Crosstalk between biotic and abiotic stress responses in tomato is mediated by the *AIM1* transcription factor

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**Summary**

Plants deploy diverse molecular and cellular mechanisms to survive in stressful environments. The tomato (*Solanum lycopersicum*) abscisic acid-induced *myb1* (*SlAIM1*) gene encoding an R2R3MYB transcription factor is induced by pathogens, plant hormones, salinity and oxidative stress, suggesting a function in pathogen and abiotic stress responses. Tomato *SlAIM1* RNA interference (RNAi) plants with reduced *SlAIM1* gene expression show an increased susceptibility to the necrotrophic fungus *Botrytis cinerea*, and increased sensitivity to salt and oxidative stress. Ectopic expression of *SlAIM1* is sufficient for tolerance to high salinity and oxidative stress. These responses correlate with reduced sensitivity to abscisic acid (ABA) in the *SlAIM1* RNAi, but increased sensitivity in the overexpression plants, suggesting *SlAIM1*-mediated ABA responses are required to integrate tomato responses to biotic and abiotic stresses. Interestingly, when exposed to high root-zone salinity levels, *SlAIM1* RNAi plants accumulate more Na⁺, whereas the overexpression lines accumulate less Na⁺ relative to wild-type plants, suggesting that *SlAIM1* regulates ion fluxes. Transmembrane ion flux is a hallmark of early responses to abiotic stress and pathogen infection preceding hypersensitive cell death and necrosis. Misregulation of ion fluxes can result in impaired plant tolerance to necrotrophic infection or abiotic stress. Our data reveal a previously uncharacterized connection between ABA, Na⁺ homeostasis, oxidative stress and pathogen response, and shed light on the genetic control of crosstalk between plant responses to pathogens and abiotic stress. Together, our data suggest *SlAIM1* integrates plant responses to pathogens and abiotic stresses by modulating responses to ABA.

**Keywords**: tomato, *Solanum lycopersicum*, ABA-induced R2R3MYB, *Botrytis cinerea*, salt stress.

**Introduction**

Plant responses to biotic and abiotic stresses involve a network of molecular mechanisms that vary depending on the nature of the pathogen or the stress signal. Plant responses to necrotrophic fungi are complex, involving diverse genetic and molecular mechanisms, and vary depending on the primary mechanism of the pathogen virulence (Wolpert *et al.*, 2002; Glazebrook, 2005). Broad host necrotrophic fungi produce toxins, cell-wall degrading enzymes (CWDEs) and reactive oxygen intermediates (ROIs) that determine the severity of disease (Edlich *et al.*, 1989; Tiedemann, 1997; Muckenschnabel *et al.*, 2002). These disease factors cause electrolyte leakage, changes in ion fluxes, cell death and other stress responses, underlining the similarities in plant responses to microbial necrotrophy and abiotic stresses.

Exposure to abiotic stress, in some cases, enhances resistance to pathogens indicative of crosstalk between biotic and abiotic stress signaling (Bowler and Fluhr, 2000). Induced resistance common to both biotic and abiotic stresses has also been documented (Zimmerli *et al.*, 2000; Jakab *et al.*, 2005; Ton *et al.*, 2005).

Distinct response pathways that regulate plant responses to diverse environmental signals have been extensively described. However, recent studies suggest a greater coordination of plant responses to pathogens and abiotic stresses, including the expression of overlapping sets of genes in response to infection and abiotic stresses (Cheong *et al.*, 2002; AbuQamar *et al.*, 2006; Fujita *et al.*, 2006). The plant hormones ethylene (ET), salicylate (SA), jasmonate
(JA) and abscisic acid (ABA) act synergistically or antagonistically to regulate plant responses to pathogens and abiotic stress factors. In addition, ROIs and secondary messengers, such as calcium, modulate plant responses to diverse environmental signals (Bowler and Fluhr, 2000). Regulatory proteins, including transcription factors, protein kinases and diverse post-translational mechanisms, regulate responses to plant hormones and ROIs, both of which are central to plant responses to biotic and abiotic stresses (Liu and Zhang, 2004; Rentel et al., 2004). The accumulation of ROIs precedes cell death and is associated with resistance to biotrophic pathogens (Lamb and Dixon, 1997). Cell death promotes plant susceptibility to some necrotrophic fungi (Govrin and Levine, 2000). ROIs also mediate abiotic stress-induced cell death (Torres et al., 2002), and Arabidopsis NADPH oxidases, primary sources of ROIs, control responses to the plant stress hormone ABA (Kwak et al., 2003). Thus, plant responses to environmental signals are regulated by a network of intracellular pathways.

Abscissic acid regulates the plant response to drought, low temperature and osmotic stress. Recently, ABA has emerged as a positive or negative regulator of disease resistance, depending on the nature of the host–pathogen interaction (Anderson et al., 2004; Lorenzo et al., 2004; Mauch-Mani and Mauch, 2005). ABA deficiency in tomato and impaired ABA responses in Arabidopsis result in increased resistance to Botrytis cinerea, and other necrotrophic pathogens, as a result of the reduced ABA signaling but increased JA- or ET-responsive gene expression (Audeaert et al., 2002). The enhanced response to ABA3 (ERA3) gene is allelic to EIN2 (Ghassemian et al., 2000), which is required for resistance to some necrotrophic fungi (Thomma et al., 1999). In addition, resistance to the necrotrophic oomycete Pythium irregulare and to the bacterial necrotroph Ralstonia solanacearum requires ABA synthesis and responses indicating a positive role for ABA in disease resistance (Adie et al., 2007b; Hernandez-Blanco et al., 2007). ABA also controls stomatal closure during pathogen invasion, thereby regulating microbially accessible to plant tissues (Melotto et al., 2006). ABA regulation of stomatal closure is dependent on ROIs (Kwak et al., 2003). Furthermore, abiotic and biotic stress responses often converge into the mitogen-activated protein kinase (MAPK) signaling in Arabidopsis. Arabidopsis MPK3 and MPK4 function in abiotic stress and basal defense responses (Nuhse et al., 2000; Asai et al., 2002; Jonak et al., 2002; Rentel et al., 2004; Veronese et al., 2006). Arabidopsis OXI1 regulates the activation of MPK3 and MPK6 by ROIs, and is also required for pathogen resistance (Rentel et al., 2004). Thus, pathogen and stress response signaling share significant regulatory mechanisms, with complex interactions between responses to plant hormones, pathogens, abiotic stresses and ROIs.

