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The *BOTRYTIS SUSCEPTIBLE1* Gene Encodes an R2R3MYB Transcription Factor Protein That Is Required for Biotic and Abiotic Stress Responses in Arabidopsis

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The molecular and cellular mechanisms involved in plant resistance to the necrotrophic fungal pathogen *Botrytis cinerea* and their genetic control are poorly understood. Botrytis causes severe disease in a wide range of plant species, both in the field and in postharvest situations, resulting in significant economic losses. We have isolated the *BOS1* (*BOTRYTIS-SUSCEPTIBLE1*) gene of Arabidopsis based on a T-DNA insertion allele that resulted in increased susceptibility to Botrytis infection. The *BOS1* gene is required to restrict the spread of another necrotrophic pathogen, *Alternaria brassicicola*, suggesting a common host response strategy against these pathogens. In the case of the biotrophic pathogens *Pseudomonas syringae* pv *tomato* and the oomycete parasite *Peronospora parasitica*, *bos1* exhibits enhanced disease symptoms, but pathogen growth is similar in *bos1* and wild-type plants. Strikingly, *bos1* plants have impaired tolerance to water deficit, increased salinity, and oxidative stress. Botrytis infection induces the expression of the *BOS1* gene. This increased expression is severely impaired in the *coi1* mutant, suggesting an interaction of *BOS1* with the jasmonate signaling pathway. *BOS1* encodes an *R2R3MYB* transcription factor protein, and our results suggest that it mediates responses to signals, possibly mediated by reactive oxygen intermediates from both biotic and abiotic stress agents.

INTRODUCTION

As a result of the continued exposure to pathogens, pests, and a variety of other environmental stresses, plants have evolved various mechanisms for survival. Biotic and abiotic stresses can result in the increased synthesis of secondary metabolites and signaling molecules, ion fluxes, an oxidative burst, and changes in the transcription of an array of genes (Bowler and Fluhr, 2000; Chen et al., 2002). Furthermore, biotic and abiotic stresses can induce the expression of both distinct and overlapping sets of genes (Chen et al., 2002; Cheong et al., 2002). These cases highlight the presence of both specialized and broad molecular mechanisms to translate environmental signals into adaptive responses depending on the nature of the perceived signal.

Plant resistance strategies to protect against pathogen infection are diverse. Some highly specific mechanisms have evolved to resist coevolved and specialized pathogen strains. These often are characterized by resistance genes that are effective against specific races of pathogen species, so-called gene-for-gene interactions (Flor, 1971). Other resistance mechanisms provide protection against a broad spectrum of fungal, bacterial, and viral pathogens. Examples of this type of resistance include systemic acquired resistance (SAR) and induced systemic resistance (Pieterse et al., 1998; Dempsey et al., 1999). Such resistance responses may be deployed singly or in combination in a single plant genotype depending on the nature of the pathogen. A variety of plant hormones, including salicylic acid (SA), jasmonate (JA), ethylene, and abscisic acid, have been implicated in mediating defense responses (Gaffney et al., 1993; Thomma et al., 1998; Audenaert et al., 2002). Changes in the status of these hormones, their perception, and signaling alter defense responses to pathogens, indicating their involvement in the execution of local and systemic resistance (Gaffney et al., 1993; Thomma et al., 1998; Audenaert et al., 2002).

SAR is an SA-dependent resistance response activated by some pathogens that cause necrosis or by cell death triggered by host resistance responses. Characteristics of SAR include resistance in tissues distant from the initial infection site, persistence for weeks to months, and protection against secondary infection by a broad spectrum of predominantly biotrophic pathogens (Métraux et al., 1990; Gaffney et al., 1993; Reuber et al., 1998). By contrast, the activation of SAR in Arabidopsis by preimmunization of plants with *Pseudomonas syringae* pv *tomato* does not protect against secondary infection by *Botrytis cinerea* (Govrin and Levine, 2002). Mutations that constitutively activate SAR promote Botrytis infection (Govrin and Levine, 2000; Kachroo et al., 2001), whereas mutations that inhibit SA signaling do not appear to affect systemic responses to Botrytis (Thomma et al., 1998). There is evidence that SA produced by the phenylpropanoid pathway may play a role in the response at the point of inoculation (Ferrari et al., 2003). Botrytis causes necrosis and the expression of SAR marker genes in Arabidopsis but does not result in the enhanced resistance characteristic of SAR (Govrin and Levine, 2002).

Resistance to some necrotrophic pathogens in Arabidopsis has been associated with signaling pathways regulated by ethylene and JA. The *PDF1-2* gene, which encodes an antifungal peptide, is activated by the necrotrophic pathogens Botrytis and *Alternaria brassicicola* and by ethylene and JA (Penninckx et al., 1998). Increased susceptibility to some necrotrophic patho-

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gens is correlated with defects in JA and ethylene accumulation and signaling and the accompanying loss or delay in *PDF1-2* activation (Thomma et al., 1999; Kachroo et al., 2001). However, the induction of *PDF1-2* by pathogens, JA, or ethylene is not affected by the inhibition of SA accumulation or SA signaling (Penninckx et al., 1998; Diaz et al., 2002). Furthermore, the application of ethylene or methyl jasmonate reduces disease severity caused by some necrotrophs in Arabidopsis and tomato (Thomma et al., 1999; Diaz et al., 2002).

Necrotrophic pathogens often promote host cell death—for example, through the generation of reactive oxygen intermediates (ROI) or the secretion of toxins into host tissue. ROI generated as a result of an oxidative burst during infection mediate hypersensitive cell death as part of the host resistance mechanism (Lamb and Dixon, 1997). The oxidative burst may enhance disease resistance in a number of ways. ROI may result in the cross-linking of cell wall proteins, rendering the cell wall more resistant to attack by fungal enzymes. ROI also can have direct toxic effects on pathogens, or they may act as second messengers for the activation of genes that encode protective proteins (Lamb and Dixon, 1997). By contrast, necrotrophic pathogens such as *Sclerotinia sclerotiorum* and Botrytis may induce toxic levels of ROI in the host to promote infection (Govrin and Levine, 2000). With Botrytis, a positive correlation between pathogenicity and the intensity of ROI has been reported (Edlich et al., 1989). The inhibition of ROI formation in the host reduces susceptibility to Botrytis (Govrin and Levine, 2000). Cell death resulting from resistance responses or other physiological functions facilitates Botrytis infection (Govrin and Levine, 2000; Kachroo et al., 2001). Accordingly, the expression of animal antiapoptotic genes in plants increases resistance to the necrotrophic pathogens *S. sclerotiorum*, Botrytis, and *Cercospora nicotiana* (Dickman et al., 2001).

The genetic control of resistance to necrotrophic pathogens in general and Botrytis in particular is poorly defined. To determine the components of the host response to necrotrophic infection, mutants with enhanced susceptibility to Botrytis were identified in a T-DNA insertion–mutagenized Arabidopsis population. One mutant, *bos1* (*Botrytis susceptible1*), was characterized further and found to be susceptible to a second necrotrophic pathogen, *A. brassicicola*, as well as to oxidative stress, salinity, and water deficit. *bos1* plants developed enhanced symptoms in response to inoculation with the biotrophic pathogens *Peronospora parasitica* and *P. syringae* pv *tomato*. In terms of the growth of these pathogens within the host tissue, however, there was no difference between *bos1* and wild-type plants. The *BOS1* gene was isolated based on a T-DNA insertion allele and found to encode an R2R3MYB transcription factor protein. R2R3MYB proteins are implicated in the regulation of plant-specific processes (Stracke et al., 2001). BOS1 is an R2R3MYB protein that regulates responses to both biotic and abiotic stresses.

RESULTS

Identification of Botrytis-Susceptible Mutants

A T-DNA–mutagenized population of Arabidopsis (McElver et al., 2001) was screened for increased susceptibility to Botrytis infection (see Methods). To facilitate the identification of recessive mutations, the T2 (segregating) generation of seeds was used in the screen. Twenty 3-week-old T2 plants per individual transgenic line were grown on soil and spray-inoculated with a suspension containing 10^5 Botrytis spores/mL. Disease was scored between 5 and 10 days after inoculation for plants exhibiting increased disease symptoms, which ranged from extensive chlorosis and necrosis to death.

The *bos1* mutant was identified among T2 segregating plants by its enhanced susceptibility to Botrytis and subjected to the detailed studies described here. Because of the killing effects of the Botrytis infection, plants exhibiting the Botrytis susceptibility phenotype were recovered by rescreening more T2 individuals from the segregating line. In this secondary screen, single leaves were drop-inoculated with the spore suspension. Disease progression was observed in the inoculated leaves at 5 days after inoculation to score for the Botrytis response phenotype. The infected leaves then were removed to ensure the survival of the Botrytis-susceptible individuals. The plants were allowed to self-pollinate and set seed. The Botrytis susceptibility identified in the primary screen was confirmed in this secondary assay. *bos1* plants were characterized by extensive tissue damage at an early stage after Botrytis infection compared with wild-type plants. These symptoms progressed and resulted in the complete decay of *bos1* plants at ~10 days after inoculation (Figures 1A and 1B) as the infection became systemic. In the wild-type plants, symptoms did not spread beyond the inoculated leaves.

