM-OE1, and *atbbd1* lines at 12 and 24 h post inoculation (B) and in wild-type, OM-OE1, *atbbd1*, and complementation lines at 24 h post inoculation (C). Callose was visualized by aniline blue staining and epifluorescence microscopy, and callose depositions were quantified by determining the number of yellow pixels (corresponding to pathogen-induced callose) per million pixels in digital photographs of infected leaf areas. Values presented are means ± SE (n = 17) obtained from three independent experiments.

explanation for this resistance is that highly induced SA responses in the mutant may drive such resistance (Clay et al., 2009). Studies on a bacterial effector, the DspA/E-deficient mutant of *Pst*DC3000 (*ΔCEL*), showed that although *ΔCEL* was able to inactivate the SA-independent basal defense of the host plant, it failed to invade aggressively into the host plant because the inoculated host plant was able to overcome the suppression of the basal defense by activating the SA-dependent basal defense pathway (DebRoy et al., 2004). It would appear that the increase in SA-dependent responses in *atbbd1* compensates for the decrease in callose deposition in response to *Pst*DC3000 and that the increase in callose deposition in OM-OE1 may provide an enhanced basal immunity in response to *Pst*DC3000. Our studies also support recent reports of ABA having important roles in the resistance to necrotroph and susceptibility to *P. syringae* through the cross talk with JA- and SA-dependent defense responses, which activate JA accumulation and suppress the SA-dependent responses (de Torres Zabala et al., 2009; Fan et al., 2009). Therefore, we suggest that OmBBD and AtBBD1 participate in the ABA-derived callose deposition that mediates the enhanced JA-related responses and the suppression of SA-related responses.

Conversely, the negative roles of ABA in defense responses against necrotrophs have also been reported (Audenaert et al., 2002; Anderson et al., 2004; Fujita et al., 2006; Asselbergh et al., 2007). In their review, Asselbergh et al. (2008) suggest that the positive and negative regulation of ABA in the plant's defense responses to pathogens may be fine-tuned by the large and complex overlap between abiotic and biotic stress signaling pathways at multiple levels. Our data show that ABA-related responses were altered in the *35S*:*OmBBD* lines and *atbbd1* (Fig. 7; Supplemental Fig. S3). To determine whether the altered ABA-related responses in the *35S*:*OmBBD* lines and *atbbd1* are involved in tolerance responses to abiotic stresses, we examined the response of these mutants to salt stress by comparing their root growth to that of the wild type

on solid Murashige and Skoog (MS) medium containing 150 mM NaCl. No clear differences were observed (data not shown), indicating that the altered ABA-related responses in these mutants are not directly involved in tolerance responses to abiotic stresses.

