Polyamines Attenuate Ethylene-Mediated Defense Responses to Abrogate Resistance to *Botrytis cinerea* in Tomato[1][C][W][OA]

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Transgenic tomato (*Solanum lycopersicum*) lines overexpressing yeast spermidine synthase (*ySpdSyn*), an enzyme involved in polyamine (PA) biosynthesis, were developed. These transgenic lines accumulate higher levels of spermidine (Spd) than the wild-type plants and were examined for responses to the fungal necrotrophs *Botrytis cinerea* and *Alternaria solani*, bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000, and larvae of the chewing insect tobacco hornworm (*Manduca sexta*). The Spd-accumulating transgenic tomato lines were more susceptible to *B. cinerea* than the wild-type plants; however, responses to *A. solani*, *P. syringae*, or *M. sexta* were similar to the wild-type plants. Exogenous application of ethylene precursors, *S*-adenosyl-Met and 1-aminocyclopropane-1-carboxylic acid, or PA biosynthesis inhibitors reversed the response of the transgenic plants to *B. cinerea*. The increased susceptibility of the *ySpdSyn* transgenic tomato to *B. cinerea* was associated with down-regulation of gene transcripts involved in ethylene biosynthesis and signaling. These data suggest that PA-mediated susceptibility to *B. cinerea* is linked to interference with the functions of ethylene in plant defense.

A complex set of host and pathogen genetic factors determine the outcome of plant responses to pathogens. Necrotrophic fungi deploy a variety of virulence factors that assist in colonization of a wide range of host species (Groll et al., 2008). *Botrytis cinerea*, a necrotrophic fungal pathogen that infects >200 plant species, is the causal agent of the gray mold disease, resulting in significant economic losses. Information on how plants combat necrotrophic pathogens such as *B. cinerea* and what signaling molecules are involved in such interactions is beginning to emerge (Abuqamar et al., 2008; Laluk et al., 2011). Host immune response to infection is mediated by diverse regulatory processes, of which, plant hormone functions have been studied extensively in relation to disease (Spoel and Dong, 2008; Bari and Jones, 2009; Pieterse et al., 2009). A number of reports suggest a significant role of ethylene (ET) in imparting resistance against *B. cinerea* and other necrotrophic fungi. These include (1) requirement of the ET response pathway and its genetic components for efficient resistance to *B. cinerea* in different plant species (Thomma et al., 1999; Díaz et al., 2002); (2) enhanced resistance to *B. cinerea* by the expression of *Ethylene response factor1* (*ERF1*), a component of ET signaling in Arabidopsis (*Arabidopsis thaliana*; Berrocal-Lobo et al., 2002), and transgenic expression of the *etr1* mutant allele in tobacco (*Nicotiana tabacum*), resulting in susceptibility to the necrotrophic oomycete *Pythium* spp. (Knoester et al., 1998); (3) induction of >30 ET-regulated transcription factors after infection of Arabidopsis with *B. cinerea* (AbuQamar et al., 2006), some of which directly contribute to resistance (Zheng et al., 2000); (4) increased resistance of *constitutive triple response1-1* and

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ethylene overproducer2 plants to *B. cinerea* (Lloyd et al., 2011); (5) ET role in pathogen-associated molecular pattern-triggered immunity to *B. cinerea* (Laluk et al., 2011); and (6) ET-regulated cell wall modifications involved in defense responses to *B. cinerea* (Lloyd et al., 2011). Additionally, a number of protein kinases have been shown to play a role in ET-dependent responses to *B. cinerea* infection. Thus, various genetic and biochemical data suggest a critical role of ET in *B. cinerea* resistance (Han et al., 2010; Lumbreras et al., 2010).

Polyamines (PAs) are polycationic, ubiquitous compounds that have essential functions in all organisms studied thus far involving regulation at both transcriptional and translational levels (Veress et al., 2000; Kasukabe et al., 2004; Yoshida et al., 2004; Alcázar et al., 2005; Igarashi and Kashiwagi, 2006; Srivastava et al., 2007; Mattoo and Handa, 2008; Handa and Mattoo, 2010). Putrescine (Put), spermidine (Spd), and spermine (Spm) are the three most prominent PAs in plants. Decarboxylation of Orn by Orn decarboxylase (ODC) or Arg by Arg decarboxylase (ADC) leads to the synthesis of Put, which is converted to Spd by Spd synthase (SpdSyn), and Spd, in turn, then converted to Spm by Spm synthase (SpmSyn; Nambeesan et al., 2008). In these reactions, both SpdSyn and SpmSyn enzymes use aminopropyl residues derived from decarboxylated *S*-adenosyl-Met, which is synthesized from *S*-adenosyl-Met (SAM) by SAM decarboxylase (SAMdc; Martin-Tanguy, 1997; Bouchereau et al., 1999; Mehta et al., 2002). SAM is also a substrate for 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, a reaction that generates ACC, the immediate precursor of ET (Fluhr and Mattoo, 1996). PAs are present in various cellular compartments, such as the vacuole, mitochondria, and chloroplast, as well as in cell wall fractions (Kaur-Sawhney et al., 2003). While both nuclear and cytoplasmic localization of ODC has been reported, the ADC pathway is predominantly localized to the chloroplast. SpdSyn has been reported to be localized both in the chloroplast and cytoplasm (Nambeesan et al., 2008).