Fungal growth in inoculated plants was assessed to determine if the *bos1* mutation affects pathogen multiplication as well as disease symptoms. The accumulation of the Botrytis *β-tubulin* gene transcript was determined at representative stages of disease progression in *bos1* and wild-type plants using RNA gel blots. The Botrytis *β-tubulin* gene was shown previously to be expressed constitutively in Botrytis during plant infection (Benito et al., 1998); therefore, the amount of its transcript is indicative of the rate of fungal growth in planta. The Botrytis *β-tubulin* mRNA accumulated as disease progressed, with a significantly greater amount of the fungal mRNA detected in inoculated *bos1* plants compared with the wild type (Figure 1E). In wild-type plants, the amount of transcript remained the same between 3 and 4 days after inoculation, whereas in *bos1* plants, fungal RNA continued to increase throughout the testing period. Thus, the *bos1* mutation resulted in increased susceptibility to Botrytis, as determined by increased growth of the pathogen in host tissue.

bos1 Is a Recessive Mutation

To determine the genetic relationship of the *BOS1* wild type and mutant alleles, 60 randomly selected single T2 plants from the segregating *bos1* line were grown and assayed for Botrytis resistance using the drop-inoculation method. T3 seeds were harvested from selfed T2 individuals, and the *bos1* phenotype was determined in the T3 families. Twelve T2 individuals had the *bos1* phenotype, and the T3 progeny of all 12 of these lines were uniformly Botrytis susceptible. The remaining 48 T2 individuals exhibited wild-type resistance to Botrytis. T3 progeny

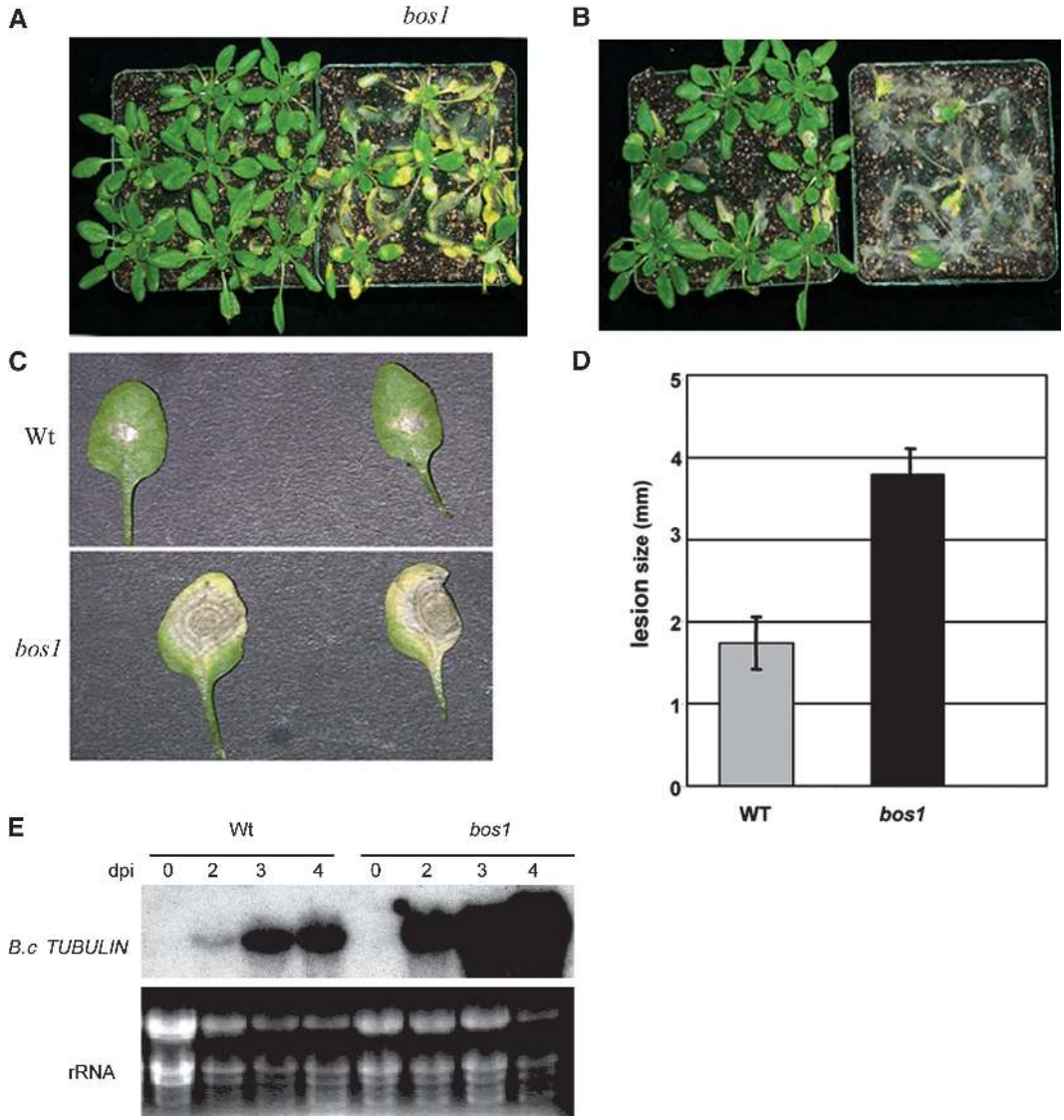

Figure 1. Progress of Disease Development in *bos1* Plants Inoculated with Necrotrophic Pathogens.

(A) and **(B)** Wild-type (left) and *bos1* (right) homozygous plants at 5 **(A)** and 10 **(B)** days after inoculation with Botrytis. Plants were inoculated by spraying spore suspension at a density of 10^5 spores/mL and kept under high humidity.

(C) Leaves from wild-type (Wt) and *bos1* homozygous plants at 5 days after inoculation with a 4-μL droplet of *A. brassicicola* spores (10^5 spores/mL).

(D) Lesion size was measured at 3 days after inoculation. Data points represent average lesion size ± SE of measurements from a minimum of 40 lesions. This experiment was repeated three times with similar results. WT, wild type.

(E) Accumulation of the Botrytis (B.c) *β-tubulin* mRNA in inoculated plants. Twenty micrograms of total RNA extracted from inoculated plants was loaded per lane. dpi, days after inoculation.

of 33 of the Botrytis-resistant T2 plants segregated for Botrytis susceptibility. The remaining 15 T3 families were uniformly Botrytis resistant. These results suggest that *bos1* is a recessive allele. This notion was confirmed by backcrossing a homozygous *bos1* plant to wild-type plants. The F1 progeny had a wild-type Botrytis-resistant phenotype. In the F2 generation, 10 of 64 individuals were Botrytis susceptible. The values obtained from the analysis of the F2 and T2 segregation do not differ significantly from a 3:1 segregation ratio ($\chi^2 = 0.2776$: 1.2604, $P \leq 0.05$). These values are consistent with the recessive nature of the mutation causing the Botrytis susceptibility phenotype.

The T2 plants and T3 families also were used to determine genetic linkage between a T-DNA insert and Botrytis susceptibility. Arabidopsis genomic DNA flanking T-DNA inserts in *bos1* were obtained by thermal asymmetric interlaced PCR (TAIL-PCR) (Liu et al., 1995; McElver et al., 2001). Two TAIL-PCR products were amplified, and the corresponding genomic fragments were located in the Arabidopsis genome by comparison with the genomic sequence. One TAIL-PCR product was

found on chromosome 1, and the second was found on chromosome 3. Pairs of PCR primers were designed to the genomic sequence flanking the T-DNA insertion sites. For each insert, the combination of one genomic primer plus a T-DNA primer gave a PCR product only when genomic DNA from plants containing that T-DNA insert were used as a template. When the two genomic PCR primers for a specific insertion site were used in combination, a PCR product was produced only when a wild-type allele was present. Heterozygous individuals give PCR products when both combinations of primers were used. Tests with these primers confirmed that PCR fragments of the expected size were amplified from plants of known *bos1* genotype. Furthermore, sequencing of the PCR products confirmed that the primers generated the expected fragments. This PCR-based analysis was used to confirm the T-DNA insertion and to determine the cosegregation of the *bos1* phenotype and the T-DNA inserts using the segregating T2 and T3 families described above.

The T-DNA insert that mapped to chromosome 3 cosegregated with the Botrytis susceptibility phenotype, suggesting that the *bos1* mutation is caused by this T-DNA insertion. All individual T2 plants that exhibited susceptibility to Botrytis gave the insert-specific PCR product and did not give the PCR product indicative of the wild-type allele. Genomic DNA from all of the T2 siblings with wild-type levels of Botrytis resistance gave the wild-type PCR band. Based on these findings and further genetic analysis supported by molecular analysis of the tagged locus in F3 populations (data not shown), it could be concluded that Botrytis susceptibility is a single recessive Mendelian trait linked to the T-DNA insertion on chromosome 3.

bos1 Affects Responses to Other Pathogens

To determine whether the disease susceptibility of *bos1* is specific to Botrytis or extends to other pathogens, we challenged *bos1* and wild-type plants with various pathogens. Three days after inoculation with *A. brassicicola*, another necrotroph, *bos1* leaves showed significantly larger disease lesions than did *A. brassicicola*–inoculated wild-type leaves (Figure 1D). At 5 days after inoculation, >50% of the leaf areas of the inoculated *bos1* leaves were killed by the infection, with concentric brown infection rings typical of *A. brassicicola* clearly visible (Figure 1C). Wild-type plants were resistant to *A. brassicicola*, exhibiting only a restricted whitish lesion that affected <25% of the inoculated leaf.

The *bos1* mutation did not affect the growth of the biotrophic oomycete pathogen *P. parasitica*. Growth of the pathogen was assayed by direct observation of stained hyphae in infected leaves and by counting the spores produced on infected leaves. Using both measurements, there was no significant difference in pathogen growth in wild-type and *bos1* plants. This was true for both virulent and avirulent isolates of *P. parasitica*. Although *bos1* did not affect the susceptibility of the plant to colonization by the pathogen, it did affect the rate of symptom development. After *P. parasitica* inoculation, leaves of *bos1* plants became chlorotic at an earlier time point than did leaves of wild-type plants (data not shown).