The possibility that the endogenous levels of ABA or the levels of pathogen-induced reactive oxygen intermediates (ROIs) may be key factors in the positive or negative fine-tuning involvement of ABA in defense responses has been discussed in several research articles (Mauch-Mani and Mauch, 2005; Torres and Dangl, 2005; Asselbergh et al., 2008; Ton et al., 2009). ROIs regulate various cellular defense responses in a dose-dependent manner: high levels of ROIs promote hypersensitive response-related programmed cell death, while low levels of ROIs induce antioxidant enzymes (Mellersh et al., 2002; Vranova et al., 2002). ABA also has two opposing roles in ROI-induced responses, which are also dose dependent: at low concentrations, ABA can cause the accumulation of ROIs to induce antioxidant-mediated defense responses, but at high concentrations, it conversely induces the oxidative burst to cause cell death (Jiang and Zhang, 2001). JA also plays an important role in the attenuation of the induced ROI production (Overmyer et al., 2000; Hu et al., 2009). These results indicate that the level of ROIs plays important roles in fine-tuning the interplay between ABA and JA. Nitric oxide is essential to the plant's defense responses against necrotrophs, causing an oxidative burst in host cells (Floryszak-Wieczorek et al., 2007; Asai and Yoshioka, 2009). It also has important roles in wounding-, ABA-, and/or JA-mediated redox regulation and in wound-induced callose deposition against the accumulation of ROIs (Paris et al., 2007; Sang et al., 2007; Arasimowicz et al., 2009; Hu et al., 2009). These results hypothesize that wounding-mediated responses are involved in ABA-mediated defense responses to necrotrophs. In accordance with this point of view, it is noteworthy that the transcript level of the *OmBBD* gene increased following the wound treatment and the inoculation of BPH or rice blast (Fig. 1A). Wound stress caused by the penetration of fungal pathogens or the chewing damage of herbivores is regarded as an important stress signal that induces the innate immunity of host plants at an initial stage of pathogenesis (Moran and Thompson, 2001; Diaz et al., 2002). Necrotrophic fungal pathogens penetrate into host cells by breaking down the cell walls at the infection site, either by mechanical or enzymatic wounding, and then cause the oxidative burst that kills the host cells (Howard and Valent, 1996; van Kan, 2006). As defense responses against the wounding and subsequent oxidative burst of necrotrophs, host plants induce JA-dependent responses to inhibit the proliferation of pathogens in their cells (Glazebrook, 2005). Wounding initiates the production of ROIs, such as hydrogen peroxide (Ruuhola and Yang, 2006), and hydrogen peroxide production is coupled with JA biosynthesis in the peroxisome. As such, this redox regulation has important roles in the plant's defense responses against pathogen attacks or wound stress (Li et al., 2005; Wasternack et al., 2006). The positive involvement of ABA in wound-induced responses has also been reported. Wound-inducible genes are not activated by wounding in ABA-deficient mutants of tomato (Pena-Cortes et al., 1996) or when JA synthesis is blocked (Pena-Cortes et al., 1993), indicating the synergistic interaction of ABA and JA in wound-induced responses. Interestingly, PMR4-mediated signaling is involved not only in necrotrophic pathogen-induced callose deposition but also in wound-induced callose deposition (Jacobs et al., 2003). Based on our results and those of earlier studies, we hypothesize that the positive roles of ABA in defense responses against necrotrophs may be closely related to wound-induced responses and not directly related to the dominant role played by ABA in the plant's responses to abiotic stress. Support for this hypothesis is provided by our results: (1) the *OmBBD* gene was induced by both wounding and rice blast; (2) OmBBD played a role in ABA-mediated callose deposition against *B. cinerea*; and (3) the *35S*:*OmBBD* lines and *atbbd1* did not respond to abiotic stresses, such as NaCl.

In rice, ABA was reported to play negative roles in SA-dependent defense responses against a hemibiotrophic fungal pathogen, *M. oryzae* (Xiong and Yang, 2003), similar to its reported role in Arabidopsis. However, the involvement of ABA in pathogen-induced defense responses has also been reported based on the transcript analysis of several defense-related genes, including mitogen-activated protein kinase (MAPK) genes, *OsSIPK* (for salicylic acid-induced protein kinase; Lee et al., 2008), *OsMSRMK2* (for multiple stress-responsive MAPK; Agrawal et al., 2002), *OmMKK1* (for *O. minuta* MAPK kinase 1; You et al., 2007), and a rice leucine-rich repeat receptor-like kinase (LRR-RLK) and *OsBBR1* (blast resistance-related gene; Peng et al., 2009). *M. oryzae*, a hemibiotrophic fungal pathogen, causes an intense transcriptional reprogramming that is mediated by auxin, ABA, and JA during switching from the biotrophic phase to a necrotroph-like phase (Schweizer et al., 1997; Ribot et al., 2008). A JA-inducible rice *myb* gene (*JAmyb*), which plays important roles in host defense responses against rice blast fungus, is rapidly induced by JA as well as by wounding (Lee et al., 2001). Collectively, those results suggest the possible roles of ABA-, wounding-, and/or JA-mediated responses in the plant's defense responses against *M. oryzae*. In parallel, the *OmBBD* gene is induced by wounding and rice blast as well as by ABA, JA, and SA, suggesting the possibility that the *OmBBD* gene may be responsive to the necrotroph-like phase of *M. oryzae*. Callose deposition in rice is induced on sieve plates by BPH feeding, where it functions as a cell wall barrier in a basal defense mechanism by blocking the digestion of phloem sap (Hao et al., 2008). The penetration of rice blast, *M. oryzae*, strongly induces callose deposition at

