The *Arabidopsis* Botrytis Susceptible1 Interactor Defines a Subclass of RING E3 Ligases that Regulate Pathogen and Stress Responses

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Abstract

We studied the function of Arabidopsis *Botrytis Susceptible 1 Interactor (BOI)* in plant responses to pathogen infection and abiotic stress. BOI physically interacts with and ubiquitinates Arabidopsis BOS1, an R2R3MYB transcription factor previously implicated in stress and pathogen responses. In transgenic plants expressing the *BOS1-GUS* transgene, GUS activity could be detected only after inhibition of the proteosome, suggesting that BOS1 is a target of ubiquitin-mediated degradation by the proteosome. Plants with reduced BOI transcript levels generated through RNA interference (*BOI* RNAi) were more susceptible to the necrotrophic fungus *Botrytis cinerea* and less tolerant to salt stress. In addition, *BOI* RNAi plants exhibited increased cell death induced by the phytotoxin α-picolinic acid, and by a virulent strain of the bacterial pathogen, *Pseudomonas syringae*, coincident with peak disease symptoms. However, the hypersensitive cell death associated with different race-specific resistance genes was unaffected by changes in the level of *BOI* transcript. *BOI* expression was enhanced by *B. cinerea* and salt stress but repressed by the plant hormone gibberellin (GA), indicating a complex regulation of *BOI* gene expression. Interestingly, *BOI* RNAi plants exhibit reduced growth responsiveness to GA. We also present data revealing the function of three Arabidopsis *BOI RELATED GENES (BRGs)* which contribute to *B. cinerea* resistance and suppression of disease-associated cell death. In sum, BOI and BRGs represent a subclass of RING E3 ligases that contribute to plant disease resistance and abiotic stress tolerance through the suppression of pathogen as well as stress-induced cell death.
Introduction

Plants recruit diverse cellular regulatory mechanisms to respond to various internal and external signals. Ubiquitin-mediated modification of proteins is a widespread regulatory mechanism that is important for normal cellular functions and plant responses to environmental signals. In Arabidopsis, nearly 6% of the proteome is devoted to ubiquitin-mediated regulatory changes (Vierstra, 2009), suggesting the importance of this pathway for normal cellular processes. Three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3), act in consecutive steps to transfer and covalently ligate the 76-aa protein ubiquitin to target proteins. E3 ligases provide the specificity by recruiting target proteins for ubiquitination (Smalle and Vierstra, 2004). The Arabidopsis genome contains >1300 E3 ligases, of which at least 469 are predicted RING-domain-containing E3 ubiquitin ligases (Stone et al., 2005). The attachment of a polyubiquitin chain of four or more ubiquitins linked via ubiquitin lysyl residue 48 are typically targeted for degradation by the 26S proteosome (Thrower et al., 2000). Modification with a lysl-63-linked polyubiquitin chain has been implicated in protein activation and stress response (Shi and Kehrl, 2003), whereas monoubiquitination of proteins regulates transcription, receptor internalization, and endosomal sorting independent of proteolysis (Liu et al., 2005). RING E3 ligases studied to date are implicated in hormone signaling, plant development, responses to abiotic and biotic stresses, and the regulation of cell death (Smalle and Vierstra, 2004; Kawasaki et al., 2005; Dong et al., 2006). However, the physiological functions of most E3 ligases are yet to be determined.

Ubiquitination-mediated protein removal or other regulatory changes have emerged as important components of host defense and counter defense by pathogens (Zeng et al., 2006). Treatment of plants with the proteosome inhibitor MG132 caused increased susceptibility to the necrotrophic fungus Plectosphaerella cucumerina (Llorente et al., 2008). The Arabidopsis RIN2 and RIN3 RING E3 ligases affect the extent of hypersensitive response (HR) mediated by RPM1 and RPS2 (Kawasaki et al., 2005). The tobacco ACRE276 and its Arabidopsis homolog PUB17, encoding U-BOX E3 ubiquitin ligases are positive regulators of HR (Yang et al., 2006). The loss of function mutation in the rice SPOTTED LEAF11 (SPL11) gene encoding an E3 ligase causes lesion mimic phenotypes and confers resistance to the hemibiotrophic pathogens Magnaporthe grisea and Xanthomonas oryzae pv oryzae, indicating that SPL11 is a suppressor of cell death (Zeng et al., 2004). A membrane-bound E3 ligase that is associated with lipid rafts of the plasma
membrane was recently described (Lin et al., 2008). The knock-down of RING1 leads to reduced sensitivity to the fungal toxin fumonisin B1, whereas overexpression of RING1 confers hypersensitivity. The RING E3 ligase HISTONE MONOUBIQUTINATION 1 contributes to resistance to necrotrophic fungi independent of proteolysis (Dhawan et al., 2009). Thus, some RING ligases play a role in pathogen defense and the suppression of cell death, although the mechanisms and their targets are not known.

Cell death is a ubiquitous phenomenon during plant pathogen interactions, occurring in both susceptible and resistant responses. Cell death also occurs in response to abiotic stresses, senescence and other physiological and developmental processes. The most common form of cell death is the HR, a plant resistance reaction against strains of biotrophic pathogens carrying effector molecules. Plant resistance proteins and pathogen effectors are key determinants of resistance-related HR, although many modulating components are known, including plant hormones and reactive oxygen species. Necrotrophic pathogens cause phytotoxin-mediated host cell death (Wolpert et al., 2002). The distinction between cell death caused by necrotrophic pathogens, HR and other forms of PCD at the molecular and symptom level is unclear.

In Arabidopsis, many genes regulate cell death associated with disease resistance and susceptibility. A common genetic control for disease susceptibility and resistance-associated cell death does exist. The plant MAPKKKα is a positive regulator of cell death associated with both plant resistance and susceptibility (del Pozo et al., 2004). The Arabidopsis LSD1 is a zinc finger protein required to limit cell death initiated by various signals (Jabs et al., 1996). Many more mutations define the genetic control of different forms of cell death (Greenberg and Yao, 2004). The contribution of cell death to the plant immune response is dependent on the nutrient acquisition strategy of the invading pathogen. As necrotrophic fungi are adapted to extract nutrients from dead cells, HR cell death enhances pathogen growth and colonization (Govrin and Levine, 2000). Plant mutants with enhanced cell death have increased resistance to biotrophic pathogens but susceptibility to necrotrophic fungi (Kachroo et al., 2001; Veronese et al., 2004).

Previously, we described the Arabidopsis BOS1 (Botrytis Susceptible 1) gene encoding an R2R3MYB transcription factor that is required for resistance to pathogens and tolerance to certain abiotic stress factors (Mengiste et al., 2003). Here we studied the role of Botrytis susceptible1 Interactor (BOI) and three BOI RELATED GENES (BRGs), encoding a small subclass of RING E3 ligases. BOI physically interacts with BOS1, has E3 ligase activity and
ubiquitinates BOS1 in vitro. BOI RNAi plants are more susceptible to B. cinerea and less tolerant to salt stress than the wild type plants, consistent with the phenotypes of the bos1 mutant allele (Mengiste et al., 2003). BOI is required to restrict the extent of cell death induced by the fungal toxin α-picolinic acid, a known inducer of cell death in animal and plant cells, as well as disease-associated cell death caused by a virulent strain of P. syringae. Ectopic expression of BOI reduced toxin-induced cell death but failed to restrict HR cell death mediated by the disease resistance genes RPM1 and RPS2, thus limiting the role of BOI to certain types of cell death. The BOI RNAi plants exhibit reduced growth responses to gibberellin (GA), suggesting a link between reduced growth responsiveness to GA and disease and stress tolerance. Together, our data suggest that Arabidopsis BOI and BOI-related RING E3 ligases contribute to plant stress and disease tolerance through the suppression of pathogen and abiotic stress-induced necrosis.