The response of *bos1* to virulent and avirulent strains of the bacterial pathogen *P. syringae* pv *tomato* also was tested. As with *P. parasitica*, there was no detectable difference in the rate of growth of *P. syringae* in *bos1* plants compared with wild-type plants for both virulent and avirulent bacterial strains (Figures 2C and 2D). However, the *bos1* plants did develop more severe chlorosis in response to infection than did the wild-type plants (Figures 2A and 2B).

bos1 Plants Are Hypersensitive to Multiple Abiotic Stress Factors

To determine whether the *bos1* mutation affected the response to abiotic as well as biotic stresses, *bos1* plants were analyzed for their tolerance to salinity, drought, cold, osmotic, and oxidative stresses. To test for altered salt tolerance, seeds were germinated on MS medium (Murashige and Skoog, 1962) plates that were kept vertical to allow seedlings to grow over the agar surface. Five-day-old seedlings were transferred to MS medium supplemented with a range of NaCl concentrations. Three weeks later, the plants were scored for visible changes, including size differences and loss of chlorophyll. The growth of *bos1* plants was reduced significantly after exposure to salt stress (Figure 3A). At 100 mM NaCl, 82% of *bos1* plants were stunted in growth and had chlorotic leaves compared with wild-type plants, in which only 9% of plants showed these symptoms. At 150 mM NaCl, all *bos1* plants were stunted and chlorotic, whereas 75% of the wild-type plants had no visible damage.

To assess sensitivity to drought stress, *bos1* and wild-type plants were grown on soil for 3 weeks with normal watering. Watering then was withheld for 10 days. At this time, most *bos1* and wild-type plants showed visible signs of wilting. Regular watering was resumed for 3 weeks, and plants were scored for recovery from the drought stress. The majority of wild-type plants recovered after the resumption of regular watering, but the recovery of *bos1* plants was reduced significantly after the 10-day dry period (Figure 3B). Only 30% of water-stressed *bos1* plants recovered, compared with 89% of wild-type plants. This finding was supported further by germination tests under reduced water potential caused by increasing concentrations of polyethylene glycol 8000. Over a range of polyethylene glycol concentrations from 5 to 16%, 20 to 40% fewer *bos1* seeds germinated than wild-type seeds (Figure 3C).

bos1 plants did not show increased sensitivity to all abiotic stresses, however. There was no significant difference between wild-type and mutant plants in response to cold and general osmotic stresses (data not shown). This finding suggests that *BOS1* is involved in responses to only a subset of abiotic stresses.

The sensitivity of *bos1* plants to oxidative stress was assessed using two different reagents that generate ROI. Rose bengal (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein) generates ROI in medium when exposed to light (Wierrani et al., 2002). When added to germination medium at 6 μM, it resulted in an almost total block in growth of *bos1* seedlings (Figure 4A). In addition, the seedlings appeared etiolated, with elongated hypocotyls and no expanded green leaves. The growth of wild-type seedlings was inhibited slightly by this treatment, but the plants developed normally. The plants also were tested for sensitivity to paraquat (methyl viologen), an agent that promotes the formation of ROI by inhibiting electron transport in the reduction of

NADP to NADPH during photosynthesis (Suntres, 2002). Wild-type and *bos1* plants were treated by placing a droplet of 10 to 100 μM paraquat or water on individual leaves. After treatment at 100 μM, *bos1* leaves were almost totally bleached, whereas wild-type leaves showed minimal damage (Figure 4B).

The production of ROI during Botrytis infection was determined using two histochemical staining approaches. The in situ formation of H_2O_2 was detected using diaminobenzidine, which polymerizes into a visible reddish-brown polymer at the site of H_2O_2 formation (Thordal-Christensen et al., 1997). The accumulation of superoxide anions (O_2^-) was tested by staining with nitroblue tetrazolium (Doke, 1983). Comparable amounts of H_2O_2 and O_2^- were detected in all Botrytis-inoculated plants at 1 day after inoculation (data not shown). At 2 days after inoculation, significantly more H_2O_2 and O_2^- were generated in plants at the site of inoculation and the surrounding areas compared with 1 day after inoculation, correlating with increased disease levels in both mutant and wild-type plants (Figure 5). At 2 days after inoculation, the *bos1* plants exhibited larger and more intensely stained areas for O_2^- and H_2O_2 than did wild-type plants (Figures 5D and 5H). This finding correlates well with the increased fungal accumulation at 2 days after inoculation in *bos1* plants and the increased tissue maceration that results in enhanced production of ROI, consistent with previous reports (Edlich et al., 1989; Tiedemann, 1997). Early disease symptoms in *bos1* include a very distinct spreading chlorosis that surrounds the infection sites. The formation and intensity of O_2^- appears to coincide with this disease symptom. In wild-type plants, the O_2^- appears to be less intense and more restricted in area (Figures 5B and 5D).

BOS1 Encodes an R2R3MYB Transcription Factor

To identify the mutation that causes the *bos1* phenotype, genomic DNA flanking the T-DNA insertion was obtained by performing TAIL-PCR (Liu et al., 1995) on genomic DNA from *bos1* plants. Comparison of the sequence of the TAIL-PCR product with the Arabidopsis genomic sequence indicated that the T-DNA inserted just 5′ of the start codon of a gene encoding a putative R2R3MYB transcription factor protein (AtMYB108; At3g06490). Analysis of the insertion site showed that in addition to the T-DNA insertion, there is a deletion of 314 bp upstream of the translation start site that includes the entire 151-bp 5′ untranslated region and 163 bp of the promoter region

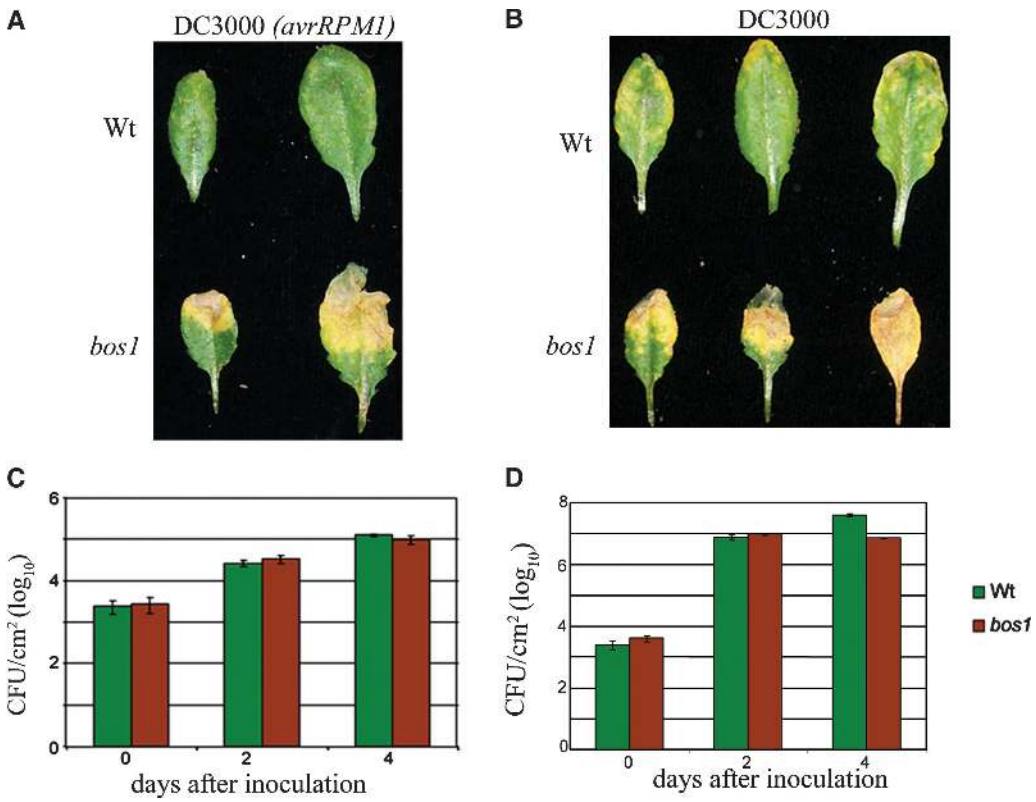

Figure 2. *bos1* Plants Show Wild-Type Levels of Resistance but Increased Symptom Development in Response to Bacterial Pathogens.

(A) and **(B)** *P. syringae* pv *tomato* avirulent strain DC3000 carrying *avrRpm1* **(A)** and virulent strain DC3000 **(B)**. Representative leaves are shown at 4 days after inoculation. Wt, wild type.

(C) and **(D)** Bacterial growth for DC3000 carrying *avrRpm1* **(C)** and DC3000 **(D)**. Four-week-old plants were infiltrated with bacterial suspension (OD_{600} = 0.001), and the number of bacteria was determined per area of leaf plotted for wild-type (green bars) and *bos1* (red bars) plants. CFU, colony-forming units.