the early stages of disease development (Kim et al., 2004; Yang et al., 2004). Although the mechanism of callose deposition in rice remains largely unknown, the results of a number of studies suggest that callose deposition is triggered by the penetration of rice pathogens and that this deposition is important in host resistance. Several research groups have found that plant defense responses are partially conserved between Arabidopsis and rice (Song and Goodman, 2001; Ding et al., 2009; Park et al., 2009). The resistance of Arabidopsis to *M. oryzae* is derived from the induction of the SA and JA/ET signaling pathways, callose deposition, and accumulation of ROIs, all of which are similar to the defense responses against the necrotrophic fungus *B. cinerea* (Park et al., 2009). We found that the ectopic expression of OmBBD in *atbbd1* complemented completely the deficient phenotype in terms of callose deposition and susceptibility to *B. cinerea*, indicating the conserved roles of OmBBD and AtBBD1 in defense responses of Arabidopsis against *B. cinerea*. Therefore, we surmise that the functions of OmBBD in rice defense responses may be similar to those in the defense responses of Arabidopsis.

In conclusion, we have established that the protein encoded by the wound-, BPH-, and rice blast-induced *OmBBD* gene is a novel bifunctional nuclease with both RNase and DNase activities. Our results from studies on Arabidopsis mutants suggest that OmBBD and its Arabidopsis ortholog, AtBBD1, have regulatory roles in ABA-derived callose deposition as an initial defense barrier against pathogenesis. Our preliminary data from yeast two-hybrid screening enables us to suggest that the regulatory roles of OmBBD and AtBBD1 may be mediated by a counterpart protein. We also propose the possible role of a BFN as a novel regulator in ABA-mediated defense responses.

MATERIALS AND METHODS

Plant Materials

Oryza minuta (accession no. 101144) and *Oryza sativa* 'Hwasungbyeo' plants were cultivated at 25°C under a 16-h/8-h (light/dark) photoperiod for 4 and 2 weeks, respectively, when the plantlets reached the four-leaf stage. All Arabidopsis (*Arabidopsis thaliana*) genotypes were grown on a Sunshine No. 5 mixture at 22°C under a 16-h/8-h (light/dark) photoperiod.

Cloning and Identification of the *OmBBD* and *AtBBD1* Genes

The putative full-length cDNA of *OmBBD* was obtained by 5′ RACE-PCR with four primer pairs (Supplemental Table S1) obtained from a previous *O. minuta* cDNA library (Frohman et al., 1988; Cho et al., 2004b). The full-length cDNA was obtained by end-to-end PCR with the primers nearest to the 5′ or 3′ end, using newly synthesized first-strand cDNA of pooled RNAs isolated from *O. minuta* leaves infested with blast and BPH. All PCR products were cloned into the pGEM-T Easy cloning vector (Promega), and three colonies were selected for sequencing. The full-length ORFs with the *Bam*HI site of *AtBBD1* were amplified from first-strand cDNA of the pooled RNAs isolated from Arabidopsis leaves treated with MeJA and ABA and then cloned into the pGEM-T Easy vector. All primers used in this study are described in Supplemental Table S1.

Construction of the *OmBBD* and *AtBBD1* Overexpression Lines and Identification of the T-DNA Insertion Line

We cloned the full-length ORFs of *OmBBD* and *AtBBD1* containing the *Bam*HI site and stop codon into the modified pCAMBIA2300 and pCAMBIA1300 vectors, respectively. All constructs were introduced into *Agrobacterium tumefaciens* GV3101 (Dityatkin et al., 1972) and then transformed into the Arabidopsis Col-0 ecotype or *atbbd1* by the floral dip method (Clough and Bent, 1998). The transgenic lines were selected on solid MS medium (Duchefa Biochemie) containing 50 mg L^{-1} kanamycin or 30 mg L^{-1} hygromycin, transferred to soil, and grown to maturity. The T-DNA insertion mutant *atbbd1* (SALK_067960) was obtained from the Arabidopsis Biological Resource Center. The homozygous T-DNA insertion mutant lines were identified by genomic PCR using primers recommended by the Arabidopsis Biological Resource Center (Supplemental Table S1).