Results

Identification of BOI RING E3 ligase as the BOS1 interacting protein

The Arabidopsis R2R3MYB transcription factor BOS1 (Botrytis Susceptible 1) is required for resistance to B. cinerea and tolerance to abiotic stress factors (Mengiste et al., 2003). To gain insights into the functions of BOS1, we screened for BOS1 interacting proteins from a yeast two hybrid (Y2H) cDNA library. Full length BOS1 autoactivated in the absence of an interacting partner in yeast cells. Deletion analysis of the BOS1 cDNA mapped the autoactivation activity to the C-terminal 50 amino acids region of BOS1. The BOS1 cDNA C-terminal deletion construct lacking autoactivation activity was used as bait for screening an Arabidopsis cDNA library constructed from B. cinerea-induced tissue. A cDNA clone corresponding to the Arabidopsis At4g19700 gene encoding a RING E3 ligase was identified as a strong Botrytis Susceptible1 Interactor (BOI). BOI encodes a protein of 304 amino acids that contains the C-terminal HCa type RING E3 ligase domain (Figure 1a). We then swapped the vectors and co-expressed full-length BOS1 cDNA in the prey vector (pAD-BOS1) with BOI in the bait vector (pBD-BOI), which resulted in growth on selective medium and β-gal activity, confirming interaction in yeast (Figure 1b). In combination with the pAD empty vector, the bait plasmid (pBD-BOI) did not activate the transcription of the β-gal reporter gene and growth on selective medium, confirming lack of autoactivation by BOI.
The BOI protein contains three distinct regions, an amino terminal unique region with limited sequence conservation, a conserved central domain we designated WRD domain, and a C-terminal RING domain (Figure 1a, c, d; Supplemental Figure 1). Four deletion constructs of BOI were tested for interaction with full-length BOS1 to determine regions required for interaction (Figure 1a). The WRD domain covering the amino acid residues from 178-214 is conserved in BOI, three Arabidopsis BOI RELATED GENES (BRGs) and a poplar (Populus trichocarpa, XP_002322357) gene encoding RING E3 ligase (Figure 1c). The functions of these E3 ligases and the WRD domain are not known. BOI cDNA with the WRD domain interacted with BOS1, but the deletion lacking this region failed to interact, suggesting that this domain represents the interaction module of BOI (Figure 1b). Consistent with this, the WRD domain is predicted to form the coiled-coil structure (Supplemental Figure 2) normally required for protein-protein interactions in other proteins. Most RING domains in E3 ligases are associated with various substrate-binding regions including coiled-coil (Stone et al., 2005). The RING domain is dispensable and not sufficient for interaction with BOS1 in Y2H.

The RING domain of BOI is closely related to the RING domain of predicted proteins from Arabidopsis, poplar and rice (Oryza sativa) (Figure 1d). In addition, BOI shares high sequence conservation to the RING domain of mammalian and drosophila inhibitor of apoptosis (IAP) proteins. IAPs are regulators of apoptosis and other cellular processes in different organisms, but their function in plants is not known (Deveraux and Reed, 1999; Vaux and Silke, 2005). Based on the nature of the metal ligand residues present and/or the number of amino acids between them (Stone and Walker, 1995), the RING domain of BOI is of the RING-HCa type and has three amino acids between the metal ligand positions 4 and 5, a spacing that is also conserved in the animal IAP proteins as shown in Figure 1d (Stone et al., 2005). The full length sequence alignment between BOI and BRGs are shown in supplemental Figure 1a and show overall identity ranging from 38 to 64% at the amino acid level. Next, the poplar sequence (XP_002322357) is the only other putative plant RING E3 ligase with high overall sequence identity of 47 % to BOI. Phylogenetic analysis further confirmed that BOI is related to diverse predicted proteins from Arabidopsis and other plants (Supplemental Figure 3). The function of these BOI-related proteins has not been determined in any other plants.

BOS1 and BOI co-localize and interact in the nucleus of the plant cell
BOS1 and BOI are both localized to the nucleus when expressed individually in *Nicotiana benthamiana* cells as GFP fusion proteins (Figure 2a). To confirm *in vivo* interaction, a bimolecular fluorescence complementation (BiFC) assay was performed. BOS1 was translationally fused with the N-terminal 173 amino acid portion of the yellow fluorescent protein (YFP) (pBOS1-nYFP), and BOI was fused with the C-terminal 155 amino acid portion of YFP (pBOI-cYFP). pBOS1-nYFP and pBOI-cYFP were introduced into *Agrobacterium* and co-infiltrated into *N. benthamiana* leaves. Microscopic examination revealed YFP fluorescence only when the two constructs were co-expressed (Figure 2b). Leaves from plants infiltrated with either of the constructs alone or in combination with the empty vector showed no fluorescence. When cells were stained with the fluorescent nuclear stain 4',6-diamidino-2-phenylindole (DAPI), YFP fluorescence was observed in the nucleus of cells co-transformed with both constructs (column with DAPI), suggesting an interaction in the nucleus. The interaction of BOS1 and BOI in the nucleus is consistent with the function of BOS1 as a transcription factor. BRG1, an E3 ligase that is closely related to BOI failed to interact with BOS1 in BiFC and Y2H assays showing the unique interaction between BOI and BOS1 proteins (supplemental Figure 4).

**BOI is required for resistance to B. cinerea**

No loss of function T-DNA insertion alleles of the *BOI* gene were available from T-DNA insertion collections to conduct functional studies (Alonso et al., 2003; Rosso et al., 2003). We generated *BOI* RNAi lines using the 5' *BOI* gene-specific region lacking any conserved domain. Two *BOI* RNAi lines with clearly reduced *BOI* gene expression were selected (Figure 3a). We also generated 35S:*BOI* transgenic Arabidopsis lines that over express the *BOI* transcript relative to the wild-type plants (Figure 3b). The expression of *BOS1* was reduced in BOI RNAi lines after *B. cinerea* infection but was comparable to the wild type level in the *BOI* over expression lines (Figure 3c). The *BOI* gene is expressed at low but detectable levels in healthy leaf tissues but is significantly induced by *B. cinerea* (Figure 3a,d). The *BOS1* gene did not regulate *BOI* gene expression as the *bos1* mutant allele showed no altered *BOI* transcript level (Figure 3d). The BOI RNAi plants show no altered growth or any other morphological changes. In addition, BOI RNAi lines have no effect on the *BRG1* gene expression (supplemental Figure 5).

The *BOI* RNAi lines were significantly more susceptible to *B. cinerea* infection with a larger disease lesion and increased fungal growth relative to the wild-type plants (Figure 4a, b).
However, the 35S:BOI plants did not exhibit altered responses to B. cinerea. BOI RNAi and wild type plants expressing the translation fusion of BOS1 and β-glucuronidase (GUS) gene under the control of the CaMV35S promoter (35S:BOS1-GUS), were analyzed to determine the functional relationship between BOS1 and BOI. Interestingly, BOI RNAi plants expressing 35S:BOS1-GUS were more resistant to B. cinerea than the wild type plants, suggesting that BOS1 is a likely direct downstream target of BOI (Figure 4c-d). Similarly, the transgenic 35S:BOS1-GUS plants, were more tolerant to oxidative stress caused by the herbicide paraquat (supplemental Figure 6). These data are consistent with the increased susceptibility or sensitivity of the bos1 T-DNA insertion allele to pathogens and abiotic stress agents (Mengiste et al., 2003).

**BOI reduces disease and toxin related cell death but not HR**

To determine the role of BOI in resistance to bacterial pathogens, the BOI RNAi and 35S:BOI lines were tested for responses to the virulent strains of P. syringae pv tomato (Pst). Bacterial growth and disease symptoms were not changed in BOI RNAi and 35S:BOI lines inoculated with a virulent strain of Pst (Supplemental Figure 7). We then measured electrolyte leakage as a sensitive assay for detecting changes in cell death (Dellagi et al., 1998; Kawasaki et al., 2005). Higher cell death normally correlates with higher electrolyte leakage. Ion leakage measured after inoculation of BOI RNAi and 35S:BOI plants with avirulent Pst DC3000 (AvrRpm1, AvrRpt2) was not different from that of the wild-type plants, suggesting that BOI has no role in RPM1- and RPS2-mediated HR cell death in Arabidopsis (Figure 5a, b). In contrast, plants inoculated with virulent PstDC3000 showed significantly increased electrolyte leakage in the BOI RNAi plants indicating increased cell death associated with disease susceptibility (Figure 5c). The increase in electrolyte leakage was coincident with the expression of disease symptoms caused by virulent P. syringae in Arabidopsis plants, thus marking cell death associated with susceptibility.