The role of PAs in plant-pathogen interactions remains elusive. Nevertheless, dramatic changes in PA metabolism have been reported in both compatible and incompatible plant-microbial interactions (Walters, 2003a). Chinese cabbage (*Brassica campestris* ssp. *pekinensis*) leaves infected with turnip yellow mosaic virus and maize (*Zea mays*) with the fungal biotroph *Ustilago maydis* showed increase in Put and Spd compared to healthy leaves (Torget et al., 1979). Barley (*Hordeum vulgare*) leaves infected with the brown rust fungus *Puccinia hordei* exhibited >6-fold increase in Spd compared to control healthy leaves and resulted in the formation of green islands surrounding the site of infection due to chlorophyll retention (Greenland and Lewis, 1984). ADC, ODC, and SAMDC activities increased in barley leaves infected with *Blumeria graminis* f. sp. *hordei* (Walters et al., 1985). Also, decreases in PAs have been observed in some plant-fungal and plant-viral infections (Bakanashvili et al., 1987; Edreva, 1997). PA conjugates, hydroxycinnamic acid amides, synthesized by the formation of an amide linkage with cinnamic acids, mainly *p*-coumaric, ferulic, and caffeic acids, have been reported to enhance plant resistance to pathogens by forming an enzymatic hydrolysis resistance phenolic barrier (Walters et al., 2001). PA catabolism by diamine and PA oxidases leading to the production of hydrogen peroxide (H_2O_2) also has been implicated in mediating programmed cell death and wall lignification (Walters, 2003b). An Spm signaling pathway has been implicated in *Tobacco mosaic virus*-induced hypersensitive response that involves ZFT1 and ZFP1, two Cys_2/His_2-type transcription factors (Uehara et al., 2005; Mitsuya et al., 2009). PAs may also induce nitric oxide, an important signaling molecule involved in disease resistance in Arabidopsis (Delledonne et al., 1998; Tun et al., 2006), although its involvement as a trigger for disease tolerance was ruled out in field-grown tomato (*Solanum lycopersicum*; Kumar et al., 2004). There have been few studies suggesting the effect of transgenic manipulation of PA biosynthesis genes during pathogen response. Transgenic tobacco plants expressing *SAMdc* under the control of 35S promoter of *Cauliflower mosaic virus* (CaMV35S) exhibited tolerance to *Verticillium dahliae* and *Fusarium oxysporum* (Waie and Rajam, 2003). Arabidopsis plants engineered to overexpress *SpmSyn* under the CaMV35S promoter also showed resistance to *Pseudomonas viridiflava* (Gonzalez et al., 2011).

We demonstrated a role of PAs in plant growth and development using high Spd-producing transgenic plants generated by expressing yeast *SpdSyn* in tomato (Nambeesan et al., 2010). Here, we investigated the response of these plants to the fungal pathogens *B. cinerea* and *Alternaria solani*, the bacterial pathogen *Pseudomonas syringae*, and the chewing insect tobacco hornworm *Manduca sexta*. These transgenic lines that accumulated higher Spd levels were more susceptible to *B. cinerea*, while showing no differences in their response to *A. solani*, *P. syringae*, and *M. sexta* compared to the wild-type plants. The increased susceptibility of the high-Spd lines to *B. cinerea* was found interrelated to the altered functions of the plant hormone ET.

RESULTS

Overexpression of *ySpdSyn* Increases Spd Levels in Tomato Leaves

Tomato cultivar OH 8245 was used to generate transgenic lines expressing the *ySpdSyn* gene under the control of the CaMV35S promoter using *Agrobacterium tumefaciens*-mediated transformation (Fig. 1A). Several independent T0 plants transformed with this chimeric construct were selected for kanamycin resistance and screened for the presence of the *ySpdSyn* gene using PCR. Kanamycin-resistant and PCR-positive plants were then screened for transgene and immunoreactive protein expression using RNA-blot and immunoblot analyses, respectively, as described