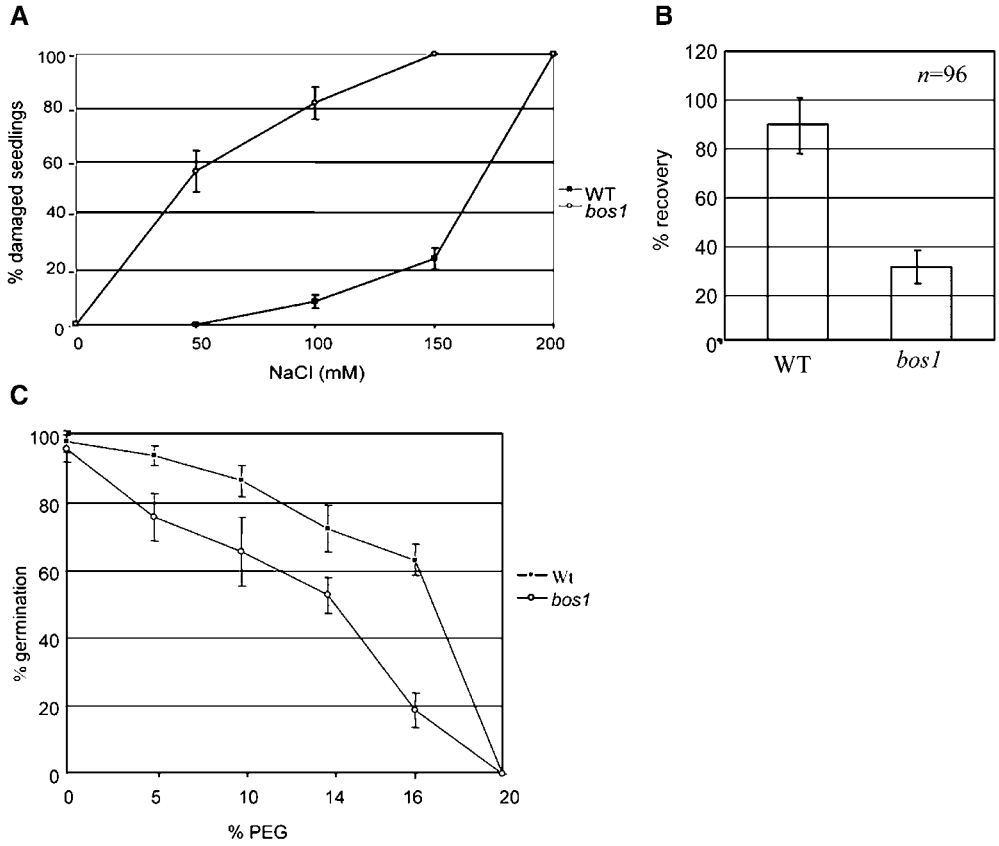

Figure 3. Impaired Abiotic Stress Tolerance in *bos1*.

(A) Sensitivity to NaCl. WT, wild type.
(B) Percentage plant survival after drought stress.
(C) Percentage germination of seeds in the presence of polyethylene glycol 8000 (PEG). Wt, wild type.
Data points represent average values ± SE.

(Figure 6A). The effect of the *bos1* mutation on the expression of the *BOS1* gene was determined. In untreated plants that were homozygous for the wild-type allele, *BOS1* was expressed at very low levels (Figure 6B). In RNA from leaves of untreated plants that were homozygous or heterozygous for *bos1*, a transcript that hybridizes with the *BOS1* probe was seen by RNA gel blot analysis. However, only plants that were homozygous for the T-DNA insertion showed the recessive *bos1* phenotype, suggesting that this is an aberrant transcript that results either in no BOS1 being produced or in the production of a nonfunctional BOS1 protein.

A genomic clone containing the *BOS1* gene flanked by 1.5 kb of a putative promoter sequence was obtained. This genomic fragment was subcloned into a binary transformation vector and used to transform *bos1* plants. Ten independent transformants were examined for the *bos1* phenotype. All of the transgenic lines had wild-type levels of resistance to Botrytis and oxidative stress (Figures 6C and 6D). The transgenic plants also were indistinguishable from wild-type plants in their response to *A. brassicicola* inoculation and salt and drought stress (data not shown). These results indicate that the *bos1* phenotype is attributable to the T-DNA insertion in the *BOS1* gene and that the mutation in this gene is responsible for all of the altered phenotypic changes observed in the *bos1* plants.

The full-length cDNA corresponding to the *BOS1* mRNA was cloned by reverse transcriptase–PCR and rapid amplification of cDNA ends. The *BOS1* cDNA encodes a putative protein of 323 amino acids with a predicted molecular mass of 37,019.8 D and a theoretical pI of 4.93. Sequence searches in databases (Altschul et al., 1990) revealed extensive identity with proteins belonging to the MYB transcription factor family of proteins (Stracke et al., 2001) (Figures 7A and 7B). The ATP/GTP binding site motif A (P-loop) (Walker et al., 1982) with a consensus sequence of [AG]-x(4)-G-K-[ST] appears in the *BOS1* protein near the N terminus (positions 51 to 59). The R2 and R3 MYB DNA binding repeats (amino acids 19 to 71 and 72 to 121, respectively) of BOS1 show high sequence conservation in all MYB proteins (Figure 7A). Particularly striking is the significant sequence similarity to dehydration-induced MYB-related proteins from the resurrection plant *Craterostigma plantagineum*, CPM7, CPM5, and CPM10 (Iturriaga et al., 1996). These CPM proteins show 76 to 80% identity to the *BOS1* protein in the 152–amino acid region covering the MYB domains and the flanking sequences.

In *C. plantagineum*, these genes are expressed in response to abscisic acid and desiccation in roots and callus tissues. In Arabidopsis, the BOS1 protein shares the highest overall sequence identity (73.74%) with AtMYB78 (At5g49620). In a segment of 129 amino acids covering the conserved *R2R3MYB* repeats, this identity increases to 93%. The R3 MYB domain and the 29 residues after it are identical in *BOS1* and *AtMYB78* except for a difference in a single amino acid residue. In addition, after the N-terminal conserved DNA binding domains is a region of high sequence identity shared between BOS1, AtMYB78, and the CPM proteins (Figure 7B). Thus, these genes may constitute a functionally related subgroup. The entire Arabidopsis *R2R3MYB* gene family was analyzed, and the Arabidopsis MYBs were subgrouped further based on the degree of conservation in the amino acid residues after the MYB domains (Stracke et al., 2001). One subgroup, containing BOS1, AtMYB78, AtMYB62, AtMYB116, AtMYB2, and AtMYB112, is highly identical in sequence after the R3 domain. AtMYB2 functions as a transcriptional activator in abscisic acid signaling (Abe et al., 2003), although no functional data exist for any of the other members of this subgroup.

Interestingly, the BOS1 protein shows a consensus sequence motif for sumolyation (ψKXE) at residues 21 to 22 and 126 to 129, including the target Lys (K) residue. This is a target sequence for protein modifications caused by SUMO, a small ubiquitin-like modifier, which is targeted to proteins by an isopeptide bond (Melchior, 2000). Sumolyation has been implicated in protein stabilization, protection from degradation, and localization to nuclear speckles (Melchior, 2000; Ballesteros et al., 2001). The presence of a target site for sumolyation suggests a possible post-translational modification of the BOS1 protein.

The promoter region of *BOS1* was analyzed for the presence of putative *cis*-acting regulatory elements (Higo et al., 1999). A number of consensus *cis*-acting elements were found in the *BOS1* promoters that are consistent with the role of the gene in stress and disease response pathways. These include consensus sequences for binding different MYB and MYC proteins involved in the regulation of various physiological responses (MYB, 5′-TAACTG-3′; MYC, 5′-CACATG-3′), sequences that mediate abscisic acid–regulated gene expression (ABRE, 5′-AACGTG-3′; AtHB6, 5′-CAATTATTA-3′; CE element, 5′-CACC-3′), and sequences that mediate pathogen-induced (5′-TTGACT-3′) and wound-induced (5′-TAATTTCAA-3′) gene expression (Table 1).

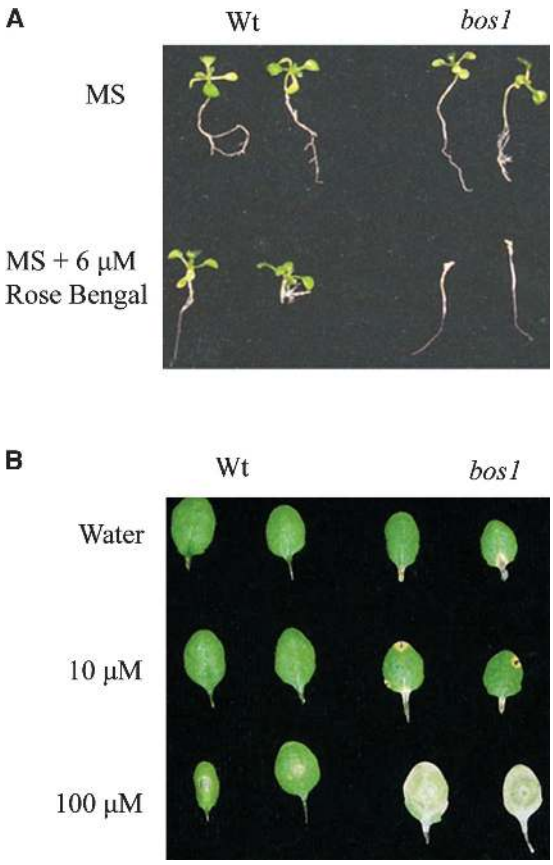

Figure 4. *bos1* Plants Have Increased Sensitivity to Compounds That Generate Oxidative Stress.

(A) Five-day-old seedlings germinated on MS medium were transferred to MS medium containing rose bengal. Photographs were taken 2 weeks after transfer to the medium containing rose bengal. Wt, wild type.

(B) Response to paraquat was assayed by depositing a single 2-μL droplet of methyl viologen dissolved in water on individual leaves. Control leaves were treated with water alone. Photographs were taken 4 days after treatment.