The 35S:BOI and BOI RNAi lines were assayed for their effects on cell death caused by α-picolinic acid (PA), a toxin produced by some fungi that induces apoptosis in animal as well as plant cells (Zhang et al., 2004; Kim et al., 2006). The BOI RNAi plants exhibited increased ion leakage following PA infiltritation, whereas the 35S:BOI plants showed reduced ion leakage, suggesting that BOI plays a key role in limiting cell death caused by PA in Arabidopsis (Figure
5d). Thus, BOI suppresses cell death caused by PA and *PstDC3000* but has no role in the HR cell death mediated by race specific interactions.

The Arabidopsis *LSD1* is a zinc-finger protein that suppresses the spread of cell death to uninfected cells surrounding HR sites (Dietrich et al., 1997). The *lsl1* mutant is impaired in the control of cell death initiated by various signals. In order to determine whether BOI has an effect on run-away cell death, we constructed *lsl1;35S:BOI* through genetic crosses between Arabidopsis *lsl1* mutant and 35S:BOI lines. The *lsl1/lsl1;35S:BOI* plants were comparable to *lsl1* in their survival under growth conditions that triggered cell death in *lsl1* plants (data not shown), suggesting that BOI failed to substitute for *LSD1* in the control of cell death. These data suggest that *LSD1* and BOI function in independent pathways.

**BOI is a functional E3 ligase and ubiquitinates BOS1 in vitro**

To determine whether the BOI protein has an E3 ligase activity, BOI was expressed in *Escherichia coli* as a fusion with a histidine tag (His). The recombinant His-BOI protein exhibited an E3 ligase activity *in vitro* and formed polyubiquitinated proteins in the presence of E1, E2, and purified His-BOI. The ubiquitination activity is depended on the presence of His-BOI as well as E1, E2 and all the components required for ubiquitination, suggesting its involvement in protein ubiquitination and that BOI is a functional E3 ligase (Fig 6a). To test the requirement of the RING domain of BOI for its ubiquitin ligase activity, the RING domain was deleted and the remaining portion was expressed as a fusion with the maltose binding protein (MBP). Although the full length MBP-BOI protein showed ubiquitination activity, the BOI protein lacking the C-terminal RING domain abolished the ubiquitination activity despite being dispensable for interaction with BOS1 (Figure 6b, Figure 1). Thus, the RING ligase domain is required for ligase activity but not for its interaction with BOS1. This is consistent with the general notion that substrate binding and the E3 ligase activity are encoded by separate regions of RING E3 ligases.

The interaction between BOS1 and the E3 ligase BOI suggests that BOS1 is ubiquitinated. Interestingly, the BOS1 protein contains the ubiquitin-interacting motif (UIM) that is defined by a conserved spacing of a hydrophobic residue, an alanine and a serine residue (Miller et al., 2004). A consensus UIM motif is present in BOS1 at I151, A154, and S158 consistent with what is described (Miller et al., 2004). Recombinant MBP-BOI, ubiquitinated
GST-BOS1 in the presence of all the components required for ubiquitination, consistent with the data on physical interaction (Figure 6c).

**BOS1 is regulated by the plant proteosome**

We reasoned that BOI functions through ubiquitin mediated-modification of the BOS1 protein leading to proteolysis or other post-transcriptional regulations. Transgenic 35S:BOS1-GUS generated in the wild type and BOI RNAi plants were analyzed for GUS activity to determine the regulatory relationship between BOI and BOS1. The RT-PCR data in Figure 6d show a typical example revealing that BOS1-GUS was expressed at constitutive levels in both wild type and BOI RNAi transgenic plants. Histochemical assays failed to detect any GUS activity in >200 BOS1-GUS transgenic plants in the wild type and 96 in the BOI RNAi background. However, after treatment of 35S:BOS1-GUS plants with the proteosome inhibitor MG132, the BOS1-GUS activity was detected at the site of MG132 infiltration in many transgenic plants (Figure 6e). These data indicate that BOS1:GUS is rapidly degraded by the 26S proteosome consistent with ubiquitination of BOS1 by BOI shown in Figure 6c. The levels of BOS1-GUS activity in the BOI RNAi did not differ significantly from activity in wild type plants based on the histochemical staining of MG132 infiltrated tissue. This may be due to the presence of residual BOI protein in the RNAi plants which may be sufficient for ubiquitination of BOS1 or to the functional redundancy with other closely related E3 ligases. Due to the lack of the T-DNA insertion alleles of BOI, it is not possible to validate this first possibility.

**Defense gene regulation and BOI gene expression patterns**

The expression of PR-1 and PDF1.2, two defense marker genes associated with resistance responses to biotrophic and necrotrophic pathogens, respectively, were tested in BOI RNAi and 35S:BOI plants using quantitative RT-PCR (qPCR). In BOI RNAi plants, PR-1 gene expression was higher than the wild type plants (Figure 7a). This may be due to the increased susceptibility and pathogen growth in the BOI RNAi plants or altered defense signaling. Expression of PDF1.2 gene was also higher in BOI RNAi but showed no changes in 35S:BOI plants (Figure 7b). BOI is normally induced by B. cinerea in sid2 and nahG plants, genotypes impaired in SA accumulation, suggesting that BOI gene expression is independent of endogenous SA levels (Figure 7c). The BOI gene is induced by 1-aminocyclopropane-1-
carboxylic acid (ACC), the natural precursor of ethylene (ET) and SA but significantly repressed by Methyl jasmonate (MeJA) (Figure 7d). Methyl viologen (paraquat), a herbicide that produces reactive oxygen species, slightly induced the BOI gene expression. GA suppressed the expression of BOI by about 8.5-fold relative to the mock controls (Figure 7e). Consistent with the repression of BOI by GA, BOI expression is higher in the GA-deficient mutant ga1-3 (Sun and Kamiya J., 1994) (Figure 7e). Exogenous application of GA in the ga1-3 mutant repressed BOI gene expression. In addition, publically available data (https://www.genevestigator.ethz.ch) reveal that BOI gene expression increases in response to PstDC3000, drought, uniconazole and palcobutrazol, suggesting an important function for BOI in mediating responses to biotic and abiotic stresses and plant hormones.

Plant lines expressing the GUS gene driven by the BOI or BOS1 promoters were generated to study the pattern of BOI and BOS1 gene expression relative to infection sites. Expression of GUS in BOI promoter:GUS (BOIPr:GUS) lines localized to PA, PstDC3000 or B. cinerea induced necrotic sites (Figure 8a). PA induced the most intense GUS staining surrounding the site of infiltration. Evans blue staining reveals that PA, PstDC3000 and B. cinerea induce cell death (Figure 8b). The necrotic sites revealed by Evans blue staining and the sites of BOI expression appear to coincide. This is particularly evident in PA treated tissue where the site of PA infiltration heavily stains with Evans blue marking cell death (Figure 8b) but remains free of GUS due to the cell death (Figure 8a,c). BOS1Pr:GUS was highly expressed surrounding the site of PA treatment similar to BOI1 expression (Figure 8c). In addition, the BOI gene is expressed in siliques, roots, and flowering tissues (Figure 8d,e). Intriguingly, comparison of expression patterns in reproductive tissues clearly show that BOI is highly expressed in the stigma tips, while BOS1 is mainly expressed in anthers (Figure 8e). Thus, both are expressed in distinct parts of the reproductive tissues, suggesting complementary functions.

**Arabidopsis BOI RELATED GENES encoding RING E3 ligases have overlapping function in suppression of cell death**

BOI is most closely related to Arabidopsis At5G45100, hereafter designated BOI RELATED GENE 1 (BRG1). BRG1 interacted weakly with BOS1 in a Y2H but failed to interact in a BiFC assay in N. benthamiana cells (Supplemental Figure 4a,b). The other two BOI-related E3 ligases (BRG2, At1g79100; BRG3, At3g12920) failed to interact with BOS1 in Y2H assays.
The recombinant BRG1 protein had E3 ubiquitin ligase activity but did not ubiquitinate the BOS1 protein, consistent with the lack of \textit{in vivo} interaction (Figure 9a,b). BOI and BRG1 show high sequence conservation yet only BOI interacted with BOS1 \textit{in vivo}. BOI and BRG1 sequences carry multiple deletions relative to BRG2 and BRG3 (Supplemental Figure 1a). At2G12290 was only recently annotated as a putative RING E3 ligase of this family and thus was not studied in detail here. At2G12290 is unique in terms of structural features with predicted protein size that is only half that of the other BRGs and devoid of many of the features of BOI and the BRGs. However, it shares the WRD domain and carries a variant RING domain with many of the invariant cysteine residues absent (Supplemental Figure 1a,b). It is likely that At2g12290 is a pseudogene or is mistakenly annotated.