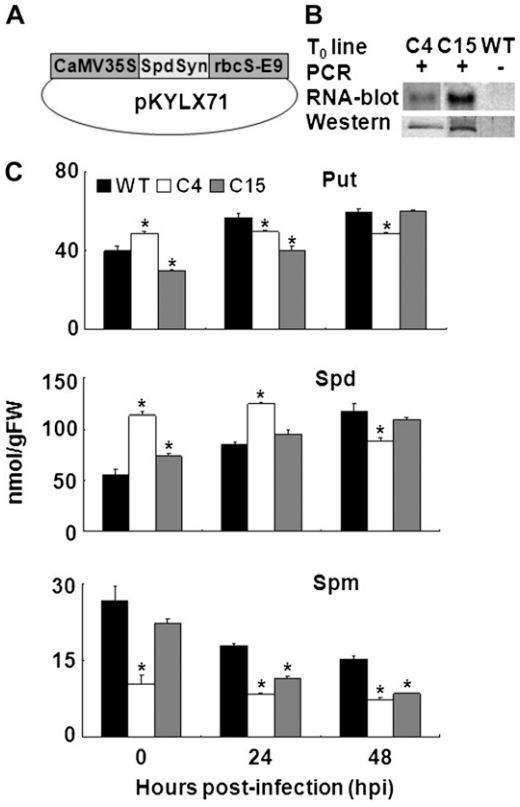

Figure 1. Changes in polyamine levels in fully expanded leaves of wild-type and transgenic (T2 homozygous C4 and C15) tomato plants expressing the yeast *SpdSyn* gene. A, The chimeric construct containing *ySpdSyn* cloned between the CaMV35S promoter and the 3′ end of a pea *rbcS-E9* gene in pKYLX71 vector was used to develop transgenic tomato plants. B, Presence (+) or absence (−) of introduced *ySpdSyn* transgene as determined by PCR; *ySpdSyn* transcript (RNA blot) and protein levels (western blot) in leaves of wild-type, T0 C4, and C15 plants. C, The levels of Put, Spd, and Spm quantified in leaves at 0, 24, and 48 hpi with *B. cinerea*. The fully expanded leaves of 6-week-old plants were drop inoculated with a conidial suspension of *B. cinerea* containing 3×10^5 spores/mL. The samples were collected at the indicated postinoculation time periods and the levels of Put, Spd, and Spm quantified using HPLC as described in "Materials and Methods." Error bars represent SE of means ($n = 3$), and asterisks indicate significant difference from the wild type ($\alpha = 0.05$).

in "Materials and Methods." Based on these analyses of the T0 transgenic lines, two independent transgenic lines, C4 and C15, were selected for studies described in this manuscript (Fig. 1B). T1 seedlings were grown, and homozygous C4 and C15 plants were generated. Spd levels in the leaf tissue were 2.1- and 1.3-fold higher in C4 and C15, respectively, compared to the wild-type cultivar (Fig. 1C; 0 h postinfection [hpi]). However, the pattern of Put levels was inconsistent, being 1.2-fold higher in C4 and 1.3-fold lower in C15 compared to wild-type plants (Fig. 1C). Spm levels were lower in leaves of both transgenic lines compared to the wild type, but C4 plants had greatly reduced Spm content than the C15 plants. These data suggest that *ySpdSyn* was functional in tomato and led to higher Spd levels.

Leaf PA Levels Increase during *B. cinerea* Infection

To determine the relationship between PA content and disease response, changes in PA levels were quantified after inoculation with *B. cinerea* (Fig. 1C). In wild-type plants, Put levels increased by 1.4-fold within 24 hpi and thereafter remained elevated. Leaves of C15 plants had a similar increase in Put levels at 24 hpi, which increased to about 2-fold at 48 hpi, while that of C4 remained unaltered during *B. cinerea* infection. Also, Spd levels in wild-type leaves continued to increase after *B. cinerea* infection, increasing >2-fold at 48 hpi. Prior to infection, Spd levels were higher in C4 (>2-fold) and C15 leaves than in the wild-type plants. Spd levels increased further (by 1.5-fold) at 48 hpi in *B. cinerea*-infected C15 but not C4 leaves. Spm levels decreased 24 hpi in wild-type and C15 lines upon infection with *B. cinerea* but were similar in C4 leaves (Fig. 1C). Collectively, these results indicate that an increase in Spd and Put levels occurs with a concomitant decrease in Spm levels during tomato infection with *B. cinerea.*