BOS1 Expression Is Induced by *Botrytis* Infection

The expression of the *BOS1* gene after Botrytis infection was studied. The *BOS1* transcript is of low abundance in RNA from wild-type tissue grown under standard conditions. However, the gene showed significant induction in tissue from Botrytis-infected plants compared with buffer-treated controls (Figure 8A). Expression was detectable by 24 h after inoculation, and the *BOS1* transcript accumulated further upon disease progression. *BOS1* expression also was examined in two other mutants that result in increased Botrytis susceptibility: *coi1*, which is insensitive to JA (Xie et al., 1998), and *ein2*, an ethylene-insensitive mutant (Guzman and Ecker, 1990). In *coi1*, the Botrytis-inducible expression of *BOS1* was delayed and reduced, suggesting a link between *BOS1* and the JA-dependent disease response pathway. The *ein2* mutant also exhibited increased susceptibility to Botrytis, but the expression of *BOS1* in response to Botrytis was not affected in this mutant. The SAR pathway does not appear to be involved directly in resistance to necrotrophic pathogens, although there is evidence for antagonistic interactions between SAR- and JA-mediated resistance pathways (Kachroo et al., 2001). Plants that express the bacterial salicylate hydroxylase (*NahG*) gene are unable to accumulate SA and do not develop SAR (Gaffney et al., 1993). The expression of *BOS1* was similar in *NahG* and wild-type plants (data not shown).

bos1 Plants Show Unaltered Expression of *PR-1* and *PDF1-2*

The *PR1* gene is a molecular marker for the SAR response, whereas expression of the defensin gene *PDF1-2* has been linked

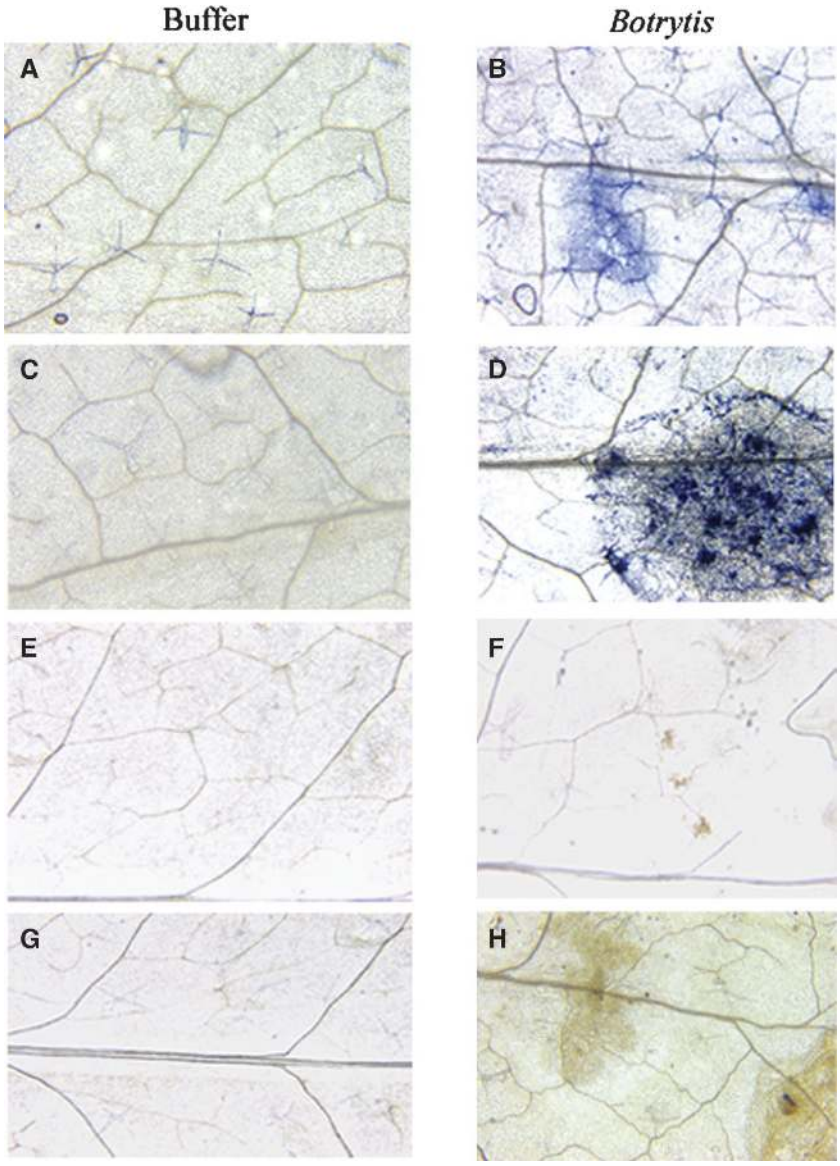

Figure 5. Generation of ROI in Botrytis-Infected Tissues.

(A) to **(D)** Production of O_2^- in wild-type (**[A]** and **[B]**) and *bos1* (**[C]** and **[D]**) plants at 2 days after inoculation after treatment with buffer (**[A]** and **[C]**) or Botrytis spore suspension (**[B]** and **[D]**).
(E) to **(H)** Production of H_2O_2 in wild-type (**[E]** and **[F]**) and *bos1* (**[G]** and **[H]**) plants treated with buffer (**[E]** and **[G]**) or Botrytis (**[F]** and **[H]**).
ROI production was assayed using nitroblue tetrazolium for O_2^- and 3,3′-diaminobenzidine for H_2O_2. O_2^- is indicated by the blue spots, and the reddish-brown coloration indicates the polymerization of 3,3′-diaminobenzidine at the site of H_2O_2 production.

to the disease resistance pathway mediated by JA and ethylene. To determine whether pathogen susceptibility in *bos1* was associated with the altered expression of either of these genes, we analyzed the expression of the genes after Botrytis infection (Figure 8B). In an RNA gel blot analysis, Botrytis infection induced *PDF1-2* expression in both wild-type and *bos1* plants. This finding suggests that some essential components of a resistance response to Botrytis infection can be mediated independently of *PDF1-2*. The expression of *PR-1* was induced more strongly in the *bos1* background than in the wild-type plants (Figure 8B).

DISCUSSION

The data presented here clearly demonstrate a role for *BOS1* in resistance to Botrytis. A mutation in the *BOS1* gene resulted in increased growth of the pathogen in infected *bos1* plants, expression of the *BOS1* gene was induced by Botrytis infection, and a wild-type copy of the *BOS1* gene restored *bos1* plants to wild-type levels of Botrytis resistance. In addition, the induction of *BOS1* expression was blocked in the *coi1* mutant. Plants with the *coi1* mutation were insensitive to JA and also had increased sensitivity to Botrytis. These findings suggest that

BOS1 may play a role in the defense response regulated by JA. It also is possible that the Botrytis susceptibility observed in *coi1* plants may be attributable at least in part to their lack of *BOS1* expression. The *PDF1.2* gene encodes a defensin protein and has been implicated in JA-mediated defense responses. The expression of *PDF1.2* was dependent on *COI1* but was unaffected in *bos1*, indicating that there are parts of the JA-mediated defense response that are BOS1 dependent and parts that are BOS1 independent. This finding also suggests that *PDF1.2* expression may not be a good molecular marker for Botrytis resistance. *PDF1-2* was induced normally by Botrytis infection in *bos1* plants.

Consistent with our findings in *bos1*, Ferrari et al. (2003) also reported that *PR-1* and *PDF1-2* expression levels do not correlate with susceptibility to Botrytis infection. By contrast, the *Alternaria*-susceptible mutant *esa1* of Arabidopsis, which also shows enhanced susceptibility to Botrytis, has delayed induction of *PDF1-2* after pathogen and oxidative stress treatments (Tierens et al., 2002). Similar to *bos1*, *esa1* plants induce *PR-1* expression more strongly than do wild-type plants. However, in both cases, the greater *PR-1* expression can be attributed to the more abundant proliferation of the pathogen in the mutants compared with the wild-type plants.

The *bos1* mutant plants also were more susceptible to a second necrotrophic pathogen, *A. brassicicola*, and to certain abiotic stresses. These include drought, salt, and oxidative stress. All aspects of the *bos1* phenotype were restored to wild-type levels in *bos1* plants expressing the wild-type *BOS1* gene, indicating that all aspects of the *bos1* phenotype were caused by the mutation in the *BOS1* gene.

One common factor in the various aspects of the *bos1* phenotype may be ROI. ROI have been implicated in signaling in response to both pathogens and abiotic stresses (Lamb and Dixon, 1997; Bowler and Fluhr, 2000; Mittler, 2002). Drought, salt, and cold stress all induce the accumulation of ROI such as O_2^-, H_2O_2, and hydroxyl radicals (Hasegawa et al., 2000).