GA significantly repressed \textit{BRG1}, similar to \textit{BOI}, but increased the expression of \textit{BRG2} and \textit{BRG3} from a very low basal level of expression (Figure 9c,d). Basal \textit{BRG1} gene expression is higher in the GA deficient mutant \textit{gal-3}. In addition, the basal and induced \textit{BRG} gene expression levels are presented in Supplemental Figure 8. \textit{BOI} was induced by \textit{B. cinerea} but \textit{BRG1} expression was unchanged during infection. However, \textit{BRG1} was induced by SA but repressed by MeJA similar to the \textit{BOI} gene. \textit{BRG2} and \textit{BRG3} are expressed at a low basal level with a slight reduction of gene expression after \textit{B. cinerea} infection. Expression of \textit{BRG3} did not change significantly upon SA, ACC, or MeJA treatment, whereas \textit{BRG2} was consistently repressed by SA, ACC and MeJA. The different plant hormones and \textit{B. cinerea} differentially regulated \textit{BRG} expression with upregulation of some but down regulation of others. The main features of the BOI and BRG proteins and their expression in response to various signals are summarized in Table 1. Collectively, the data suggest that \textit{BOI} and the \textit{BRGs} have overlapping and contrasting expression patterns in response to pathogen infection and plant hormones.

\textbf{BOI related genes are required for full resistance to \textit{B. cinerea}}

In order to study the function of \textit{BRG} genes, two T-DNA insertion alleles for each of the \textit{BRGs} were isolated from SALK and SAIL T-DNA lines obtained from Arabidopsis Biological Resource Center (Ohio State University, Columbus). All of the mutants have a T-DNA insertion in an exon causing loss of function mutations. The \textit{brg} mutant alleles exhibit wild-type morphology, suggesting that the \textit{BRGs} are dispensable for normal plant development under the growth conditions tested. After challenge with \textit{B. cinerea}, compared to the wild type plants, a
statistically significant increase in disease lesion size was observed for all the *brg* mutants (Figure 10a). Fungal growth also increased in all the *brg* mutants except for the *brg1* where increased susceptibility was not accompanied by increased fungal growth (Figure 10b). Thus, *BRGs* have overlapping roles in disease resistance but may function through different mechanisms. BOI functions through interaction with BOS1 but BRGs may function independently of BOS1.

Similarly, the *brg1, brg2, brg3* alleles were tested for disease related cell death caused by inoculation with *PstDC3000* or treatment with PA. All of the *brg* mutant alleles showed a significant increase in ion leakage at 2 dpi with *PstDC3000* indicative of an enhanced cell death (Figure 10c). PA treatment also caused a statistically significant increase in cell death relative to wild-type plants (Figure 10d). Thus, members of *BOI* and the *BRG* gene family share a common role in the control of cell death.

**BOI and its closely related E3 ligase are involved in GA and abiotic stress responses**

*BOI* RNAi lines and T-DNA insertion lines of *BRGs* were assayed for altered responses to stress and defense-mediating plant hormones. Seedling growth or seed germination was not altered in media containing abscisic acid (ABA), ET, MeJA and SA for *BOI* RNAi or any of the *brg* mutants (data not shown). However, in the presence of GA, overall growth and hypocotyl elongation of the *BOI* RNAi and the *brg1* mutant were reduced, relative to the wild-type plants (Figure 11a,b). The mean hypocotyl lengths in *BOI* RNAi and *brg1* mutants were significantly reduced relative to the wild type, *brg2* and *brg3* seedlings. The *brg2* and *brg3* mutants exhibited wild type GA responses. GA did not affect germination of seeds from *BOI* RNAi and the *brg* mutant plants.

Due to the increased sensitivity of the BOS1 mutant alleles to various abiotic stress factors, we evaluated the *BOI* RNAi and *brg* mutants for their role in NaCl-induced stress tolerance. *BOI* RNAi and *brg1* mutant plants have reduced growth in media containing NaCl relative to wild-type plants (Figure 11c). *BOI* RNAi seedlings turned brown and necrotic on media containing NaCl. The 35S:*BOI* was less sensitive to salt compared to the wild type plants. Interestingly, all the mutant lines varied in their sensitivity to salt and differed from sensitivity of the wild type plants. The *BOI* RNAi lines and *brg1* mutants exhibited the most severe salt sensitivity.
symptoms followed by the brg2 and brg3 mutants. Thus, BOI and BRG1 contribute to survival under abiotic stress conditions.

Discussion

We describe the function of BOS1 Interactor (BOI) in the suppression of cell death, fungal resistance, and responses to salt stress. BOI physically interacts with BOS1 in yeast and plant cells. Consistent with this, BOI is a functional E3 ligase that ubiquitinates BOS1 in vitro and possibly in vivo, revealing the regulatory relationship between the two proteins. In transgenic plants expressing the BOS1-GUS fusion construct, the GUS activity could be detected only after treatment with the proteosome inhibitor MG132, suggesting that BOS1 has a rapid turnover rate and is a target of ubiquitin-mediated degradation. In addition, the resistance of the BOI RNAi lines that express BOS1-GUS to B. cinerea and tolerance to stress provide a strong genetic evidence for the interactions between BOI and BOS1. Further, we provide evidence for the function of BRGs through the phenotypic analysis of T-DNA insertion mutants and gene expression studies. The BOI and BRG genes all contribute to disease resistance and stress tolerance through mostly overlapping and yet some distinct mechanisms involving complex regulation of gene expression and interaction with BOS1. The overall structure and the order of domains are conserved between BOI and BRGs. The primary sequence in the WRD and RING domains also are highly conserved. However, outside these regions, the level of amino acid conservation is very low. Structurally, many RING E3 ligases have a common C-terminal RING domain but also carry other domains that are thought to contribute to substrate binding. Thus, BOI represents a subclass of RING E3 ligases with overlapping functions and structural features, including a substrate binding domain predicted to form a coiled-coil structure.

Arabidopsis RNAi lines with reduced BOI levels show increased susceptibility to the necrotrophic fungus B. cinerea and reduced tolerance to salt stress. In addition, the BOI RNAi plants have increased cell death triggered by the fungal toxin, α-picolinic acid, or inoculation with a virulent P. syringae strain. Interestingly, overexpression of BOI was sufficient to reduce PA induced cell death and increase salt stress tolerance but failed to improve resistance to B. cinerea, most likely due to the complex virulence strategy of this fungus. Besides physical interaction, the BOS1 and BOI genes are coregulated during B. cinerea infection and by agents that promote cell death including toxins, confirming their common function in planta.
Interestingly, \( BOI \) is induced by SA and ACC but is repressed by MeJA and GA, suggesting that a complex regulation is required to maintain normal levels of \( BOI \) in wild-type plants. \( B. \) \textit{cinerea} infection is known to increase accumulation of SA, ET, MeJA and ABA in wild type plants. \( B. \) \textit{cinerea} induced \( BOI \) expression is clearly independent of endogenous SA levels. It is also possible that \( BOI \) expression during \( B. \) \textit{cinerea} infection is a result of stress rather than an effect of increased hormone levels. \( BOI \) and \( BOS1 \) are both expressed in reproductive tissues with \( BOI \) expressed more on the stigma while \( BOS1 \) is expressed in the anther. These observations suggest that BOS1 and BOI may also have complementary functions in developmental processes.

We also describe the function of three \textit{BOI RELATED GENES (BRGs)} encoding RING E3 ligases that display overlapping and distinct gene expression and biological functions with \( BOI \). BRGs also function in suppression of cell death, a central mechanism of tolerance to necrotrophic fungi. Among the \textit{BRGs}, \textit{BRG1} shares the highest sequence identity and the most overlapping function and co-expression with \( BOI \). The \( BOI \) and \( BRG1 \) genes are induced by NaCl but repressed by GA and MeJA, although the extent of induction varies significantly between the two genes. Despite their role in resistance to \( B. \) \textit{cinerea} and suppression of cell death, \textit{BRG2} and \textit{BRG3} have different levels of basal and induced expression compared to \( BOI \) and \textit{BRG1}. \textit{BRG2} and \textit{BRG3} have a very low basal level of expression but are further repressed by \( B. \) \textit{cinerea} and SA, whereas \( BOI \) is induced. \textit{BRG2} and \textit{BRG3} are divergent sharing only 47\% and 38\% overall identity, respectively, with the \( BOI \) protein. All BRGs have a common function in conferring resistance to \( B. \) \textit{cinerea} and suppressing cell death. However, the BRGs do not interact with BOS1, and \textit{brg2} and \textit{brg3} loss-of-function mutants exhibit no altered responses to GA, suggesting that \textit{BRG2} and \textit{BRG3} function in disease resistance through mechanisms different from \( BOI \). \textit{BRG2} and \textit{BRG3} are suppressed by \( B. \) \textit{cinerea}, but their mutants show increased susceptibility rather than increased resistance as would be predicted. Thus, rather than the transcript abundance and regulation, the post-transcriptional regulation may be more important in the case of these two genes.