The genetic factors and molecular mechanisms that mediate biotic and abiotic stress responses of tomato (Solanum lycopersicum) are not known. Here, the role of tomato abscisic acid-induced MYB1 (AIM1) transcription factor (SIAIM1) in pathogen and abiotic stress responses is described. Tomato AIM1 RNA interference (RNAi) plants, with a reduced expression of SIAIM1, show an increased susceptibility to B. cinerea, a sensitivity to salt stress, but a reduced sensitivity to ABA. Overexpression of SIAIM1 enhanced ABA sensitivity, and the tolerance to salt and oxidative stress, but did not improve resistance to B. cinerea. These phenotypes suggest that B. cinerea resistance is a complex trait in tomato, consistent with the multiplicity of the B. cinerea disease factors. Interestingly, elemental profiling of leaf tissues reveals that SIAIM1 RNAi plants exposed to high root-zone salinity levels accumulate more Na+, whereas the overexpression line has reduced concentrations. Thus, SIAIM1 may control an Na+ removal or exclusion mechanism. Our data suggest that SIAIM1 mediates responses to biotic and abiotic stresses, and links plant responses to plant hormones, ROIs, microbial infection and abiotic stress factors.

Results

Identification and characterization of the tomato SIAIM1 gene

The tomato SIAIM1 transcription factor was cloned based on sequence homology to the MYB DNA binding domains of Arabidopsis Botrytis-susceptible 1 (BOST1), BOST1 encodes an R2R3MYB transcription factor required for resistance to necrotrophic pathogens, and for tolerance to some abiotic stresses (Mengiste et al., 2003). SIAIM1-specific gene expression is undetectable in leaf tissues grown under normal conditions, but is induced by B. cinerea in tomato leaves that are indicative of a disease resistance function (Figure 1a). Expression was detectable by 24 h after inoculation, and increased further after 48–72 h. SIAIM1 was also induced by the bacterial pathogen Pseudomonas syringae, with a significant increase starting at 12 h after inoculation (Figure 1b). SA and the natural precursor of ethylene synthesis, 1-aminocyclopropane-1-carboxylic acid (ACC), failed to induce SIAIM1. ABA and NaCl induced SIAIM1 significantly, whereas paraquat, a herbicide that causes oxidative stress, and MeJA only slightly induced SIAIM1 (Figure 1c). Infiltration with buffer alone did not induce the SIAIM1 transcript and, thus, the induction is specific to pathogens, a subset of plant hormones and stress factors.

SIAIM1 is predicted to be a member of a large gene family in tomato, based on the available knowledge from other genomes. A DNA blot of tomato genomic DNA digested with different restriction enzymes and hybridized to a gene-specific region of SIAIM1, shows a single hybridizing band consistent with a single-copy gene in the tomato genome (Figure 1d). In addition, the full-length SIAIM1 was transla-
tionally fused with green fluorescent protein (GFP), and the chimeric protein was transiently expressed in *Nicotiana benthamiana* leaf tissues through *Agrobacterium* infiltration, to determine the subcellular localization of SlAIM1. The SlAIM1-GFP fusion protein only localized to the nucleus when it was expressed in *N. benthamiana* epidermal cells, consistent with its predicted DNA-binding functions, whereas cells expressing GFP alone (control, top row) exhibited a diffuse signal in both the cytosol and the membrane (Figure 1e). Thus, the data suggest that SlAIM1 is a nuclear protein and that the gene is regulated by plant hormones, pathogens and oxidative stress, indicative of a function in stress response signaling.

Sequence and phylogenetic analyses of tomato AIM1 and its relationship with other R2R3 MYB transcription factors

The SlAIM1 cDNA contains a 732-bp-long open reading frame (ORF) encoding a protein of 244 amino acids. The predicted SlAIM1 protein contains the R2R3MYB DNA binding domains close to the N-terminal sequence (positions 19–71 aa and 72–121 aa), and a C-terminal region of unknown function (Figure S1). SlAIM1 also contains the SANT domain, a putative DNA binding module recently found in diverse proteins with functions in chromatin remodeling (Zhang *et al.*, 2006). The SANT domain falls within the predicted R2 MYB repeat, and its functional significance in plants is not clear. SlAIM1 shares high sequence identity to Arabidopsis MYB78 (At5g49620), BOS1 (MYB108, At3g06490), MYB112 (At1g48000), MYB2 (At2G47190) and two dehydration-induced MYB-related proteins, CPM5 and CPM10, from the resurrection plant *Craterostigma plantagineum* (Iturriaga *et al.*, 1996) (Figure S1a,b). The R2 and R3 DNA binding domains and the short segment following the R3 domain show high sequence conservation, with 62–84% identity shared between SlAIM1 and the related MYB proteins. SlAIM1 shows the highest sequence relatedness to Arabidopsis MYB78 (84% identity), followed by CPM10 (83%) and BOS1 (82%) in the N-terminal conserved region covering the MYB domains. The SlAIM1 protein carries multiple deletions compared with the closely related R2R3 MYB proteins. SlAIM1 encodes the smallest protein (29.4 kDa), whereas AtBOS1 encodes the largest protein (37 kDa), of the closely related MYBs (Figure S1b) (Stracke *et al.*, 2001). The C-terminal sequence of SlAIM1 has no significant sequence identity to BOS1 and the other related MYBs. The Arabidopsis BOS1 autoactivates in yeast two-hybrid assays, with the activation domain of BOS1 mapped to the C-terminal 50 amino acid sequence (H. Luo, F. Song, K. Laluk, T. Mengiste, unpublished data). SlAIM1 shows no autoactivation (data not shown), suggesting that its transcriptional regulatory mechanism is different from BOS1, and that the activation domains of BOS1 and SlAIM1 are divergent.