Increased Spd Levels in Tomato Leaves Enhance Susceptibility to *B. cinerea*

Leaves from C4, C15, and wild-type plants were inoculated with *B. cinerea* spores, and the development of lesions on the leaf was followed. Visible disease symptoms were apparent within 24 hpi in wild-type leaves, but the size of the lesion was similar to the size of the drop inoculums, indicating limited progression of the disease. At 24 hpi, the lesion size in transgenic C4 and C15 plants was larger than that in wild-type leaves (Fig. 2A). At 48 hpi, average lesion diameter in wild-type plants was 5.4 cm, while it was significantly higher, by 100% and 72%, respectively, in C4 and C15 plants (Fig. 2B). Tissue maceration and extensive necrosis in the transgenic plants occurred within 72 hpi (data not shown). In each independent experiment, the C4 line was found to be more susceptible than the C15 line. Susceptibility of C4 and C15 plants to *B. cinerea* was associated with higher accumulation of fungal biomass, as was indicated by higher steady-state levels of *B. cinerea ActinA* transcripts at 24 and 48 hpi in *ySpdSyn* leaves compared to the wild type (Fig. 2C). Steady-state levels of *ySpdSyn* transcripts in *B. cinerea* and mock-inoculated C4 and C15 plants confirmed continued expression of this gene during infection (Fig. 2D). Since an increase in Spd levels was noted in wild-type plants during infection, its level at the time of infection may be important for the progression of the disease.

Inhibitors of PA Biosynthesis Reverse Susceptibility of Transgenic Plants to *B. cinerea* Infection

Leaves of wild-type, C4, and C15 plants were pretreated for 24 h in the absence and presence of 1 mM Spd, 1 mM difluoromethyl-Orn (DFMO), or 1 mM

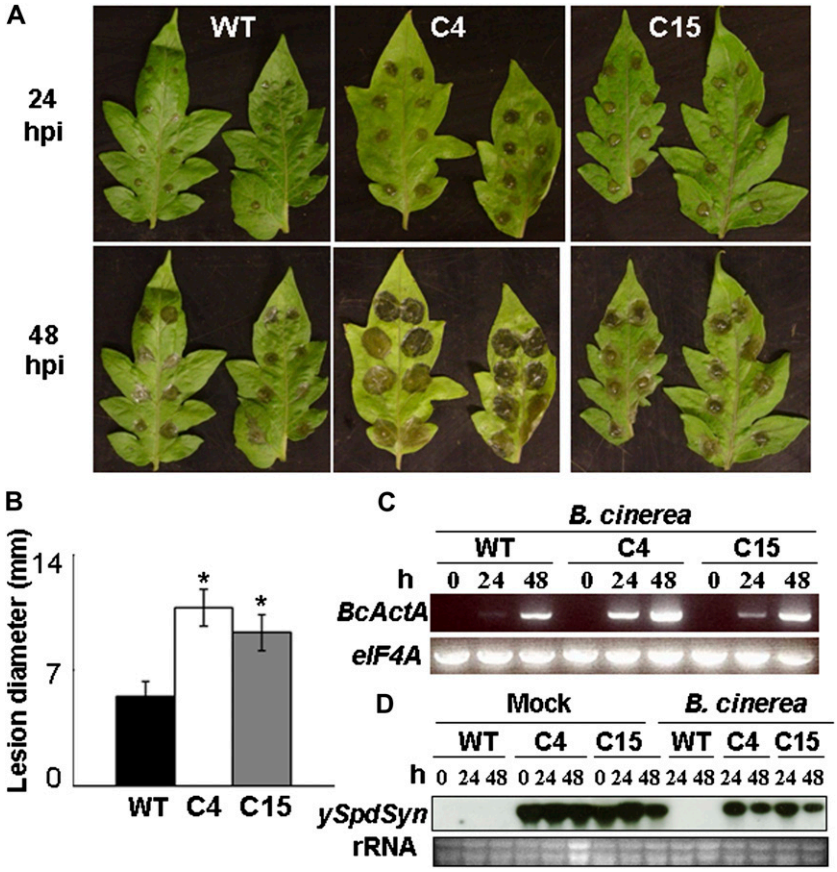

Figure 2. Expression of *ySpdSyn* increases tomato leaf susceptibility to *B. cinerea*. A, Response of wild-type and T2 homozygous C4 and C15 leaves to *B. cinerea* inoculation. Leaves were spot inoculated with 5 μL of 3×10^5 spores/mL of *B. cinerea*. B, Mean lesion size at 48 hpi in wild-type, C4, and C15 leaves. Error bars represent SE of means ($n = 60$), and asterisks indicate significant difference from the wild type ($\alpha = 0.05$). C, RT-PCR showing the expression of *B. cinerea* actinA (*BcActA*) and tomato eukaryotic initiation factor4A (*eIF4A*) in *B. cinerea*-infected wild-type, C4, and C15 leaf tissues. D, An RNA blot showing the expression of *ySpdSyn* transgene in water-treated and *B. cinerea*-inoculated leaves with increasing time period. rRNA bands are shown to indicate total RNA loading. [See online article for color version of this figure.]