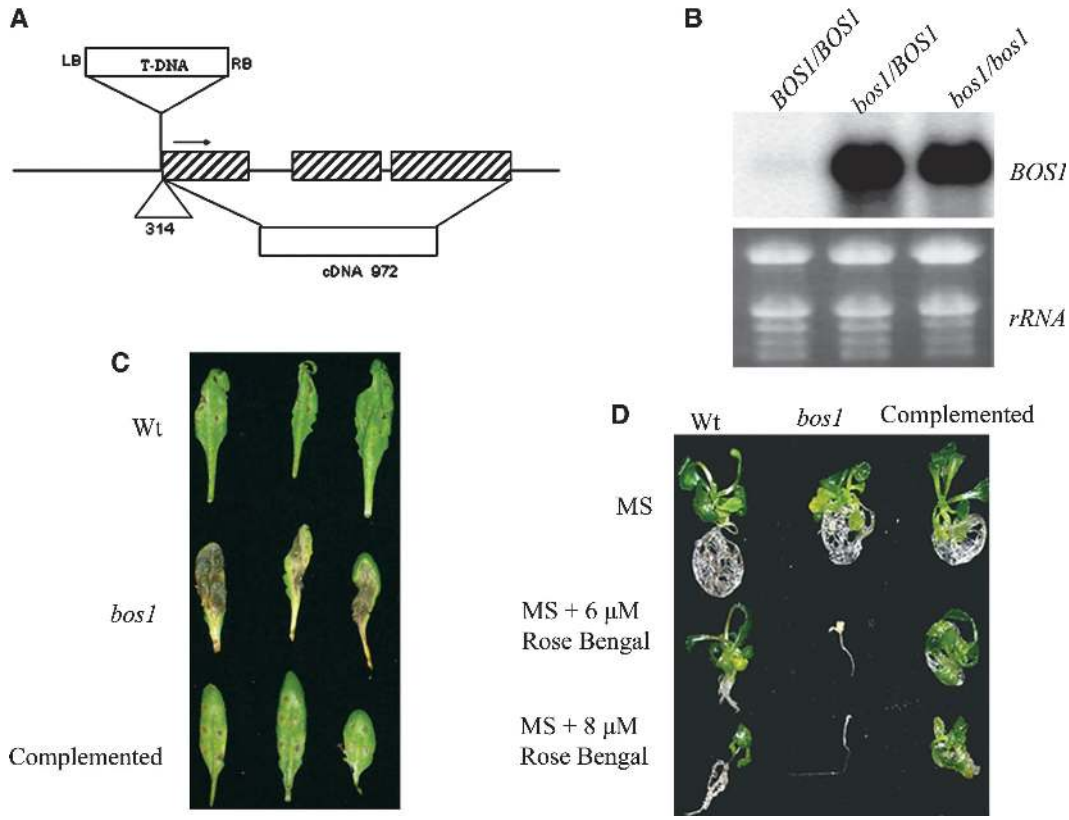

Figure 6. Genomic Structure of the *bos1* Mutant Allele, Expression of the BOS1 Gene, and Complementation of the *bos1* Phenotype.

(A) Structure of the *BOS1* gene and position of the T-DNA insertion in the *bos1* mutant allele. Exons are shown as hatched boxes. The arrow shows the predicted direction of transcription. LB, left border of the T-DNA; RB, right border of the T-DNA.

(B) *BOS1* expression in homozygous wild-type (*BOS1*/*BOS1*), heterozygous mutant (*BOS1*/*bos1*), and homozygous mutant (*bos1*/*bos1*) plants. mRNA was extracted from leaf tissue from 3-week-old plants and separated on a formaldehyde agarose gel. An RNA gel blot from the gel was hybridized to a probe representing the 3′ part of the *BOS1* gene.

(C) and **(D)** The genomic clone containing the *BOS1* gene rescues Botrytis susceptibility **(C)** and oxidative stress sensitivity **(D)** of *bos1* plants. Transgenic plants were generated by transforming *bos1* plants with constructs containing the *BOS1* genomic clone, including the 1.5-kb promoter region. The Botrytis assay was performed by drop inoculation (see Methods). The oxidative stress assay was performed using rose bengal. Plants were photographed 3 weeks after transfer to medium containing rose bengal. Wt, wild type.

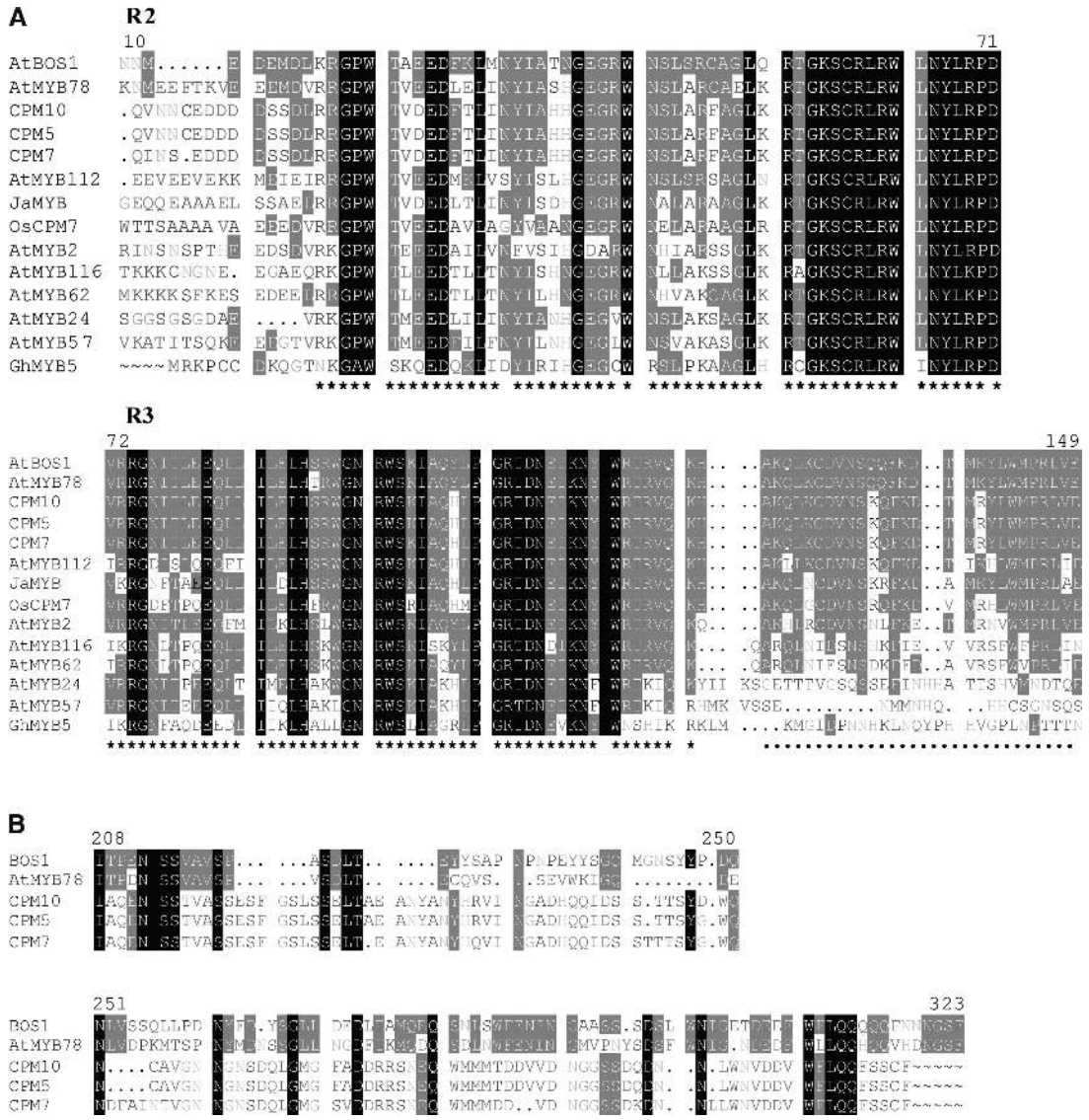

Figure 7. Sequence Comparison between BOS1 and Other Plant MYB Proteins.

(A) Alignment showing the amino acid sequences of the R2 and R3 repeats and flanking regions. The repeats are indicated with asterisks. The conserved region after the R3 repeat is shown with dots.

(B) Sequence alignment showing C-terminal amino acid sequences.

Sequences shown are from Arabidopsis (AtBOS1, AtMYB78, AtMYB112, AtMYB62, AtMYB116, AtMYB2, AtMYB57, and AtMYB24), *Craterostigma plantagineum* (CPM10, CPM7, and CPM5), *Oryza sativa* (JaMYB and OsCPM7), and *Gossypium hirsutum* (GhMYB5). Numbers above the alignments correspond to amino acid positions in BOS1. Black shading indicates amino acids conserved in all entries, and gray shading indicates amino acids identical to the BOS1 sequence.

These ROI may be signals that induce scavengers of reactive oxygen and other protective mechanisms, as well as damaging agents that contribute to stress injury in plants (Prasad et al., 1994). ROI may activate downstream signal cascades via Ca^{2+} and also can be sensed directly by key signaling proteins, such as a Tyr phosphatase, through the oxidation of conserved Cys residues (Rhee et al., 2003).

Although ROI have been implicated in the response to biotrophic as well as necrotrophic pathogens, of the pathogens tested on *bos1*, enhanced pathogen growth was observed only during infection by necrotrophic pathogens. The *bos1* plants showed enhanced disease symptoms but retained their ability to limit biotrophic pathogen growth. These data suggest a key role for *BOS1* in resistance to necrotrophic infection. However, ROI may play significantly different roles in the responses to these two classes of pathogens. ROI have been implicated in cell death in plant defense responses, and host cell death may play an important role in resistance to biotrophic pathogens (Lamb and Dixon, 1997). With necrotrophic pathogens, however, cell death may promote pathogen growth (Govrin and

Levine, 2000). The increased sensitivity to ROI in *bos1* is consistent with its increased susceptibility to necrotrophic pathogens.

Consistent with previous findings (Tiedemann, 1997), the production of ROI was increased during Botrytis infection. Although ROI production was detectable as early as 1 day after inoculation, significant differences between inoculated *bos1* and wild-type plants became apparent only as disease symptoms began to appear at ~2 days after inoculation or later. The amount of ROI increased as disease progressed. This difference may be attributable to the increased fungal growth and tissue maceration in mutant plants. This finding is consistent with the increased ROI formation in Botrytis-infected tissues that correlates with fungal biomass and disease severity (Edlich et al., 1989).