Recombinant \( BOI \) ubiquitinated BOS1 \textit{in vitro} and the BOS1-GUS fusion protein could be detected only after treatment of plants with MG132, a proteosome inhibitor, which suggested that BOS1 is normally degraded rapidly. The levels of BOS1-GUS did not vary between \( BOI \) RNAi and wild-type plants as would be expected if BOS1 ubiquitination results in its degradation by the 26S proteosome. This lack of differences may be either due to the
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requirement for additional factors or to functional redundancy. Also, the BOI RNAi lines may retain BOI protein levels sufficient to ubiquitinate BOS1. Although BOS1 is likely degraded by the proteosome and is rapidly turned over, it is possible that during salt stress, oxidative stress and *B. cinerea* infection, BOS1 may become stabilized to confer tolerance to stress. Consistent with this, the 35S:*BOS1* and 35S:*BOS-GUS* plants are more tolerant to oxidative stress and resistant to *B. cinerea*. Stress-induced stabilization of proteins is a documented regulatory mechanism that promotes stress tolerance. The Arabidopsis RGA, a DELLA protein, is stabilized during NaCl stress and promotes plant survival under stress (Achard et al., 2008). The mammalian p53 protein is normally rapidly degraded by the proteosome. Upon DNA damage, p53 levels must increase rapidly; therefore, the ubiquitination of p53 is rapidly terminated to allow the accumulation of p53 (Brooks and Gu, 2003).

Plant responses to pathogens have been studied extensively in connection with plant hormone synthesis, accumulation and signaling. Altered hormone levels and signaling promote resistance or susceptibility depending on the nature of the pathogens. Hormonal imbalances create a range of plant pathological conditions and are exploited by pathogens (Grant and Jones, 2009). Some plant hormones affect plant defense due to their role in potentiating cell death while others regulate the activation of protective mechanisms and/or the synthesis of antimicrobial compounds. Consequently, many genes involved in regulation of cell death and hormone biosynthesis and signaling are also components of the plant immune response and stress tolerance. The roles of ET and JA signaling as positive regulators of resistance to necrotrophic fungi have been firmly established based on genetic data from Arabidopsis and tomato mutants (Thomma et al., 1999; Abuqamar et al., 2008). SA is the major positive regulator of defense against many biotrophic fungi but can suppress resistance to some necrotrophic fungi (Veronese et al., 2006; Spoel et al., 2007). JA and ET act synergistically to promote resistance to necrotrophic fungi but ABA suppresses or promotes defense depending on the specific plant-pathogen interaction (Mauch-Mani and Mauch, 2005). Recent reports suggest that gibberellins are suppressors of resistance to necrotrophic fungi. GAs activate a plant growth response pathway that results in the degradation of DELLA proteins, known suppressors of GA-mediated plant growth responses (Harberd et al., 2009). The DELLA triple/quadruple mutant lacking four of the five DELLA genes is susceptible to *B. cinerea* likely due to the increased accumulation of reactive oxygen species that promote cell death and susceptibility to *B. cinerea* (Achard et al.,
The quadruple DELLA mutant is also susceptible to *A. brassicicola* but resistant to *P. syringae*, attributed to the attenuated JA-dependent defense and enhanced cell death, respectively (Navarro et al., 2008). In the case of *BOI* RNAi, reduced GA growth responses correlated with susceptibility to *B. cinerea* independent of changes in the JA-mediated defense response pathway deduced from the normal expression of the JA-regulated *PDF1.2* gene.

The BOI RING E3 ligase was recently described as a target of DELLA proteins based on global microarray analysis and chromatin immunoprecipitation experiments (Zentella et al., 2007). However, both the-loss-of function triple DELLA mutant (*rga-24gait6ga1-3*) and the dominant DELLA mutant protein that stabilizes the DELLA protein RGA express *BOI* at elevated levels relative to the wild type plants, making it unclear whether *BOI* is a direct downstream target of DELLAs. Nevertheless, the *BOI* and *BRG1* genes are both significantly repressed by GA, and the *BOI* RNAi and the *brg1* mutant have reduced seedling growth and hypocotyl elongation in response to GA. It is not clear whether the GA-mediated suppression of *BOI* and *BRG1* is through removal of DELLAs by GA or indirectly. It is possible that GA-mediated removal of DELLA protein RGA results in the recruitment of other regulatory factors that then suppress *BOI* and *BRG1* gene expression. Plants that carry a dominant mutation that stabilizes DELLA and *BOI* RNAi have attenuated responses to GA, suggesting that they function in an antagonistic fashion in plant responses to GA. However, BOI and BRG1 may still act as positive factors of GA responses downstream of DELLA consistent with the suppression of their gene expression by GA as well as the reduced GA responses in the *BOI* RNAi and the *brg1* mutant. Interestingly, our data demonstrate that *BOI* and *BRG1* are required for resistance to *B. cinerea* and tolerance to salt stress consistent with the role of DELLAs in defense and stress tolerance (Achard et al., 2006; Achard et al., 2008; Navarro et al., 2008).

*BOI* and *BOS1* define components of host defense that restrict the extent of necrosis and hence are positive regulators of *B. cinerea* resistance and stress tolerance. The susceptibility of *BOI* RNAi and the *brg* mutants is most likely due to impaired ability to restrict initial pathogen-induced necrosis leading to increased ion leakage. The phenotypes of the *bos1* allele are consistent with an impaired control of necrosis during infection and salt-induced stress (Mengiste et al., 2003). Interestingly, the *BOI* gene has a limited effect on the HR cell death mediated by the Arabidopsis disease resistance genes *RPM1* and *RPS2* and the suppressor of cell death, *LSD1*, despite the similarities of these different forms of cell death at the molecular level.
These data indicate that the genetic control of the various types of cell death may be different. Future progress on the identification and characterization of toxin receptors and their signal transduction components may shed light on the genetic control of phytotoxin and necrotrophic pathogen-mediated cell death. The BOI RNAi plants and brg mutants do not show a lesion mimic phenotype and the altered responses are most likely due to loss of an active mechanism that occurs after pathogen infection. Similarly, in the presence of NaCl, the BOI RNAi and brg2 mutants exhibit increased browning and necrosis of the cotyledons and emerging leaves. This, combined with the significant impact of loss-of-function and gain-of-function alleles of BOI and the T-DNA insertion alleles of brg mutants on toxin- and disease-induced cell death, suggest that this subclass of E3 ligases is crucial in disease and stress tolerance in Arabidopsis.

Plant disease resistance pathways responding to pathogens of different life styles function in a synergistic or antagonistic manner. The salycilate- mediated and JA/ET-mediated defense pathways interact antagonistically (Spoel et al., 2007). Many Arabidopsis mutants show contrasting defense gene expression and plant responses to biotrophic and necrotrophic pathogens (Glazebrook, 2005; Veronese et al., 2006). In the BOI RNAi plants, expression of the PRI and PDF1.2 genes are both slightly enhanced, suggesting that the altered disease response is not related to defense signal antagonism. Despite the high frequency of Arabidopsis mutations that reveal antagonism between defense pathways, many other genes regulate defense without apparent antagonistic interactions (Berrocal-Lobo et al., 2002; Coego et al., 2005; Dhawan et al., 2009) consistent with the function of BOI. Most such pathways are likely to function independently of hormone-mediated responses.