The Arabidopsis genome contains ~135 R2R3MYB genes, with 22 subfamilies defined based on the conservation of the MYB DNA binding repeats, and a short sequence that is
C-terminal to the R3 domain (Stracke et al., 2001). One subfamily representing a phylogenetic clade contains Arabidopsis BOS1, MYB78, MYB112, MYB116, MYB2 and MYB62 (Stracke et al., 2001). Phylogenetic analysis places SlAIM1 in this clade (Figure S1c). Among the genes in this clade, Arabidopsis T-DNA insertion alleles in MYB78 and MYB112 show no altered responses to B. cinerea and Alternaria brassicicola, whereas bos1 shows increased susceptibility (Mengiste et al., 2003) (Figure S2). The disease resistance function of MYB112, MYB62 and MYB2 are not determined. MYB2 regulates Arabidopsis responses to salinity, ABA and drought (Abe et al., 2003), and shares 67% identity to SlAIM1 in the R2 and R3 MYB domains. CPM5 and CPM10 genes are induced by drought stress and ABA in C. plantagineum tissues, and have been associated with plant stress responses (Iturriaga et al., 1996). Arabidopsis BOS1, MYB2 and tomato AIM1 genes are all induced by an overlapping set of factors, including ABA, NaCl, B. cinerea and P. syringae (Table S1). Thus, SlAIM1 may be functionally related to Arabidopsis BOS1, MYB2 and C. plantagineum CPM10.

Tomato AIM1 is required for resistance to B. cinerea

To determine the function of SlAIM1, tomato lines with reduced SlAIM1 gene expression were generated by expressing double-strand RNA (dsRNA) corresponding to the gene-specific region of SlAIM1. SlAIM1 expression was reduced in several transgenic tomato lines relative to wild-type plants (Figure 2a). Tomato SlAIM1 RNAi lines 1 and 8 had the greatest reduction in SlAIM1 gene expression, and were used for the experiments described in this report. The SlAIM1 RNAi plants do not show any developmental growth defects when grown under normal horticultural conditions. However, 2 days after challenge with B. cinerea, SlAIM1 RNAi plants showed an increased susceptibility, with larger disease lesions than the CastlemartII wild-type plants (Figure 2b,c). This was accompanied by a slight increase in fungal growth, as measured by the levels of the B. cinerea ActinA gene transcript (Benito et al., 1998) (Figure 2d). In addition, we suppressed the SlAIM1 gene expression through virus-induced gene silencing (VIGS) (Liu et al., 2002) in the cherry tomato cultivar Micro-Tom. In whole-

Figure 2. SlAIM1 is required for the full resistance of tomato plants to Botrytis cinerea.
(a) RT-PCR showing SlAIM1 transcript levels in SlAIM1 RNAi lines.
(b) SlAIM1 RNAi plants show increased susceptibility to B. cinerea.
(c) Disease lesion size in B. cinerea-inoculated leaves at 2 days after inoculation.
(d) RNA gel blot showing accumulation of B. cinerea ActinA mRNA as a measure of fungal growth in inoculated tomato leaves.

In (a), RNA samples were extracted from leaves at 3 days after inoculation with B. cinerea. In (d), the total RNA (15 μg) was loaded per lane. In (c), the data represent the mean ± SE from a minimum of 60 lesions. Both an analysis of variance (ANOVA) and Duncan’s multiple range test were performed to determine the statistical significance of the mean disease lesion sizes using SAS software (SAS, 1999). Bars with different letters are significantly different from each other (P = 0.05). Experiments were repeated at least three times with similar results. The disease symptoms in (b) are representative of SlAIM1 RNAi lines 1 and 8. The data from the SlAIM1 RNAi line 1 is presented in panels (c) and (d). Ethidium bromide staining of rRNA or the amplification of the tomato elongation factor 4A (elF4A) were used as controls. Abbreviations: BcActinA, Botrytis cinerea ActinA gene; h, hours post-inoculation.
plant disease assays. SIAIM1 VIGS plants showed severe disease symptoms, with extensive tissue damage at 3 days post-inoculation (dpi) with B. cinerea, compared with the wild-type cultivar, thereby confirming the data from the RNAi plants (Figure S3).

SIAIM1 RNAi plants were tested for responses to the virulent strain of the bacterial pathogen P. syringae pv. tomato. Plants were inoculated by infiltration, spray inoculation or seedling incubation with bacterial suspension, as described by Zipfel et al. (2004) and Uppalapati et al. (2008). There was no difference observed in both symptom development and bacterial growth between SIAIM1 RNAi and the wild-type plants, regardless of the inoculation method (data not shown). Thus, the disease resistance function of SIAIM1 does not extend to P. syringae.

Tomato lines that overexpress SIAIM1 (35S:SIAIM1) were generated in the Micro-Tom cultivar, and two transgenic lines (35S:SIAIM1 lines 4 and 7) that have high SIAIM1 gene expression were selected (Figure S4a). The 35S:SIAIM1 tomato plants were comparable with the wild-type plants in the level of B. cinerea resistance (Figure S4b).