The *esa1* mutant of Arabidopsis (Tierens et al., 2002) was identified from a genetic screen for enhanced susceptibility to *A. brassicicola*. The nature of the *ESA1* gene was not determined. Similar to the *bos1* mutation, *esa1* affects resistance to necrotrophic pathogens. In contrast to *bos1*, which affects disease symptoms during biotrophic infection, the *esa1* mutation appears to have no effect on responses to biotrophic pathogens. The effect of the *esa1* mutation on abiotic stress responses, including sensitivity to ROI, was not reported. However, attenuated camalexin accumulation and *PDF1-2* induction in response to ROI-generating compounds were suggested to be the causes of the disease susceptibility in *esa1*. Based on these data, it was suggested that *ESA1* is involved in the transduction of signals generated by ROI. The normal induction of *PDF1-2* in *bos1* during Botrytis infection suggests that, although both *esa1* and *bos1* mutations affect functions related to ROI signaling, the two may regulate different effector molecules. The sensitivity of *bos1* to two compounds that generate ROI and biotic and abiotic stresses suggests a role for BOS1 in mediating responses to signals from both biotic and abiotic sources.

Table 1. Putative *cis*-Acting Elements in the *BOS1* Promoter

cis Element[a]	Position[b] (strand)	Sequence[c]
MYB binding sites		
MYB	1247 (+)	TAACTG
MYC	810 (+), 94 (+)	CACATG
MYBGAHV	1476 (−), 774 (+)	TAACAAA
MYBST1	363 (+)	GGATA
Pathogen/elicitor response		
W box	684 (+), 638 (+)	TTTGACT
ELRECORE	1336 (−)	TTGACC
Abscisic acid responsiveness		
ATHB6CORE	307 (−)	CAATTATTA
ABRE	917 (+)	AACGTG
HDZIP2HB2	584 (+)	TAATCATTA
Coupling element	1100 (+), 915 (+), 350 (+)	CACC
Wound induction	595 (+)	TAATTTCAA

[a] Details are described in the text and in the references cited therein.
[b] Positions of the *cis* elements are with respect to the translation start sites.
[c] Sequences are indicated from 5′ to 3′.

The observed responses of *bos1* to multiple biotic and abiotic stresses suggest the presence of similar intermediate signaling molecules that are used in diverse stresses. Intracellular networks rather than linear pathways may operate in such cases. A limited number of signaling intermediates may interact in a combinatorial manner to allow specific cellular responses to numerous signals. How physiological specificity can be generated and how a single factor such as *BOS1* mediates responses to multiple environmental cues are important questions that need to be addressed. BOS1 may regulate cellular and molecular mechanisms that provide cross-tolerance to biotic and abiotic stresses. Similar *trans*-acting factors involved in both biotic and abiotic stress signal transduction pathways have been reported previously (Park et al., 2001). Overexpression of the *TOBACCO STRESS-INDUCED GENE1*, an EREPB/AP2-type transcription factor gene, resulted in improved tolerance to salt and pathogens. Similarly, constitutive overexpression of the *ETHYLENE RESPONSE FACTOR1* gene in Arabidopsis resulted in enhanced resistance to several necrotrophic pathogens, including Botrytis (Berrocal-Lobo et al., 2002).

Plant resistance to Botrytis is determined by multiple host and environmental factors. In contrast to responses to biotrophic pathogens, which are governed by gene-for-gene interactions, resistance to Botrytis likely requires the contributions of multiple loci for full resistance. Plant responses to necrotrophic infection may share common cellular and molecular mechanisms with plant responses to abiotic stresses, wounding, cell death, and senescence. The plant hormones SA, JA, ethylene, and abscisic acid all have been implicated in these various plant responses (Rao et al., 2000, 2002; Borsani et al., 2001; Turner et al., 2002; Xiong et al., 2002).

The role of SA in plant defense responses to Botrytis infection is not clear. Increased levels of SA and constitutive SAR result in plant susceptibility to Botrytis. Mutants impaired in SA signaling, such as *npr1* and *pad4*, have no effect on resistance (Ferrari et al., 2003). However, *nahG* plants that do not accumulate SA and inhibition of Phe ammonia lyase–dependent biosynthesis of SA cause enhanced disease symptoms after Botrytis infection (Govrin and Levine, 2002; Ferrari et al., 2003), suggesting that SA may be required for a local response to Botrytis at the point of infection. This is in contrast to mutations that affect JA and ethylene response pathways that are required for both local and systemic resistance against Botrytis (Ferrari et al., 2003).

BOS1 is a member of a large class of genes that contain one or more MYB domains (Stracke et al., 2001). MYB proteins are categorized further into subfamilies depending on the number of conserved repeats of the MYB domain they contain. MYB proteins from animals generally contain three repeats (R1, R2, and R3). In plants, a distinct subfamily is characterized by two related helix-turn-helix motifs called the R2 and R3 repeats (i.e., *R2R3MYB*), which may regulate plant-specific processes (Stracke et al., 2001). Approximately 135 genes encode R2R3MYB transcription factor proteins in the Arabidopsis genome. The functions of most of these R2R3MYBs have not been determined, although they have been implicated in a range of functions, including the regulation of cell death (Daniel et al., 1999; Lee et al., 2001; Vailleau et al., 2002), the phenyl-

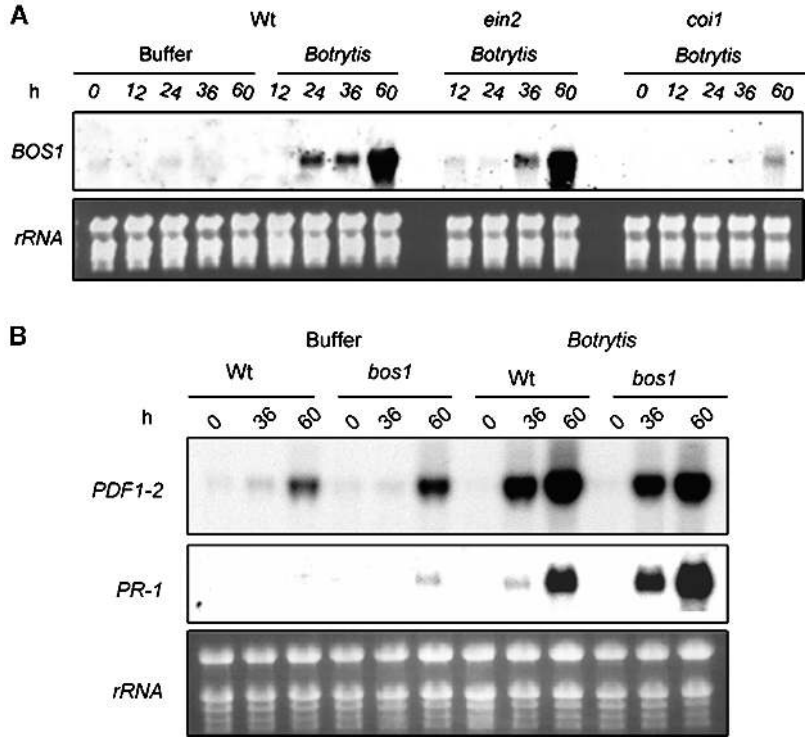

Figure 8. Expression of *BOS1* and Defense Response Marker Genes.

RNA gel blot analysis of *BOS1* **(A)** and *PDF1-2* and *PR-1* **(B)** expression in Botrytis-infected tissues. Wild-type (Wt), *ein2*, *coi1*, and *bos1* plants were inoculated by spraying Botrytis at 10^5 spores/mL or buffer, and tissue was frozen for RNA extraction. Twenty micrograms of total RNA was loaded per lane. Numbers indicate hours after inoculation. The bottom gels show total RNA as a loading control. The experiment was repeated two times with similar results.

propanoid pathway (Borevitz et al., 2000), Trp biosynthesis (Bender and Fink, 1998), and drought stress tolerance (Urao et al., 1993). They may function as activators or repressors of transcription (Jin et al., 2000).

In database searches, *BOS1* is found to be most similar to a small family of *MYB* genes from the resurrection plant *C. plantagineum*. These genes are induced by abscisic acid and drought stress; therefore, they may have some overlap in function with *BOS1*. The most closely related gene in the Arabidopsis genome is *AtMYB78*. These genes share overall identity of 74% at the amino acid level and 93% identity in the MYB domains, although based on the mutant phenotype of the *bos1* plants, either these two genes perform different functions or they have nonoverlapping expression patterns.

Consistent with a role for BOS1 in response to stresses, a number of consensus stress response *cis*-acting sequences were found in the *BOS1* promoter region (Table 1). These include abscisic acid and dehydration response elements as well as W boxes, which have been implicated in the regulation of pathogen response genes (Rushton et al., 1996; Shen et al., 1996; Maleck et al., 2000). The physiological significance of the *cis*-regulatory sequences identified in the *BOS1* promoter region remains to be determined experimentally, but their presence suggests the possible regulation of *BOS1* expression by various environmental cues and plant growth regulators.

Understanding the genetic control of pathogen and abiotic stress responses has a bearing on rational crop breeding. Plants could be bred to resist specific or nonspecific stress conditions based on the knowledge of the molecular regulation of physiological responses. The function of the *BOS1* gene could provide valuable insights for generating crop varieties with increased tolerance to necrotrophic pathogens and abiotic stresses.