cyclohexylamine (CHA), compounds that inhibit ODC (Rajam and Galston, 1985) and SpdSyn (Saftner et al., 1997), respectively. Then, these plants were challenged with *B. cinerea*, and the lesion development was monitored. Pretreatment with DFMO or CHA did not alter the response of wild-type plants to *B. cinerea* but eliminated the enhanced susceptibility of the transgenic leaves to this pathogen (Fig. 3, A–C). At 72 hpi, the lesion sizes in wild-type, C4, and C15 leaves treated with DFMO or CHA were of the same extent (Fig. 3E), suggesting that inhibitors of PA biosynthesis decrease susceptibility of transgenic leaves to *B. cinerea*. Treatment with Spd significantly increased lesion diameter in wild-type leaves at 72 hpi, which was similar to that seen in C4 and C15 plants (Fig. 3, F–H). Thus, these data support above results that elevated levels of Spd increase susceptibility of tomato to *B. cinerea*.

Spd-Induced Susceptibility of Tomato to *B. cinerea* Is Mediated by Down-Regulation of *SlACS1* and *SlERF1B*

Different defense pathways mediated by plant hormones interact in plant responses to infection. ET and salicylic acid (SA) have antagonistic roles in determining plant responses to *B. cinerea* infection in Arabidopsis (Glazebrook, 2005). *B. cinerea* exploits the antagonistic interactions between jasmonic acid (JA)- and SA-mediated defense pathways to establish disease in tomato. The production of exopolysaccharide by *B. cinerea*, which elicits the SA pathway and suppresses the JA signaling pathway, has been implicated in enhancing disease caused by this pathogen in tomato (El Oirdi et al., 2011). To understand the involvement of these signaling pathways in Spd-mediated susceptibility of tomato to *B. cinerea*, we analyzed the transcript levels of tomato genes involved in ET biosynthesis (ACC synthase; *SlACS*) and that of SA (pathogenesis-related 1; *SlPR-1*) mediated defense responses (Fig. 4). Indeed, the expression of *SlACS* increased by 15-fold in response to *B. cinerea* infection in wild-type leaves at 24 hpi, suggesting an activation of the ET pathway. However, in the transgenic C4 leaves, only a 2-fold increase in *SlACS* expression was observed, while C15 registered a 10.6-fold increase in its expression, suggesting attenuation of ET biosynthesis (Fig. 4A). This pattern of gene expression is interesting since PAs and ET are synthesized from common upstream substrates, and overexpression of *ySpdSyn* may have interfered with the expression of *SlACS*. By contrast, the expression of the SA response marker gene *SlPR1* showed a minor increase in *B. cinerea*-inoculated wild-type leaves but a significantly higher expression in both the transgenic lines at 24 hpi (Fig. 4B). These data indicate that Spd levels in transgenic leaves modulate ET biosynthesis pathway and thereby may affect ET-regulated processes during *B. cinerea* infection. The elevated levels of *SlPR1* expression