Expression of tomato defense genes during B. cinerea infection and wounding

The expression of tomato defense genes was studied to determine whether the B. cinerea susceptibility of SIAIM1 RNAi plants is related to tomato defense pathways affecting B. cinerea resistance (Schlimmiller and Howe, 2005; AbuQamar et al., 2008). The uninfected tomato plants express low levels of tomato protease inhibitor-II (PI-II) and Allen oxide synthase 2 (AOS2) genes, normally associated with wound and JA responses. The PI-II and AOS2 genes were strongly induced in response to B. cinerea infection and mechanical wounding, independent of SIAIM1 (Figure 3a). Thus, the enhanced susceptibility of SIAIM1 RNAi plants to B. cinerea is independent of at least part of the JA/wound response pathways leading to the expression of these genes. The ACC synthase (ACS) gene expression is induced to the same level in both the wild-type and SIAIM1 RNAi plants in response to B. cinerea and wounding, suggesting that SIAIM1 acts independently of the ET-dependent defense response pathway in tomato. The SIAIM1 gene is also not required for the B. cinerea and wound induced expression of the pathogenesis related protein 1 (PR1) gene, a molecular marker for SA-mediated defense responses.

The tomato JA and wound response mutants suppressor of prosystemin-mediated responses 1 and 2 (spr1 and spr2), jasmonate-insensitive 1 (jai1), defenseless 1 (def1) and acyl-CoA oxidase (acx1) show impaired JA and wound responses, and/or increased susceptibility to B. cinerea (Schlimmiller and Howe, 2005; AbuQamar et al., 2008). SIAIM1 was expressed at wild-type levels in these mutants in response to B. cinerea infection (Figure 3b). Thus, the data suggest that the expression of SIAIM1 during B. cinerea infection and its B. cinerea resistance function is likely to be independent of JA and/or wound response pathways in tomato.

SIAIM1 is required for responses to ABA but not to other plant hormones

Hormone sensitivity of seedlings was assayed to determine if impaired defense in the SIAIM1 RNAi plants results from impaired hormone-related functions mediating plant defense and abiotic stress responses. SIAIM1 RNAi plants show no altered sensitivity to ET and MeJA (data not shown). In contrast, the SIAIM1 RNAi seedlings show reduced sensitivity of root elongation to ABA (Figure 4a,b). The SIAIM1 RNAi seeds also showed reduced germination and growth when directly plated on media containing ABA (Figure 4c; data not shown). By contrast, the 35S:SIAIM1 plants showed increased sensitivity to ABA (Figure 4d). ABA mediates plant responses to biotic and abiotic stresses, and these data suggest that SIAIM1 is required for ABA responses and B. cinerea resistance.

SIAIM1 is sufficient for increased tolerance to salinity and oxidative stress

The SIAIM1 RNAi plants were tested for sensitivity of seed germination and seedling growth to increased salinity.
Compared with the wild type, the SlAIM1 RNAi plants have significantly reduced seed germination and growth on medium containing high salt concentrations (Figure S5a). At 50 mM NaCl, both the wild-type and SlAIM1 RNAi plants fully germinated, but the SlAIM1 RNAi plants had a drastically reduced shoot and radical growth after germination. At 100 mM, the germination of SlAIM1 RNAi seeds was mostly inhibited. When seedlings pre-germinated on MS media were transferred to media containing 125 mM NaCl, the root growth of SlAIM1 RNAi seedlings was significantly reduced relative to wild-type plants (Figure S5b,c).

The salinity response assay was repeated under glasshouse conditions (see Experimental procedures). SlAIM1 RNAi seedlings exposed to high salinity show a clear pattern of salt sensitivity, with reduced shoot and root biomass, chlorosis and tissue collapse, in a dose-dependent manner (Figures 5 and S6). At 150 and 250 mM NaCl, stress-induced symptoms were visible as early as 5 days after salt treatment (Figure S6a). At 10 days, the SlAIM1 RNAi plants watered with 250 mM NaCl were dead. At 14 days, SlAIM1 plants watered with 200 mM NaCl exhibited reduced growth, and increased necrosis and chlorosis (Figure S6b). At 21 days, at most concentrations tested, the RNAi plants show chlorotic symptoms, total collapse of tissue and reduced shoot biomass (Figure 5a,b). A similar dose-dependent reduction in total root biomass was observed in SlAIM1 RNAi plants, as compared with the wild-type plants (Figure 5c,d).

The 35S:SlAIM1 plants generated in the Micro-Tom genetic background were assayed for increased tolerance by extended exposure to salt stress. In the absence of stress, the tomato 35S:SlAIM1 plants show reduced growth compared with the wild-type cultivar (Figure 6). When exposed to salt, the biomass accumulation of the wild-type plants was reduced in a dose-dependent manner (Figure 6a,b). By contrast, the growth of 35S:SlAIM1 plants was less affected by increasing salt concentrations. The wild-type plants show significant and subsequent reductions in shoot growth starting at 100 mM NaCl, relative to untreated controls, whereas the 35S:SlAIM1 plants exhibited no significant decline in shoot biomass over most NaCl concentrations.
tested. When grown at salinity concentrations of 200 and 250 mM, the wild-type Micro-Tom had significantly reduced root and shoot biomass, whereas the 35S:SlAIM1 was less affected (Figure 6a–c). Interestingly, 35S:SlAIM1 plants showed an increasing root length under increasing salt stress (Figure S7). In a different assay, plants were continuously grown under different concentrations of salinity, starting at 10 days after germination. The wild-type plants were completely killed between 25–30 days, whereas the overexpression line survived (Figure S8). Thus, SlAIM1 is sufficient to confer increased resistance to high salinity.

The pictures in (a) and (c) are from representative samples of SlAIM1 RNAi lines 1 and 8, and were taken at 21 days after the start of the salt treatment. The measurements from SlAIM1 RNAi line 1 are presented in (b), (d) and (e). In (b), (d) and (e), an analysis of variance (ANOVA) and a Duncan’s multiple range test were performed to determine the statistical significance of differences of the mean dry weights using the SAS software (SAS, I, 1999). The bars with different letters are significantly different from each other (P = 0.05). In (e), the asterisk indicates statistically significant differences in the mean Na⁺ concentrations between wild-type and SlAIM1 RNAi plants (P = 0.05).