METHODS

Plant Maintenance

The *Arabidopsis thaliana* T-DNA insertion collection used in this screen has been described (McElver et al., 2001). The T-DNA lines generated in the Columbia ecotype were chosen for screening. Plants were grown on soil (Super Fine Germinating Mix; Conrad Fafard, Agawam, MA) under fluorescent light (200 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 22 $\pm$ 4°C with 60% RH and a 12-h-light/12-h-dark cycle. Plants were watered by subirrigation.

Fungal Culture

Botrytis cinerea was grown on 2XV8 agar (36% V8 juice, 0.2% $CaCO_3$, and 2% Bacto-agar) at 20°C. Fungal cultures were initiated by transferring pieces of agar containing mycelium to fresh 2XV8 agar and incubated at 20 to 25°C. Conidia were collected from 10-day-old cultures by

placing agar slices containing fungal material in 1% Sabouraud Maltose Broth buffer (Difco, Sparks, MD) and vortexing to release the spores. The suspension was passed through Miracloth to separate the fungal material from pieces of agar. *Alternaria brassicicola* was grown on potato dextrose agar, and spores were collected by the same procedure but resuspended in distilled water.

Disease Assay

To infect plants, the fungal spore density was adjusted to 10^5 spores/mL in Sabouraud Maltose Broth buffer for Botrytis or in sterile water for *A. brassicicola* and sprayed using a Preval sprayer (Valve Corp., Yonkers, NY). Single leaf inoculations were performed by placing 2- to 3-μL droplets of spore suspension of Botrytis or 3- to 4-μL droplets of *A. brassicicola* on individual leaves of soil-grown plants. After inoculation, plants were kept under a transparent cover to maintain high humidity and transferred to a growth chamber with 21°C day and 18°C night temperatures for Botrytis and 24°C temperature with a 12-h-light/12-h-dark cycle for *A. brassicicola*. The light intensity was ~200 $\mu E \cdot m^{-2} \cdot s^{-1}$. To test plants for resistance responses to *Peronospora parasitica*, 3-week-old plants were sprayed with a conidial suspension (10^5 spores/mL) and kept under high RH.

Bacterial Pathogen Assay

Fully expanded leaves of 4-week-old plants were infiltrated with suspensions ($OD_{600} = 0.001$ in 10 mM $MgCl_2$) of *Pseudomonas syringae* pv *tomato* strain DC3000 or strain DC3000 harboring a plasmid carrying the *avrRPM1* gene. Inoculation was performed by pressing a 1-mL syringe without a needle against the abaxial side of the leaves and forcing the bacterial suspension through the stomata into the intercellular spaces. To determine bacterial growth, infected leaves were collected at 0, 2, and 4 days after inoculation. At each time point, 10 leaves were collected from each wild-type and *bos1* plant, leaf discs of the same size were made using a hole puncher, and bacterial titers from those leaves were determined.

Abiotic Stress Assays

Seeds were surface-sterilized by treating them sequentially in 70% ethanol for 2 min and 30% Clorox solution containing 0.01% Tween for 10 min and rinsing four times in sterile water. Seeds were plated on medium containing the inorganic salts of MS medium (Murashige and Skoog, 1962), 3% sucrose, and 0.8% (w/v) purified agar. Plates were kept at 4°C for 48 h to synchronize germination, transferred to growth chambers with fluorescent lights, and maintained under the same environmental conditions described above for plants grown in pots. Plates were incubated vertically to facilitate the transfer of germinated seedlings. For abiotic stress assays, seedlings were transferred individually to multiple-well plates (BD Biosciences, Franklin Lakes, NJ) with each well containing 0.5 mL of MS liquid medium supplemented with NaCl (0, 50, 100, 150, 200, and 250 mM), rose bengal (0, 2, 4, 6, and 8 μM) (Sigma), or mannitol (0.1, 0.2, 0.4, 0.8, and 1.0 M). Data on abiotic stress sensitivity were scored after 2 weeks in culture. The cold stress assay was performed by keeping plants at 4°C from 2 to 7 days and observing plant recovery under standard growth conditions. A detached leaf assay was used to determine the sensitivity of plants to photooxidative damage induced by methyl viologen (paraquat). Leaves from soil-grown plants were placed on half-strength solidified MS medium without sucrose, and a single drop of 2 μL was placed on each leaf from methyl viologen (Sigma) solutions containing 0, 10, 100, and 500 μM.

Germination on Polyethylene Glycol

Seeds were placed on 9-cm filter paper saturated with 1.5 mL of water or polyethylene glycol 8000 solution from 10 to 20% concentration (Sigma). Seeds were kept at 4°C for 48 h to synchronize germination. Seedling establishment was scored as the appearance of green seedlings after 5 days. Each treatment was performed in triplicate with ~50 seeds per treatment. The experiment was repeated three times.

Thermal Asymmetric Interlaced PCR

Plant genomic sequences adjacent to the T-DNA inserts were recovered by thermal asymmetric interlaced PCR (TAIL-PCR) (Liu et al., 1995) with minor modifications (McElver et al., 2001). Primers specific to the T-DNA borders used were as follows: LB1 for 1° TAIL (5′-GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC-3′), LB2 for 2° TAIL (5′-GCTTCCTATTATATCTTCCCAAATTACCAATACA-3′), and LB3 for 3° TAIL (5′-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′). The following degenerate primers were used: 5′-NGTCGASWGANAWGAA-3′, 5′-TGWGNAGSANCASAGA-3′, 5′-GWGNAGWANCAWAGG-3′, and 5′-WGTGNAGWANCANAGA-3′.

Cosegregation between the *bos1* Phenotype and the T-DNA Insert

Plants from putative segregating T-DNA populations were inoculated with Botrytis by depositing 3 μL of the spore suspension on approximately four leaves per plant. Botrytis resistance was recorded for each plant at 5 days after inoculation. DNA extracted from these plants was subjected to PCR amplification. The T-DNA LB3 primer (5′-TAGCATCTGAATTTCATAACC-3′) and the *BOS1* primer (5′-GTCTTAACCTTCACGCACATAAAA-3′) when used in combination generate a PCR product of ~500 bp that is used as a marker for the T-DNA insertion that causes the *bos1* mutation. The segregation of the disease phenotype was compared with the PCR-amplified band.

Cloning of the cDNA

The genomic sequence from TAIL-PCR of the *bos1* mutant was used as the basis for cloning the wild-type *BOS1* cDNA and genomic clones. The 5′ end of the *BOS1* cDNA was determined by rapid amplification of cDNA ends (RACE) according to the SMART protocol (BD Biosciences). Briefly, poly(A) RNA was isolated using the PolyATract mRNA isolation system (Promega, Madison, WI) according to the manufacturer's instructions and reverse-transcribed using oligo(dT) primers provided with the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). This was used as a template to amplify *BOS1* cDNA using the sense and antisense gene-specific primers 3′RACE Primer 1 (5′-GACGTCCGCCGTGGAAACATTACACTT-3′), 3′RACE Primer 2 (5′-GGAAGAACGGACAACGAGATCAAGAAC-3′), 5′RACE Primer 1 (5′-TAGTACTCCGTTAAGTCTGACGCCGGAGA-3′), and 5′RACE Primer 2 (5′-ATGCAAGATGACGTGCCGGCTGAT-3′). Once the 5′ and 3′ ends of the *BOS1* cDNA were determined by RACE, gene-specific primers were designed to amplify the entire cDNA from the start to stop codons of the cDNA using Advantage 2 polymerase mix HF2 polymerase (BD Biosciences). The *BOS1* genomic region was amplified using a high-fidelity PCR system (Roche Diagnostics, Mannheim, Germany) using gene-specific primers designed to include the 1.5-kb region upstream of the start codon and a reverse primer designed to include the stop codon. *BOS1* genomic forward primer (5′-TGCACCAAACCAAGTAACAAGAGG-3′) and reverse primer (5′-CTAGCTAGCTCAGAAGCTACCATTATTGTT-3′) were used.

RNA Gel Blot Analysis

RNA was extracted from harvested leaf tissue frozen in liquid nitrogen using a hot phenol/chloroform method followed by lithium chloride precipitation (Verwoerd et al., 1989). RNA was separated on formaldehyde agarose gels. The gels then were blotted onto Hybond N^+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Probes were

labeled with ^{32}P by random priming using a commercial kit (rediPrime II; Amersham Pharmacia Biotech). Hybridization of probe and subsequent washings were performed as described (Church and Gilbert, 1984). The Botrytis *β-tubulin* gene (Benito et al., 1998) was amplified from the Botrytis genomic DNA and used as a template for random prime labeling. The primers 5′-ACTCATATGTTGGAGATGAAGCGCA-3′ and 5′-AATGTTACCATACAAATCCTTACGGACA-3′ were used to amplify the *β-tubulin* gene fragment for random prime labeling.

Detection of Reactive Oxygen Species

Staining for the presence of H_2O_2 via the 3,3′-diaminobenzidine (DAB) uptake method was performed as described (Thordal-Christensen et al., 1997). Leaves were detached and placed in 1 mg/mL DAB-HCl, pH 3.8 (D-8001; Sigma). In the presence of endogenous peroxidase activity, DAB generates a reddish-brown DAB polymer that could be detected at the site of H_2O_2 formation. The solution for the in situ detection of superoxide (O_2^-) contained 1 mg/mL nitroblue tetrazolium in 10 mM NaN_3 and 10 mM phosphate buffer, pH 7.8 (Doke, 1983). Plant samples were detached and immersed for 30 min in 5 mL of nitroblue tetrazolium staining solution to detect the presence of O_2^-. In both cases, after staining, leaves were cleared in 96% boiling ethanol and observed with a microscope.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Tesfaye Mengiste, mengiste@purdue.edu.

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