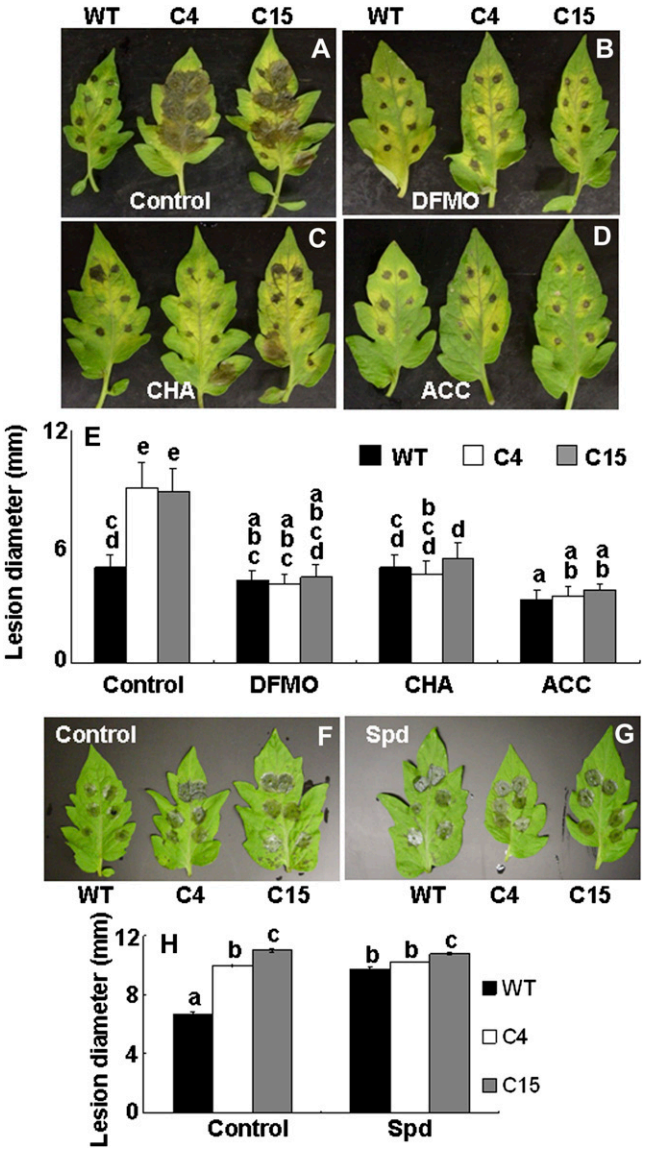

Figure 3. Spd, PA biosynthesis inhibitors, and ET precursor alter susceptibility of wild-type and T2 homozygous *ySpdSyn* transgenic tomato leaves to *B. cinerea* infection. A to D, F, and G, Detached leaves were pretreated for 24 h with water (A; mock control), 1 mM DFMO (B), 1 mM CHA (C), 100 μM ACC (D), water (F; control), and 1 mM of Spd (G) and spot inoculated with of 3×10^5 spores/mL of *B. cinerea*. E and H, Disease lesion diameter determined 72 hpi. Error bars indicate SE ($n \geq 50$). Statistical significance was determined using ANOVA and Tukey's honestly significant difference test for the separation of mean disease lesion size. Letters on top of the bars indicate significantly different values from each other at $\alpha = 0.05$. [See online article for color version of this figure.]

may be a function of higher fungal growth in transgenic lines or its positive regulation, and possibly of the SA signaling pathway, by PAs in tomato.

The role of ET signaling in regulating Spd-mediated disease symptoms was further examined by following the expression of genes in the ET response pathway. The gene transcripts examined included the tomato transcription factors *SlEIL1* and *SlEIL2* (*EIN3-like1* and *2*), which are positive regulators of ET responses. EIN3, EIL1, and EIL2-like transcription factors are members of a redundant gene family in tomato with complex functions throughout plant development (Tieman et al., 2001) and in turn regulate *ERF1*, a transcription factor required for activation of defense response genes (Solano et al., 1998; Guo and Ecker, 2004). As shown in Figure 4C, a 6- and 4-fold increase in the expression of *SlEIL1* occurred in C4 and C15 transgenic plants, respectively, by 24 d postinfection, which was not observed in the wild type. The expression pattern of *SlEIL2* was variable: In the wild type and C4, these increased similarly, 2.5-fold, upon infection, but in C15 leaves, they were suppressed by 1.3-fold during infection (Fig. 4D). The expression of *SlERF1B* increased approximately 59-fold in the wild type compared to a relatively moderate 14- and 4-fold increase in C4 and C15 leaves, respectively, at 24 hpi, which is consistent with the disease susceptibility phenotypes of the transgenic lines (Fig. 4E). These results support our contention that impaired or altered expression of components of ET biosynthesis and signaling pathways account for enhanced susceptibility of high-Spd tomato plants to *B. cinerea*. Interestingly, transcripts of *β-1,3-glucanase*, another *PR* gene, increased in both the C4 and C15 transgenic plants upon *B. cinerea* infection, but this did not induce resistance (Fig. 4F). It is therefore likely that higher PA levels may contribute to general perturbations of cellular homeostasis that could subsequently cause a general up-regulation of PR proteins and disease susceptibility.

Treatment with ET Precursors Reverses Spd-Mediated Susceptibility of Tomato to *B. cinerea*

Another approach used to establish ET involvement in *B. cinerea-ySpdSyn* plant interaction was to treat the plants with precursors of ET biosynthesis and then challenge them with *B. cinerea*. Accordingly, C4 and C15 leaves were pretreated with 100 μM ACC or 200 μM SAM and then inoculated with *B. cinerea*. Results indicated that treatment with either ACC or SAM effectively mitigated the enhanced susceptibility of C4 and C15 leaves to *B. cinerea* (Figs. 3, A, D, and E, and 5). These data confirm that ET is an important factor in this interaction. With respect to plant growth responses, ET is known to produce a characteristic seedling growth response called the triple response (Guzmán and Ecker, 1990). However, in the presence of exogenous ACC, such a response was not observed with the transgenic C4 and C15 seedlings compared to the wild type (Supplemental Fig. S1). This observation suggests that PA-mediated changes in plant defense are separate from the function of ET in plant development.