The elemental profiling of leaf tissue (Lahner et al., 2003) revealed that SlAIM1 RNAi leaves accumulate significantly higher concentrations of Na⁺ than the corresponding wild-type plants (Figure 5e). The 35S:SlAIM1 plants show significantly reduced Na⁺ concentrations, consistent with the increased tolerance of the 35S:SlAIM1 line to high salinity (Figure 6d). All other elements measured did not vary significantly between the RNAi, 35S:SlAIM1 and wild-type plants (Figures S9 and S10).

In addition, in media containing 3 mM hydrogen peroxide (H₂O₂), SlAIM1 RNAi plants show a severe reduction in seedling growth. At 5 mM H₂O₂, SlAIM1 RNAi plants failed to result from ionic effects of the salinity stress, rather than osmotic effects.

Figure 5. SlAIM1 is required for tolerance to salt stress.
(a) Salinity-induced stress symptoms in vegetative tissues.
(b) The dry weight of shoot matter (n = 9).
(c) Salinity-induced changes in root biomass.
(d) Weight of root dry matter (n = 9).
(e) Leaf tissue Na⁺ concentrations of CastlemartII wild-type and SlAIM1 RNAi plants (n = 10) exposed to salt.

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grow normally following germination, producing only a small radical with no shoot emerging, whereas the wild-type plant had fully germinated, with some shoot and limited root growth (Figure 7a). The 35S:SlAIM1 seedlings were more resistant to H$_2$O$_2$ than the wild-type plants (Figure 7b). The data suggest that SlAIM1 is sufficient to confer tolerance to oxidative stress caused by H$_2$O$_2$.

The SlAIM1 RNAi and 35S:SlAIM1 plants do not show altered expression of the tomato gene NHX1, involved in intracellular K$^+$ and Na$^+$ transport (Venema et al., 2003), superoxide dismutase, implicated in tolerance to various environmental stresses (Seong et al., 2007), or the tomato ABA- and salt-inducible dehydrin gene TAS14 (Godoy et al., 1990). The tomato Na$^+$/H$^+$ antiporter SOS1 gene was also normally expressed in the SlAIM1 RNAi and 35S:SlAIM1 plants, suggesting that SISOS1 either acts upstream of SlAIM1 or is independent of SlAIM1. Thus, SlAIM1 appears to regulate ion homeostasis through an uncharacterized pathway.

Discussion

We have identified a genetic regulator, SlAIM1, which integrates pathogen and abiotic stress responses. The function of tomato SlAIM1 in pathogen and abiotic stress responses has been determined. First, tomato plants with reduced expression of SlAIM1 generated through RNAi or VIGS result in increased susceptibility to B. cinerea infection, suggesting that SlAIM1 is required for tomato resistance to B. cinerea. Second, SlAIM1 RNAi plants are sensitive to increased salinity and oxidative stress, but are insensitive to ABA. Interestingly, the overexpression of SlAIM1 was sufficient for the increased tolerance to high salinity and oxidative stress, but was not sufficient for increased B. cinerea resistance. SlAIM1 also regulates Na$^+$ homeostasis, as revealed from the increased Na$^+$ accumulation in the SlAIM1 RNAi, and the reduced accumulation in the 35S:SlAIM1 plants. Thus, SlAIM1 is required for tomato pathogen and abiotic stress responses, possibly by contributing to ABA signaling.

Figure 6. SlAIM1 is sufficient for increased resistance to salt stress.
(a) Responses of Micro-Tom wild-type and 35S:SlAIM1 plants to various concentrations of salt.
(b) Shoot (% = 9) and (c) root dry weights (% = 9), and (d) Na$^+$ concentration in leaf tissue (% = 10), of 35S:SlAIM1 plants.
The pictures in (a) are from representative samples of 35S:SlAIM1 lines 4 and 7, at 33 days after the initiation of salinity treatment. The measurements taken from transgenic line 35S:SlAIM1 line 7 are presented in (b), (c) and (d). Statistical analysis was performed as described in the legend for Figure 5. In (b) and (c), the bars with different letters are significantly different from each other ($P$ = 0.05). In (d), the asterisks indicate that the mean values are significantly different from each other ($P$ = 0.05).
The specific molecular function of *SlAIM1* is still unknown. The predicted protein sequence of *SlAIM1* and its nuclear localization is consistent with a DNA-binding function, and its role as a disease and ionic/oxidative stress tolerance factor has clearly been established with this study. The *SlAIM1* RNAi plants are susceptible to *B. cinerea*, and are sensitive to salt and oxidative stress, which is accompanied by a reduced sensitivity to ABA. The altered pathogen and stress responses may result from the impaired ABA-dependent activation of *SlAIM1*-controlled protective mechanisms. Consistent with this, the *SlAIM1* transcript accumulates in response to the exogenous application of ABA, pathogens, and salt and oxidative stress. These data suggest that *SlAIM1* regulates the crosstalk between biotic and abiotic stress responses in tomato. The tomato mutant *tos1*, affected in ABA signaling, shows hypersensitivity to osmotic and salt stresses, but is insensitive to exogenous ABA (Borsani *et al.*, 2002). The tomato *TOS1* gene has not been identified, and the *B. cinerea* resistance of the mutant was not determined, but its ABA and abiotic stress responses suggest a similarity with the function of *SlAIM1*.