Overall, we provide strong evidence for the role of BOI and BRGs in plant resistance to B. cinerea and tolerance to salt stress. BOS1 and BOI may integrate plant responses to diverse signals in a manner comparable to DELLA proteins that are also required for stress tolerance as well as resistance to necrotrophic fungi (Achard et al., 2008; Navarro et al., 2008). The reduced GA responses of BOI RNAi and brg1 seedlings as well as the dramatic suppression of BOI and BRG1 genes by GA and their upregulation by salt suggest that these E3 ligases contribute to plant immunity and salt stress tolerance through a function that involves the GA response pathway. A complex transcriptional and post-transcriptional regulation and additional genetic factors likely govern the functions of BOI, BRG1 and BOS1 in plant defense and stress.
tolerance. The exact points of actions of BOI, BOS1 and the BRG proteins in GA-dependent and independent defense responses need to be defined further.

Materials and Methods

Plant growth conditions and diseases assays

Plant growth conditions, growth media and disease assays were as described (Dhawan et al., 2009). *B. cinerea* disease assays were performed on soil-grown plants by drop inoculation (4-5 µl) of 2.5×10^5 spores/ml. As a measure of *B. cinerea* growth in inoculated plants, the levels of the *B. cinerea* ActinA DNA were determined by qPCR using primers shown in the Supplemental Table 1. The DNA levels concentration calculated by the comparative cycle threshold method (Applied Biosystems, Foster City, CA, USA) with the Arabidopsis Actin2 as the endogenous reference for normalization as described (Bluhm and Woloshuk, 2005).

The culture and disease assay for *P. syringae* pv tomato were as described (Zheng et al., 2006). Procedures for measuring electrolyte leakage were described (Kawasaki et al., 2005; Dhawan et al., 2009). Briefly, a bacterial suspension (OD_{600}: 0.01) or α-picolinic acid (1 mg/ml) was infiltrated into leaves. Six leaf disks (0.8 cm diameter) were collected from the infiltrated area and washed with water for 50-60 min, and then placed in a tube containing 8 ml of water. Conductivity of five replicates for each treatment was measured with conductivity meter (Model AB30, Accumet® BASIC, Fisher scientific) following the procedure described (Kawasaki et al., 2005).

Generation of transgenic lines and identification of mutant alleles

To generate BOI overexpression lines, full length BOI cDNA was cloned after the CaMV35S promoter into a modified version of binary vector pCAMBIA 1200. The binary vector was then transferred into *Agrobacterium* strain GV3101 and transformed into Arabidopsis (Clough and Bent, 1998). Transgenic plants were selected on medium containing hygromycin and the lines over expressing BOI were identified by RNA-blots hybridized to the full length BOI cDNA. To generate BOI RNAi lines, the first 250-bp fragment of the BOI cDNA starting from the translation initiation codon was amplified using the primer pairs (supplemental Table 1) and cloned into the RNAi vector pGSA1252 (http://www.chromdb.org/rnai/order_vectors.html). BOI RNAi lines with reduced BOI gene expression were selected based on the levels of RNA by
hybridizing to the 3’ terminus of the BOI gene. BOI promoter GUS lines were generated by cloning 1.5 kb fragment of the BOI or BOS1 upstream sequences into the binary vector pCAMBIA1391 carrying the GUS gene and transformants were selected on MS medium containing hygromycin.

We identified the mutant alleles of BOI related genes from a segregating population of T-DNA insertion lines obtained from the ABRC using T-DNA and gene-specific primers (supplemental Table 1). The mutant alleles for At5g45100 (brg1-1, Salk_010178; brg1-2, SAIL_1214_E04), At3g12920 (brg2-1, SAIL_302_F07; brg2-2, SAIL_261_G05), At1g79110 (brg3-1, SAIL_95_F06; brg3-1 SAIL_73_H10) all carry a T-DNA insertion in an exon.

RNA-blot and RT-PCR

For RNA blot and RT-PCR experiments, total RNA was isolated as described (Lagrimini et al., 1987) or with Trizol reagent according to the manufacturer’s instructions (Invitrogen). For RNA blots, total RNA was separated on 1.2% agarose-formaldehyde gels and blotted to Hybond N+ nylon membranes by a standard protocol. Probes were labeled with 32P by random priming with a commercial kit (Sigma-Aldrich). Hybridization of probe and subsequent washings were performed as described (Church and Gilbert, 1984). For qPCR, total RNA (2 µg) was reverse transcribed with oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen). The cDNA was then subjected to qRT-PCR with gene-specific primers. The expression levels were calculated by the comparative cycle threshold method (Applied Biosystems) with Arabidopsis Actin2 (At3G18780) as the endogenous reference for normalization. Primer sequences for qRT-PCR expression of BOI and BRGs are provided in Supplemental Table1. Induced expressions were from plants treated by spraying of ACC (0.5 mM), SA (5 mM), MeJA (1mM), MV (50 µM) on soil-grown leaves or in vitro grown seedlings with 100 µM GA for 3 h or 200 mM NaCl for 6 or 12 hours.

RT-PCR for BOS1-GUS transgenic plants was performed after DNase treatment of RNA and first strand cDNA synthesis. cDNA was synthesized from both control and treated samples with equal amounts of total RNA (2 µg), AMV reverse transcriptase (Promega), and oligo(dT15) primers according to standard protocols. The PCR was performed for 28 cycles with 2.5 µL of cDNA as a template and specific primer pairs (94°C for 30 s, 52°C for 30 s, and 72°C for 1 min).
Yeast two-hybrid screen

Yeast two-hybrid assays were performed with the GAL4 system according to the manufacturer’s instructions (Stratagen). The full length BOS1 cDNA and various deletion constructs were cloned into pBD-GAL4 to generate DNA binding domain bait protein fusion. The partial BOS1 cDNA without the 50 amino acid sequence lacking the transcriptional activation of LacZ was used for screening the Y2H library. We built a cDNA library from B. cinerea-infected Arabidopsis tissue in the HybriZAP-2.1 vector according to the manufacturer’s instructions (Stratagene). At least $10^6$ yeast colonies were screened by transformation into the YRG-2 yeast strain (Stratagene) expressing BOS1 cDNA. Interacting proteins were initially selected for complementation of His auxotrophy on selective medium lacking His, Leu and Trp. The putative interactors were then tested by assaying for the lacZ reporter gene activation by performing the filter lift assay as described in the Stratagene protocol. Interactions were retested for His3+, Trp+, and Leu+ auxotrophy and LacZ reporter activity (β-galactosidase assay). The plasmids from the positive clones were then isolated, sequenced and reintroduced into the original yeast bait and control bait strains to verify interaction.

Bimolecular fluorescence complementation assays

Two vectors pCAMBIA-N-YFP and pCAMBIA-C-YFP were generated based on the pCAMBIA1200 for use in the BiFc analysis (Dhawan et al., 2009). The BOI full length cDNA was inserted into pCAMBIA-C-YFP to generate the C-terminal in-frame fusions with C-YFP (pBOI-cYFP), whereas BOS1 was introduced into pCAMBIA-N-YFP to form N-terminal in-frame fusion with N-YFP (pBOS1-nYFP). After sequence verification, the constructs were verified by sequencing and the plasmids introduced into Agrobacterium tumefaciens (strain GV3101). The Agrobacterium carrying the appropriate plasmids was expressed in N. benthamiana leaf tissue by Agroinfiltration. In vivo interaction was observed with an epifluorescence microscope (Nikon Eclipse E800).

Recombinant protein purification and ubiquitination assay

Arabidopsis BOI, BOI257-304, BOI1-257 and BOS1 cDNA were cloned into pRSET A (Invitrogen), pMAL-c2x(New England Biolabs) and pGEX 4T-1(GE Healthcare) to produce the fusion protein plasmids named as pRSET–BOI, pMAL-BOI, pMAL- BOI257-304, pMAL-BOI1-257 and
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pGEX-\textit{BOS1}. The expression and the purification of the fusion proteins were performed as described in the product manuals.

\textit{In vitro} ubiquitination assays were performed in a reaction volume 30 µl that contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 2 mM ATP, 2 mM DTT, 10 mM phosphocreatine, 2 unit of creatine kinase (Sigma-Aldich), 10 µg ubiquitin, 200 ng of yeast E1 (Boston Biochem), 400 ng E2 UbcH5b (Boston Biochem), and approximately 1 µg of HIS-BOI, MBP- BOI, MBP-BOI\textsuperscript{257-304} or MBP-BOI\textsuperscript{1-257} and/or GST-BOS1. The reactions were incubated at 30 °C for 6 h and stopped by addition of 6 xSDS-PAGE sample buffer (125mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 0.2% bromophenol blue) at 100 °C for 5 min and analyzed by SDS-PAGE electrophoresis followed by protein gel blotting using anti-Ub antibodies (Cell Signaling), anti-His (Amersham Biosciences), anti-GST (Amersham Biosciences) and anti-MBP antibodies (New England Biolabs).