Plant responses to *B. cinerea* also involve other plant hormones, such as abscisic acid (ABA) and JA (Anderson et al., 2004; Mauch-Mani and Mauch, 2005). Tomato and Arabidopsis mutants deficient in

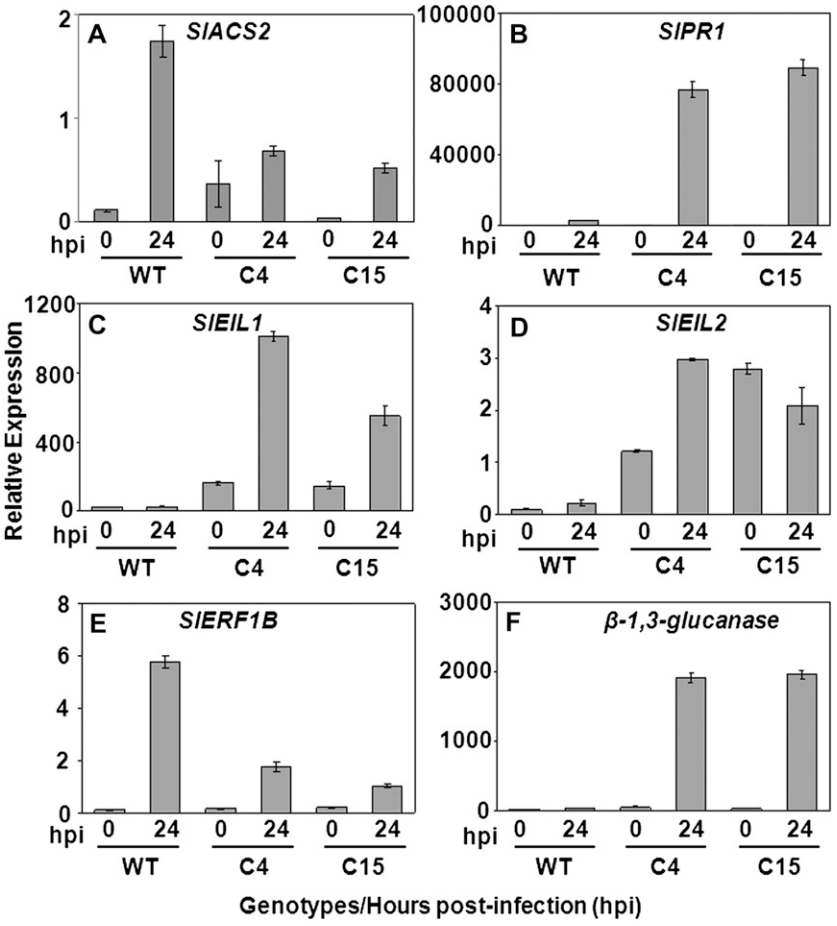

Figure 4. Expression of the *ySpdSyn* gene alters expression of the ET biosynthesis gene ACC synthase 2 (*SlACS2*; A), SA response marker gene *SlPR1* (B), ET signaling genes *SlEIL1* (C), *SlEIL2* (D), *SlERF1B* (E), and ET-dependent pathogenesis-related gene *β-1,3-glucanase* (F) in *B. cinerea*-inoculated and mock (water-treated) wild-type and T2 homozygous C4 and C15 leaves at 0 and 24 hpi. Quantitative RT-PCR analysis was used to quantify transcript levels of tomato. Relative expression was calculated by the ΔΔCt (cycle threshold) method using actin as the reference gene. Error bars represent SE ($n = 3$).

ABA were resistant to *B. cinerea* (Audenaert et al., 2002) and *P. syringae* (Thaler and Bostock, 2004; de Torres-Zabala et al., 2007), whereas inhibition of JA responses enhances susceptibility to *B. cinerea* (Kunkel and Brooks, 2002). Therefore, we tested wild-type and transgenic tomato seed germination for altered hormone sensitivity. Wild-type, C4, and C15 seeds were plated on medium containing methyl-JA (MeJA) or ABA, and their germination and/or root elongation was analyzed (Supplemental Fig. S2). Neither C4 nor C15 transgenic plants showed germination defects or differences in root elongation compared to the wild type in medium containing either hormone, suggesting that increased Spd does not interfere with responses to ABA or MeJA.

Spd-Accumulating Transgenic Lines Are Similar to the Wild Type in Responses to *A. solani*, *P. syringae*, and Tobacco Hornworm

The C4 and C15 transgenic plants were also tested for responses to other virulent pathogens, such as *A. solani* (the causal agent of early blight), *P. syringae* (the bacterial speck disease), as well as tobacco hornworm *M. sexta*. Inoculation with *A. solani* did not result in enhanced lesion development in transgenic C4 and C15 leaves compared to the wild type (Fig. 6A). Infiltration of leaves with *P. syringae* and subsequent analysis of bacterial growth revealed no significant changes in bacterial titer 3 d after infiltration (Fig. 6B). In plants exposed to tobacco hornworm larvae, increased defoliation was not observed (data not shown), but slight, though insignificant, differences in larval weight between the wild-type and the transgenic leaves were apparent. The leaves of C15 transgenic line were relatively more inhibitory to larval growth (Fig. 6C).