*SlAIM1* shares high sequence identity with the Arabidopsis *bos1, MYB78* and *MYB2* in the R2 and R3 MYB DNA-binding domains. The C-terminal region of *SlAIM1* is unique, and shares no significant sequence similarities with the related MYB proteins. Blast searches against the Arabidopsis genome using *SlAIM1* return Arabidopsis MYB78 and *BOS1*, and with the highest scores. However, because the whole genome sequence of tomato is not yet available, we cannot exclude the possibility that another tomato gene more closely related to At*bos1* or At*MYB78* exists. *SlAIM1* and *BOS1* are both induced by *B. cinerea* and other stress factors, and are required for resistance to *B. cinerea*, salt and oxidative stresses. Among the closely related MYBs, *myb78* and *myb112* mutants show wild-type levels of *B. cinerea* and *A. brassicicola* resistance. This, coupled with the phenotypes of Arabidopsis *bos1* and tomato *SlAIM1* RNAi plants, suggest *SlAIM1* is the functional homolog of Arabidopsis BOS1. Interestingly, Arabidopsis MYB2 has been implicated in osmotic, ABA and drought tolerance (Abe *et al.*, 2003). The ectopic expression of MYB2 causes reduced plant growth, similar to 35S:*SlAIM1*. MYB2 shows significant sequence similarity with *SlAIM1* around the conserved domains, but, overall, it is divergent from the other BOS1-related MYBs. Expression of *C. plantagineum* CPM10 in Arabidopsis increased salt tolerance (Villalobos *et al.*, 2004). Thus, *SlAIM1*, CPM10, Arabidopsis BOS1 and MYB2 perform similar functions.

Arabidopsis BOS1 and tomato AIM1 also show differences in the extent of their disease resistance functions, regulatory mechanisms and the genetic requirements for their expression. The *B. cinerea*-induced expression of *BOS1* requires functional JA responses (Mengiste *et al.*, 2003), whereas the *SlAIM1* expression was unaffected by the tomato mutation *jai1* (*coi1*) and other JA response mutants. The autoactivation of BOS1 in yeast two-hybrid assays, but the lack of autoactivation in *SlAIM1* and the complete divergence of the C-terminal sequences, may suggest significant differences in their regulatory functions. *SlAIM1* is not required for responses to *P. syringae*, whereas Arabidopsis *bos1* shows increased disease symptoms after inoculation with *P. syringae* (Mengiste *et al.*, 2003).

The plant stress hormone ABA has been recognized as a regulator of disease resistance through its interactions with other defense-mediating hormones. In tomato, the lack of ABA synthesis increases resistance to *B. cinerea* as a result
of increased SA-regulated defense gene expression, faster accumulation of H2O2 and the associated cell wall modifications (Audenaert et al., 2002; Asselbergh et al., 2007). The Arabidopsis ABA-insensitive abi1-1 and abi2-1 mutants show an increased susceptibility to the necrotrophic bacterial pathogen R. solanacearum, but show resistance to the necrotrophic fungus P. cucumerina (Hernandez-Blanco et al., 2007). The ABA insensitivity and B. cinerea susceptibility of SlAIM1 RNAi plants is similar to the role of ABA in resistance to R. solanacearum, P. irregular and A. brassicicola in Arabidopsis (Adie et al., 2007a; Hernandez-Blanco et al., 2007), but contradicts some of the observations showing ABA as a negative regulator of disease resistance (Anderson et al., 2004). Thus, ABA is either required for resistance or suppresses resistance depending on the specific pathogen involved, rather than whether the pathogen is a necrotroph or biotroph. ABA also mediates responses to ROS, a common factor in pathogen and abiotic stress (Laloi et al., 2004). The Arabidopsis NADPH-dependent respiratory burst oxidase homolog genes, AtRbohD and AtRbohF, are required for reactive oxygen species (ROS) generation, ABA-induced stomatal closure and the hypersensitive response to avirulent pathogens (Torres et al., 2002; Kwak et al., 2003). Impaired SlAIM1 function causes impaired ABA signaling and enhances the susceptibility to B. cinerea, suggesting a positive role for ABA in resistance B. cinerea.

Intriguingly, the ectopic expression of SlAIM1, although conferring resistance to oxidative stress and salinity, failed to increase resistance to B. cinerea, indicative of a complex mechanism of plant resistance to B. cinerea, consistent with the multiplicity of Botrytis virulence factors, including CWDEs, ROIs and toxins. Interestingly, the increased resistance to H2O2 and salinity, but not to B. cinerea, in the SlAIM1 overexpression tomato lines suggests that oxidative stress caused by B. cinerea during infection may not be the critical factor in disease development. B. cinerea strains impaired in the generation of ROS show reduced virulence (Edlich et al., 1989). In other plants, B. cinerea is known to cause an oxidative environment and contributes to disease development (Muckenschnabel et al., 2002), and the scavenging of ROIs was suggested as a resistance mechanism (Edel, 1992).

Expression of the SlAIM1 gene is tightly regulated, with an undetectable level of basal expression. High ectopic expression of SlAIM1 results in reduced plant growth compared with control plants under normal growth conditions. However, under conditions of high salinity, the 3SS:SlAIM1 plants grow significantly more than the wild-type controls, and only marginally lower than the 3SS:SlAIM1 plants grown without stress. These data suggest that SlAIM1 controls an energy-demanding protective mechanism in the 3SS:SlAIM1 plants, which in the absence of stress, confers a growth disadvantage. In Arabidopsis, various genes are required for salt tolerance, and some of these were sufficient for tolerance when overexpressed (Shi et al., 2003). In the case of tomato, although salt-sensitive mutants were identified, no genes were identified. Transgenic tomato plants overexpressing the Arabidopsis NHX1 gene, encoding the vacuolar Na+/H+ antiporter, were tolerant to high levels of salinity (Zhang and Blumwald, 2001). SlAIM1 differs from these proteins, and is a transcription factor that is likely to have a regulatory role in the activation of defense against pathogens and abiotic stress, including the regulation of antiporters and ion channels. Transmembrane ion fluxes represent important early stages in pathogen-induced necrosis or HR cell death (Hahlbrock et al., 1995). The involvement of ion channels in defense has been established by genetic data from Arabidopsis dnd1 and hlm1 mutants, which have altered disease responses that are caused by perturbations in the cyclic nucleotide-gated ion channels (CNGCs) (Clough et al., 2000; Balague et al., 2003). Arabidopsis HLM1 (CNGC4) is permeable to both K+ and Na+. Interestingly, the Arabidopsis dnd1 mutant impaired in CNGC2 shows an increased resistance to B. cinerea (Govrin and Levine, 2000). The necrosis and tissue collapse that occurs in SlAIM1 RNAi plants in response to Botrytis and high salinity could result from unregulated ion fluxes early in infection, or exposure to abiotic stress.