\textbf{Hormone sensitivity assay}

Effects of various plant hormones on seed germination were tested by directly plating seeds on media containing plant hormones and evaluating changes in the percent of germination. In addition, seedlings germinated on plain MS medium were transferred to media containing various concentrations of SA, MeJA, ACC, NaCl, and GA to determine effect on growth. Surface-sterilized seeds were sown on 1.0% (w/v) agar medium containing MS salts, 2% (w/v) sucrose, MES (0.5 g/L) and different concentrations of MeJA, ACC, 2,4-D, or IAA, pH 5.7. The plant hormones were added to the autoclaved medium from filter-sterilized stock solutions. The effects of the plant hormones on overall seedling growth and hypocotyl elongation were evaluated. The hypocotyl length was measured after 7 d of growth in culture. In each case, 25 randomly selected seedlings were measured. The experiments were repeated at least twice on different seed lots.

\textbf{Accession Numbers}

Sequence data for the genes described in this study can be found in the GenBank/EMBL data libraries under the following accession numbers: \textit{BOS1} (AT3G06490), \textit{BOI} (At4g19700 and \textit{BOI} related genes, \textit{BRG1} (At5g45100) \textit{BRG2} (At1g79110), \textit{BRG3} (At3g12920), \textit{PRI} (At2g14610) and \textit{PDF1.2} (At5g44420).
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Figure legends

**Figure 1. BOI interacts with BOS1 in yeast.**
(A) Overall structure of BOI and deletion constructs used in interaction studies.
(B) Interactions between BOS1 and BOI proteins in yeast two-hybrid assays.
Yeast strains containing full-length BOS1 in the prey vector (pAD-BOS1FL), full length BOI or deletion derivatives of BOI in the bait (pBD-BOI) were assayed for growth on selective medium (-Leucine, -Tryptophan, -Histidine) (right) and β-galatosidase activity (left). β-galactosidase activity was assayed from yeast cells grown on synthetic complete medium. Positive (+) and negative (-) controls from the Stratagen Y2H kit were assayed in parallel.
(C) Sequence comparison between BOI and related plant proteins covering the conserved central WRD domain
(D) Comparison of the RING domains of BOI and animal Inhibitor of Apoptosis Proteins.

The dark shaded residues are identical in all the proteins compared and gray shading indicates residues identical to the BOI protein. In (D), the numbers show the conserved metal ligand positions. Dashes (-) denote gaps introduced to maximize alignment. Sequences used in the alignments are from *Arabidopsis* (AT4G19700, AT5G45100, AT3G12920, AT1G79110), *Populous trichocarpa* (XP_002322357, XP_002332154), *Vitis vinifera* (XP_002272807, XP_002266864), *Physcomitrella patens* subsp. *patens* (XP_001764877, XP_001752458), *Oryza sativa* (Os04g0402500, Os04g0403200), *Solanum chacoense* (AAS76633), Human (U54878, U45879, U45880), *Xenopus laevis* (NP_001082290) and *Drosophila melanogaster* (AAC46988).

**Figure 2. BOI and BOS1 proteins interact in the nucleus of plant cells.**
(A) BOS1 and BOI are localized to the nucleus when individually expressed as GFP fusion proteins.
(B) Bimolecular fluorescence complementation (BiFC) assay showing interaction between BOI and BOS1 in the nucleus of plant cells.

In (B) pBOI-cYFP and pBOS1-nYFP were transiently co-expressed or co-expressed with a respective empty vector in *Nicotiana benthamiana* leaf cells. YFP fluorescence was detected when pBOI-cYFP was co-expressed with pBOS1-nYFP. Cells were examined under brightfield (left column), fluorescence (YFP), and as a merged image (right) showing either no interaction or interaction in the nucleus. DAPI is the nuclear stain 4,6-diamidino-2-phenylindole.

**Figure 3. BOI RNAi and over expression lines and their effects on BOS1 gene expression.**
RNA blot showing (A) *BOI* RNAi lines with reduced *BOI* gene expression and (B) 35S:*BOI* lines with increased *BOI* expression.
Quantitative RT-PCR showing expression of (C) *BOS1* in *BOI* RNAi and 35S:*BOI* lines and (D) *BOI* in the *bos1* mutant in response to *B. cinerea*.

For RNA blots, total RNA (15 µg) was loaded per lane. Quantitative reverse transcriptase PCR was performed as described in the methods. The qRT–PCR data represent expression levels calculated by the comparative cycle threshold method (Applied Biosystems, Foster City, CA, USA) with the *Arabidopsis* *Actin2* gene as the endogenous reference for normalization. Experiments were repeated at least two times with similar results. h, hours after inoculation. rRNA was used as a loading control.
Figure 4. **BOI and BOS1 contribute to Arabidopsis resistance to B. cinerea.**

(A) Mean size of disease lesion and 
(B) Accumulation of B. cinerea ActinA DNA as a measure of fungal growth in BOI RNAi and 35S:BOI plants inoculated with B. cinerea. 
(C) Mean size of disease lesion and 
(D) Disease symptoms in 35S:BOS1 and 35S:BOS1-GUS plants inoculated with B. cinerea.

In (A-D) i-4, i-6, are BOI RNAi lines; i-4 1, i-4 67, Wt2, Wt5 are BOI RNAi or wild type plants expressing the 35S:BOS1-GUS construct, respectively; 2, 11 are transgenic 35S:BOI lines. Plants were inoculated with a single drop (4 µl) of conidial suspension (2.5x10^5 B. cinerea spores/ml) per leaf. Data points represent average ± SE from a minimum of 40 disease lesions. B. cinerea growth was determined by qPCR amplification of B. cinerea Actin A DNA (Bc ActinA) relative to the Arabidopsis Actin2 DNA as the endogenous reference for normalization using the comparative cycle threshold method (Applied Biosystems, Foster City, CA, USA) with (Bluhm and Woloshuk, 2005). In (A-C) analysis of variance was performed to determine the statistical significance of the differences between mean values using SAS software (SAS Institute, 1999). Means with different letters are significantly different from each other (P = 0.05). Disease assays were repeated at least three times. Bc Actin A, Botrytis cinerea Actin A gene; At Actin, Arabidopsis Actin2 gene.

Figure 5. **BOI is a suppressor of toxin and disease associated cell death but not HR cell death.**

Electrolyte leakage in BOI RNAi and 35S:BOI plants induced by treatment or inoculation with
(A) PstDC3000 (avrRpm1), 
(B) PstDC3000 (avrRpt2) 
(C) PstDC3000 and 
(D) α-picolinic acid

Plants were inoculated with a bacterial suspension (OD_600:0.01) or infiltrated with PA toxin (1 mg/ml) and electrolyte leakage measured as described (Dhawan et al, 2009). Experiments were repeated at least three times. i-4 and i-6 are BOI RNAi #4 and 6, respectively.

Figure 6. **Recombinant BOI protein shows E3 ligase activity and BOS1 accumulates after inhibition of the plant proteosome.**

(A) Recombinant His-BOI protein shows ubiquitin E3 ligase activity 
(B) The RING domain of BOI is essential for its ubiquitin E3 ligase activity 
(C) Recombinant MBP-BOI ubiquitinates recombinant BOS1 in vitro 
(D) RT-PCR data showing the expression of BOS1-GUS in 35S:BOS1-GUS transgenic plants 
(E) Histochemical assay showing GUS activity in transgenic BOS1:GUS plants before and after treatment with the proteosome inhibitor MG132.

In (E), plants were infiltrated with the MG132 or buffer and leaves were collected and stained with x-gluc for GUS activity 6 hours after treatment. Ponceau S, a protein stain used to determine the amount of BOS1 in the reaction; dc, deletion constructs; Actin, Arabidopsis Actin 2 gene.