Pathogen Inoculation

A conidia suspension of the rice blast *Magnaporthe oryzae* (5×10^4 conidia mL^{-1} in 0.1% Tween 20; a mixture of four races of *M. oryzae*, namely KJ101, KJ102, KJ197, and KI409), which causes severe disease symptom in *O. sativa* but not in *O. minuta*, was sprayed onto the leaves of 4-week-old *O. minuta* seedlings, as reported previously (Shim et al., 2004). At least 10 larvae of the BPH (*Nilaparvata lugens*) Korean biotype1 were placed on each plant (Cho et al., 2005). For the wound treatment, the entire leaf area was punctured, except for the main vein, using needles.

Botrytis cinerea was cultured on potato dextrose broth agar medium (Becton, Dickinson and Company) at 25°C, and conidia were collected from 15-d-old cultures (24 g L^{-1}). Leaves of 4-week-old plants were either inoculated with 10 μL of a *B. cinerea* spore suspension (8×10^4 spores mL^{-1} in potato dextrose broth) or sprayed with a spore suspension (5×10^5 spores mL^{-1} in 0.001% Triton X-100). After 5 d, the sizes of the lesions on the leaves were measured, and susceptibility to *B. cinerea* was evaluated by macroscopic observation of the diameter of the symptoms.

Chemical Treatments

Concentrated liquid stock solutions of the various plant hormones (10×) were added to MS liquid media to final concentrations of 100 μM MeJA, 5 mM ETP, 200 μM SA, and 100 μM (±)-ABA (Sigma). Germinated seedlings of *O. minuta* and *O. sativa* at the four-leaf stage were then cultured in the different MS liquid media for 2, 4, 6, or 12 h.

For the experiments with Arabidopsis seedlings, 100 μM MeJA, 5 mM ETP, 100 μM SA, or 100 μM (±)-ABA, all dissolved in 0.001% Triton X-100, was sprayed in abundance onto the leaves of 2-week-old seedlings. A mock control group consisted of seedlings sprayed with only the 0.001% Triton X-100 solution. After treatment, plants were kept at 100% relative humidity and then harvested at the indicated times.

RNA Isolation and RT-PCR

Total RNA was extracted using TRIzol reagent (GibcoBRL). RT was performed using the SuperScriptIII RT kit (Invitrogen). The RT reaction conditions consisted of 5 min at 25°C (annealing), 80 min at 50°C (extension), and 10 min at 65°C (enzyme inactivation). The PCR conditions were 95°C for 5 min, followed by 21 to 30 cycles of 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s. The RT-PCR products of *eIF4A1* (for eukaryotic initiation factor 4A-1) and *EF1α* (for elongation factor 1-α) were used as loading controls for RT-PCR amplification (van Hoof et al., 2002; Nicot et al., 2005). All primers are listed in Supplemental Table S1.

Subcellular Localization of the OmBBD Protein

The coding region of *OmBBD* without the stop codon was amplified using primers containing the *Bam*HI site (Supplemental Table S1). The resultant product was cloned to be fused in the frame to the N terminus of smGFP in the *Bam*HI site of the modified pCAMBIA2300 vector containing the cassette of *35S:smGFP* from the psmGFP vector (Davis and Vierstra, 1998). The *OmBBD-smGFP* fusion construct was introduced into onion (*Allium cepa*) epidermal cells using a helium biolistic PDS-1000 gene delivery system (Bio-Rad) with 1,100-p.s.i. rupture discs and 1.0-μm gold microcarriers, as described previ-

ously (Shieh et al., 1993). After bombardment, samples were incubated on solid MS medium (Duchefa) at 25°C in the dark for 24 h. The onion epidermal cells were washed once by phosphate-buffered saline buffer and then incubated for 3 min in DAPI staining solution (1 μg mL^{-1} DAPI in phosphate-buffered saline buffer) for staining of the nuclei. The DAPI fluorescence images were detected by UV illumination of the specimen and a band-pass filter, and green fluorescence signals were obtained by blue light illumination and a band-pass filter. The subcellular distribution of the OmBBD-smGFP fusion proteins was observed with an Olympus BX51 fluorescence microscope (Olympus) with XF116-2, XF06, and XF111-2 filter sets (Omega), and the images were processed using Olympus DP Manager and DP Controller software.