In conclusion, SlAIM1 regulates pathogen and abiotic stress responses by modulating events common to biotic and abiotic stress. SlAIM1 may be required for ABA signaling, which in turn controls protective mechanisms. Future studies should focus on the molecular functions of SlAIM1 and potential targets that effect tomato responses to pathogen and stress signals.

Experimental procedures

Plant growth

Tomato cultivars CastlemartII and Micro-Tom were grown in plastic pots containing compost soil mix in a glasshouse, with a photoperiod that was extended to 15 h under fluorescent lights (160 μmol m−2 s−1) at a day/night temperature of 22/18°C ± 4°C. The tomato mutants spr1, spr2, def1, acx1, jai1 and SlAIM1 RNAi plants are in the CastlemartII background. All plants were fertilized twice weekly before any biotic or abiotic stress experiment.

Fungal and bacterial disease assays

The culture of the B. cinerea strain BOS-10 used for the disease assays and the preparation of conidial spore suspension were described previously (Mengiste et al., 2003). Tomato and Arabidopsis disease assays and determination of fungal growth were performed as described by AbuQamar et al. (2008). Bacterial disease assays were carried out using the standard leaf infiltration protocol, essentially as described by Mengiste et al. (2003), spray inoculation (Zipfel et al., 2004) or seedling inoculation (Uppalapati et al., 2008). Leaves of 6-week-old tomato plants were infiltrated
with suspension (OD_{600} ≈ 0.001 in 10 mM MgCl₂) of the bacterial strain P. syringae pv. tomato DC3000 (a generous gift of Greg Martin, Cornell University). To determine bacterial growth, leaf discs from infected leaves were collected at 0, 2, and 4 dpi. Each experiment for the bacterial growth assay was performed in three replicates. At each time point, two leaf discs were collected from wild-type and SlAIM1 RNAi plants for each replicate. Leaf discs of the same size were made using a hole puncher, and the bacterial titer per leaf area was determined. In parallel, plants were inoculated by spraying with bacterial suspension. Bacterial cultures grown overnight were collected, washed once and resuspended in sterile water containing 0.04% Silwet L-77 (Lehle Seeds, http://www.arabidopsis.com), and the solution was then sprayed on plants. Leaves from the spray-inoculated plants were harvested and surface sterilized (30 sec in 70% ethanol, followed by 30 sec in sterile distilled water), and were then used to determine bacterial growth. In addition, tomato seedlings were assayed for bacterial responses, as recently described (Upalapati et al., 2008). Seedlings (5-days old) containing 2-3-cm-long hypocotyls that were germinated under axenic conditions were inoculated by flooding MS agar plates with a bacterial suspension, until the seedlings were completely submerged (OD₆₀₀ = 0.1). The seedlings were exposed to bacterial suspension for 2-3 min with gentle mixing. The bacterial suspension was then discarded, inoculated seedlings were incubated in a growth room with a 12-h photoperiod and the disease responses were observed for up to 7 days after inoculation.

RT-PCR

For RT-PCR, cDNA was synthesized from both control and treated samples using equal quantities of total RNA (2 μg), AMV reverse transcriptase (Promega, http://www.promega.com) and oligo (dT)₁₅ primers, according to standard protocols. The PCR was performed for 35 cycles using 2.5 μL of cDNA as a template and specific primer pairs (94°C 30 sec, 52°C 30 sec, 72°C 1 min). The amplified products were separated on 1.5% agarose gels and visualized under UV light after staining with ethidium bromide. The SlAIM1 (forward, 5'-CTCGTTGCGCAATAGGTGCTCAA-3'; reverse, 5'-CGTTACAC-TAGAAATTCCCGGTGG-3') primers were used for RT-PCR. The tomato translation initiation factor (eIF4A) gene was amplified as a control to demonstrate the relative quantity of cDNA. The other primer sequences used for RT-PCR were recently described by AbuQamar et al. (2009).

Hormone and wounding treatments

Six-week-old tomato seedlings were used to confirm the SlAIM1 expression in response to abiotic stresses. For hormone and paraquat treatments, a concentration of 100 μM of paraquat (methyl viologen), ABA, MeJA, ACC or SA was sprayed. For the salt treatment, a concentration of 200 mM NaCl was applied. Mechanical wounding was performed by wounding the main veins of apical leaflets of compound leaves with dented forceps.

DNA and RNA blots

For DNA blots, genomic DNA was extracted from wild-type tomato leaves as described by Dellaporta et al. (1983). A 10-μg genomic DNA was digested with restriction enzymes and subsequently separated on a 0.8% (w/v) agarose gel. Total RNA from tomato leaf tissues was extracted from tissues frozen in liquid nitrogen, as described by Lagrimini et al. (1987). RNA was separated on 1.2% formaldehyde agarose gels. The gels were then blotted onto Hybond N⁺ nylon membranes (Amersham Pharmacia Biotech, now part of GE Healthcare, http://www.ge lifesciences.com). Probes were labeled with ³²P by random priming using a commercial kit (Sigma-Aldrich, http://www.sigmaaldrich.com). The hybridization of the probe and subsequent washings were performed as described by Church and Gilbert (1984).