Figure 7. **Basal and induced BOI and defense gene expression.**

Expression of (A) PATHOGENESIS RELATED PROTEIN1 (PR-1), 
(B) Plant defensin PDF1.2, and 
(C) BOI during B. cinerea infection. Expression of BOI in response to
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(D) ACC, MeJA, paraquat (MV), salicylic acid and (E) Gibberellin

Plants were treated with the various compounds for the durations shown as described in the methods. Experiments were repeated at least two times. Quantitative RT–PCR data was calculated as described in the legend for Figure 3. The qRT-PCR data represent the mean ± SE from three replicates. ACC, 1-aminocyclopropane-1-carboxylic acid; MeJA, methyl jasmonate; MV, Methyl viologen; SA, Salicylic acid.

Figure 8. BOI gene expression enhanced at disease- and toxin-induced necrotic sites.
(A) BOI gene expression and 
(B) Evans blue staining for cell death in response to PA, P. syringae and B. cinerea.  
(C) BOS1 gene expression induced by PA 
(D) Expression of BOI Pr:GUS (left) and BOSPr:GUS (right) in reproductive tissues. 
(E) Expression of BOIpr:GUS in siliques and roots.

In (A-B), the circles mark the site of PA infiltration revealing lack of GUS activity (A,C) but intense Evans blue staining (B). Plants were infiltrated with buffer (MgCl$_2$), PstDC3000 (Pst), α-picolonic acid (PA) or spray inoculated with B. cinerea and stained at the times indicated. Evans blue staining was performed as described (Watanabe and Lam, 2006). h, hours after infection or treatment.

Figure 9. E3 Ubiquitin ligase activity of BRG1 and expression of BOI-RELATED GENES in response to Gibberellin and salt.
Expression of BOI-RELATED GENES (BRG1-BRG3) in response to 
(A) Recombinant BRG1 protein has ubiquitin ligase activity  
(B) Recombinant BRG1 protein does not ubiquitinate BOS1 
(C) Expression of BRG1 in wild type and the GA-deficient ga1-3 mutant. 
(D) Expression of BRG1-BRG3 in response to GA

Quantitative RT-PCR was performed as described previously (Dhawan et al., 2009) and in the methods section. Experiments were repeated at least two times with similar results.

Figure 10. BOI-RELATED GENES contribute to B. cinerea resistance and the suppression of PA- and bacterial-induced cell death.
(A) Mean size of disease lesion and 
(B) Fungal growth in B. cinerea inoculated leaves.  
Electrolyte leakage after 
(C) PstDC3000 inoculation and 
(D) PA toxin treatment. 

Disease assays, sample treatment and electrolyte leakage measurements were performed as described (Dhawan et al., 2009). In (B), fungal growth was determined based on the relative PCR amplification of the B. cinerea ActinA and Arabidopsis Actin2 genes. Analysis of variance and t-test (LSD) were performed to determine the statistical significance of the differences between the mean values using SAS software (SAS, 1999). The mean values followed by different letters are significantly different from each other ($P=0.05$).
**Figure 11.** *BOI* is required for full GA-responsive growth and abiotic stress tolerance.

(A) Reduced hypocotyl and overall seedling growth and
(B) Mean hypocotyl length in *BOI* RNAi and *brg* mutants in response to GA.
(C) Sensitivity of *BOI* RNAi and *brg* mutants to salt stress

Seedlings were germinated on plain MS media and transferred to media supplemented with GA or NaCl. In (B) Analysis of variance and t-test were performed to determine the statistical significance of the differences between the mean values using SAS software (SAS, 1999). The mean values followed by different letters are significantly different from each other (*P* = 0.05). In (C) the total number of seedlings tested and percent of seedlings showing salt-induced damage including necrotic or chlorotic symptoms are shown below the images.
### Table 1. Summary of the main features of BOI and BOI RELATED GENES.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Interaction with BOS1</th>
<th>ORF, MW (kD)</th>
<th>pI</th>
<th>Identity to BOI</th>
<th>expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bc</td>
</tr>
<tr>
<td>BOI</td>
<td>Strong</td>
<td>304 aa, 33.50</td>
<td>5.51</td>
<td>100</td>
<td>+</td>
</tr>
<tr>
<td>BRG1</td>
<td>No</td>
<td>294 aa, 32.54</td>
<td>5.56</td>
<td>64</td>
<td>=</td>
</tr>
<tr>
<td>BRG2</td>
<td>No</td>
<td>358 aa, 40.08</td>
<td>6.73</td>
<td>47</td>
<td>-</td>
</tr>
<tr>
<td>BRG3</td>
<td>No</td>
<td>335 aa, 37.70</td>
<td>6.22</td>
<td>38</td>
<td>-</td>
</tr>
<tr>
<td>At2g12290</td>
<td>No</td>
<td>133 aa, 15.44</td>
<td>8.93</td>
<td>80*</td>
<td>Nt</td>
</tr>
</tbody>
</table>

BRG1, At5G45100; BRG2, At1G79110; BRG3, At3G12920; Nt, not tested; Bc, Botrytis cinerea; +, significant induction; -, significant repression; =, expression unchanged; NI, not induced; *, the identity is limited to the C-terminal region.

PA, α-picolinic acid; GA, gibberellin; SA, Salicylate; ACC, (1-aminocyclopropane-1-carboxylic acid)
References


del Pozo O, Pedley KF, Martin GB (2004) MAPKKKalpha is a positive regulator of cell death associated with both plant immunity and disease. Embo J 23: 3072-3082


Shi CS, Kehrl JH (2003) Tumor necrosis factor (TNF)-induced germinal center kinase-related (GCKR) and stress-activated protein kinase (SAPK) activation depends upon the E2/E3 complex Ubc13-Uev1A/TNF receptor-associated factor 2 (TRAF2). J Biol Chem 278: 15429-15434


Spoel SH, Johnson JS, Dong X (2007) Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. Proc Natl Acad Sci U S A 104: 18842-18847


A  

\[
\begin{array}{cccccc}
E1 & - & + & + & + & + \\
E2 & + & - & + & + & + \\
BOI & + & + & - & + & + \\
Ub & + & + & + & - & + \\
\end{array}
\]

B  

\[
\begin{array}{cccccc}
E1 & + & + & + & + & + \\
E2 & + & + & + & + & + \\
E3 & - & BOI & BOI & & \\
Ub & + & + & + & + & + \\
MBP & & BOIdc1 & BOIdc2 & BOI & \\
\end{array}
\]

C  

\[
\begin{array}{cccccc}
E1 & + & + & + & + & + \\
E2 & + & + & + & + & + \\
E3 & - & BOI & BOI & & \\
Ub & + & + & + & + & + \\
BOS1 & + & + & + & - & \\
\end{array}
\]

D  

\[
\begin{array}{cccccc}
Wt; 35S:BOS1-GUS & & & & & \\
BOI RNAi; 35S:BOS1-GUS & & & & & \\
\end{array}
\]

\[
\begin{array}{cccccc}
Wt; & 1 & 2 & 1 & 2 & \\
BOS1-GUS & & & & & \\
Actin & & & & & \\
\end{array}
\]

E  

\[
\begin{array}{cccccc}
Wt; 35S:BOS1-GUS & & & & & \\
BOI RNAi; 35S:BOS1-GUS & & & & & \\
\end{array}
\]

\[
\begin{array}{cccccc}
& - & + MG132 & + MG132 & & \\
\end{array}
\]

\[
\begin{array}{cccccc}
Wt; 35S:BOS1-GUS & & & & & \\
BOI RNAi; 35S:BOS1-GUS & & & & & \\
\end{array}
\]

\[
\begin{array}{cccccc}
& - & + MG132 & + MG132 & & \\
\end{array}
\]
### Table A

<table>
<thead>
<tr>
<th></th>
<th>E1</th>
<th>E2</th>
<th>BRG1</th>
<th>Ub</th>
<th>BOS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

### Diagram A

- **Ab: Anti-Ub**
- **Ab: Anti-MBP**
- **Ab: Anti-GST**

### Diagram B

- Protein bands for **BRG1**, **Ub**, and **BOS1**.

### Diagram C

**BRG1 expression**

<table>
<thead>
<tr>
<th>Time (h)</th>
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<th>ga1-3</th>
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</thead>
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<tr>
<td>0</td>
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<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>1.6</td>
<td>0.2</td>
</tr>
</tbody>
</table>

### Diagram D

**Expression level**

<table>
<thead>
<tr>
<th>Gene</th>
<th>0 h</th>
<th>3 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRG1</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>BRG2</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>BRG3</td>
<td>0.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>