RNase and DNase Activity Assays

The coding region of *OmBBD* was amplified and cloned into the Gateway vector pDEST15 (Invitrogen), which is a GST tag plasmid, using the primers containing the recombination sites (Supplemental Table S1). The resulting in-frame fusion plasmid was transformed into *Escherichia coli* BL21 (DE3)-pLys, and expression in *E. coli* was then induced by the addition of 0.5 mM isopropyl-β-D-thiogalactoside at 30°C for 3 h. The recombinant OmBBD-GST proteins were purified using Glutathione Sepharose 4B (Amersham Pharmacia Biotech) and 10 mM reduced glutathione (Sigma) dissolved in 50 mM Tris-HCl (pH 8.0).

RNase and DNase activities were detected on a polyacrylamide gel containing 2.4 mg mL^{-1} torula yeast RNA (Sigma) or 0.4 mg mL^{-1} salmon sperm DNA (Sigma), respectively, as described by Yen and Green (1991). After electrophoresis, the gel was washed with 25% isopropanol in 0.01 M Tris-HCl. For the detection of RNase activity, the gel was incubated in 0.1 M Tris-HCl buffer for 1 h at 56°C. For the detection of DNase activity, the gel was incubated in 0.1 M Tris-HCl buffer containing 50 μM $MnCl_2$, 50 μM $CaCl_2$, 50 μM $ZnCl_2$, and 50 μM $MgCl_2$ at 45°C for 3 h. The gels were then stained with 0.2% toluidine blue O in 0.01 M Tris-HCl (Sigma) for 20 min and destained in 0.01 M Tris-HCl. For the nucleic acid degradation assay, 15 μg of total RNA, circular plasmid, digested linear plasmid, or genomic DNA prepared from leaves of *O. minuta* was incubated with the recombinant GST-OmBBD proteins for 2 h at 56°C. Degraded nucleic acids were electrophoresed on a 1.0% agarose gel for RNA or a 0.8% gel for DNA and then stained with ethidium bromide (Bantignies et al., 2000).

Histochemical Assay to Detect Callose Deposition

For visualization of the fungal hyphae and callose deposition following fungal infection, the inoculated leaves were collected 5 d after inoculation, boiled in lactophenol-aniline blue, washed with lactophenol and 70% ethanol, and then kept in saturated chloral hydrate for excess decolorization, as reported previously (Koch and Slusarenko, 1990). The stained fungal hyphae were detected by white light, and callose was detected by UV light with the Olympus BX51 fluorescence microscope (Olympus) with XF116-2, XF06, and XF111-2 filter sets (Omega). The images were processed using Olympus DP Manager and DP Controller software. The pathogen-induced callose deposition was quantified as described by Ton and Mauch-Mani (2004) using an image-analysis program (Adobe Photoshop CS3 Extended).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers ABI79452 (OmBBD) and NP_849890 (AtBBD1).

Supplemental Data

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

Supplemental Figure S1. Expression patterns of *OmBBD* homologs in response to plant stress-related hormones.

Supplemental Figure S2. Phylogenetic analyses of OmBBD and other homologous proteins containing the DUF151 domain from bacteria and plants.

Supplemental Figure S3. Differential expression patterns of signaling marker genes in Arabidopsis wild-type (Col-0), OM-OE1, *atbbd1* plants.

Supplemental Figure S4. Comparison of disease development and callose accumulation in wild-type (Col-0), OM-OEs, and *atbbd1* plants upon inoculation of *Pst*DC3000.

Supplemental Figure S5. Interaction between OmBBD and a partial protein containing the zf_C3HC4 domain in yeast.

Supplemental Table S1. List of primer pairs used in this study.

Received September 17, 2009; accepted December 8, 2009; published December 16, 2009.

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