Rapid amplification of cDNA ends (RACE)

The RACE amplification were performed using by the BD SMART™ RACE cDNA Amplification Kit (cat. no. 634914; BD Biosciences, http://www.bdbiosciences.com). The remaining steps and modifications were described previously by AbuQamar et al. (2008).

Vector construction

To make 35S:SlAIM1 overexpression constructs, the SlAIM1 full-length cDNA was amplified by PCR from the RACE-Ready cDNA with primers SlAIM1/LP (5'-TCCCGCGGATGATTAATTTCA-TCAGAAG-3', with the Sacl site underlined) and SlAIM1/RP (5'-CGGATCCCTATAGACCAATGTTCTTCAAAT-3', with the BamHI site underlined). Restriction-digested PCR products were cloned into the pCAMBIA 99-1 vector (a modified version of the binary vector pCAMBIA 1200) behind the double cauliflower mosaic virus (CaMV) 35S promoters between the Sacl and BamHI sites.

To generate the SlAIM1 RNAi construct, 250 bp from the 3' end of the cDNA, including part the 3' untranslated region (3'UTR), of SlAIM1 were amplified by PCR from the RACE-Ready cDNA, with primers SlAIM1 RNAi/LP (5'-CGACTAAGTCATGCGAACATATA-ATTGTTCAA-3', with the SpeI and Ncol sites underlined), and SlAIM1 RNAi/RP (5'-CGGATCCCTATAGACCAATGTTCTTCAAAT-3', with the BamHI and Ascl sites underlined). The inverted repeat is assembled directly in the binary vector by a two-step cloning process using the introduced restriction enzyme sites, as described by AbuQamar et al. (2008).

Plant transformation and regeneration, and virus-induced gene silencing

Tomato transformation was carried out as described by Howe et al. (1996), and the specific modifications and details for the tomato transformation and regeneration were described in detail by AbuQamar et al. (2008). The SlAIM1 silencing was performed using the TRV vector system, as described by Liu et al. (2002).

Salt stress experiment

For experiments in tissue culture, tomato seeds were surface-sterilized with 35% (v/v) commercial bleach for 30 min, and were then washed several times with sterile water. For the germination experiments, seeds were germinated on MS medium with 3% (w/v) sucrose and 0.7% (w/v) agar, containing 0, 50, 100 and 150 mM sodium chloride (NaCl). For the root growth experiment, between 30 and 40 seeds from each genotype were germinated on basal MS medium. Seedlings (4-days old) with ~2-cm-long roots were transferred from vertical basal MS plates onto other plates of MS medium, containing 125 mM NaCl (Borsani et al., 2002). For root length growth measurements, the root lengths of 10 seedlings were measured per treatment, and three replicates were run for each treatment. Changes in root length were measured after 2 days of treatment.

For glasshouse experiments, seeds were sown in D40 deepots (Stuewe & Sons, Inc., http://www.stuewe.com) containing surface...
calcined clay (Profile Products, http://www.profileproducts.com). Deepots were placed in a greenhouse with a photoperiod extended to 15 h using incandescent and fluorescent lights (160 μmole m⁻² s⁻¹) at a day/night temperature of 26/18°C ± 4°C. Seeds were mist-watered for 16 sec every 10 min. After the seedlings germinated, deepots within blocks were randomized. Plants were subirrigated as needed with purified water containing 0 mM NaCl. After 4 days, plants were subirrigated for 2 h daily with fertilizer solution that contained 0, 50, 100, 150, 200 or 250 mM NaCl. The fertilizer solution contained (in mg per liter) 200 N, 29 P, 167 K, 67 Ca, 30 Mg and micronutrients supplied from a commercial fertilizer formulation (Miracle Gro® Excel® 15-5-15 Cal-Mag; The Scotts Co., http://www.scotts.com). Three-week-old seedlings of the CastlemartII wild-type cultivar and Scotts Co., http://www.scotts.com. Three-week-old seedlings of the CastlemartII wild-type cultivar and S. A. I. M. 1 RNAi line, or the 5-week-old Micro-Tom wild-type cultivar and the 35S:SlAIM1 line, were harvested. Shoot and root tissues were collected separately, dried at 65°C and weighed.

Tissue Na⁺ quantification
Leaf tissue (5 ± 1.5 mg dry weight) was sampled into Pyrex tubes (16 × 100 mm) and dried at 92°C for 20 h. After cooling, five samples per replicate per treatment from each genotype were weighed, digested and elemental analysis was performed with an ICP-MS for Li, B, Na, Mg, P, K, Ca, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Mo and Cd, as described by Rus et al. (2006).

Phylogenetic analysis
The conserved regions in the S. A. I. M. 1 and related R2R3 MYBs were used for constructing the SlAIM1 phylogenetic tree. Sequences were aligned using ClustalW (Thompson et al., 1994) with default gap penalties, and the alignment was manually adjusted where necessary. Mean character distances were used with default gap penalties, and the alignment was manually adjusted where necessary. Statistical support of the branches was tested with 1000 bootstrap replicates using the Felsenstein (1993) from the PHYLP v3.67 package. Mean character distances were used with default gap penalties, and the alignment was manually adjusted where necessary. Mean character distances were used with default gap penalties, and the alignment was manually adjusted where necessary. Statistical support of the branches was tested with 1000 bootstrap replicates using the Felsenstein (1993) from the PHYLP v3.67 package. Mean character distances were used with default gap penalties, and the alignment was manually adjusted where necessary. Statistical support of the branches was tested with 1000 bootstrap replicates using the Felsenstein (1993) from the PHYLP v3.67 package.

Data deposition
DNA sequences of the tomato AIM1 and the predicted amino acid sequences have been deposited in GenBank under accession no. EU934734.

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Supporting Information
Additional Supporting Information may be found in the online version of this article:

References