Increase in Spd Levels Does Not Alter Responses to Oxidative Stress

The response of wild-type, C4, and C15 seedlings to oxidative stress was measured by determining seedling growth in the presence and absence of H_2O_2. As shown in Supplemental Figure S2, seedling growth of all the three genotypes was lower in the presence of H_2O_2. However, no differences were apparent in root or shoot growth between the wild-type, C4, and C15 seedlings (Supplemental Fig. S2). Additionally, treatment of leaves with methyl viologen did not exhibit any difference in response among the three genotypes (Supplemental Fig. S3). We interpret these data to

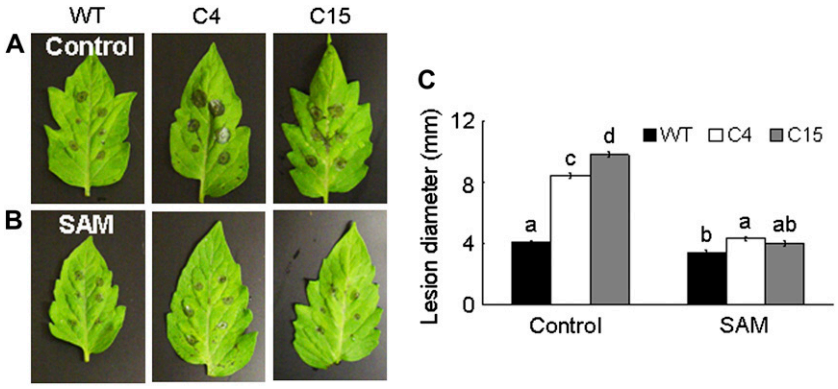

Figure 5. Pretreatment with SAM decreases susceptibility of T2 homozygous *ySpdSyn* transgenic tomato leaves to *B. cinerea* infection. A and B, Detached leaves were pretreated for 24 h with water (mock control; A) or 200 μM SAM (B) and spot inoculated with 3×10^5 spores/mL of *B. cinerea*. Leaves were photographed 72 hpi. C, Disease lesion diameter determined 72 hpi. Error bars indicate SE ($n \geq 50$). Statistical analysis was performed using ANOVA and Tukey's honestly significant difference test for the separation of mean disease lesion size. Letters on top of the bars indicate significantly different values from each other at $\alpha = 0.05$. [See online article for color version of this figure.]

suggest that increased Spd in transgenic leaves does not alleviate oxidative stress caused by H_2O_2 or methyl viologen.

DISCUSSION

We present molecular and pharmacological evidence suggesting that Spd plays a significant and specific role in tomato response to the necrotrophic fungal pathogen *B. cinerea*. We further show that the Spd-mediated enhanced susceptibility of transgenic tomato plants to *B. cinerea* is associated with reduced expression of *SlACS*, a key regulatory gene in ET biosynthesis. The suppression of ET biosynthesis gene is associated with the attenuated expression of the ET response gene *SlERF1B* in the two transgenic plants upon challenge with *B. cinerea*.

Two transgenic tomato lines engineered to accumulate Spd showed enhanced susceptibility to *B. cinerea*, with the C4 line being more prone to this pathogen than the C15 line. Increases in the Spd levels in the transgenic lines were moderate (1.3- to 2-fold higher), yet the susceptibility to *B. cinerea* was greatly increased. Moderate changes in PA levels, especially increases in Spd, have been shown to cause significant physiological changes in transgenic Arabidopsis, rice (*Oryza sativa*), and pear (*Pyrus communis*) plants overexpressing *SpdSyn* and *SAMdc* genes (Kasukabe et al., 2004; Wen et al., 2008; Peremarti et al., 2009). Unsuccessful attempts to obtain stable transgenic potato plants expressing a potato *SAMdc* under the CaMV35S promoter, while *SAMdc* antisense lines led to a range of stunted phenotypes also support this contention (Kumar et al., 1996). PAs play important roles during meiosis, sporulation, and cell division and thus have been suggested to modulate fungal development. Depletion of PAs is lethal for fungi (Tabor, 1981; Rajam and Galston, 1985; Walters, 1995). Use of DFMO and CHA, inhibitors of ODC and SpdSyn proteins, respectively, were effective in mitigating the growth of various fungal species, such as *B. cinerea* (Rajam and Galston, 1985; Saftner et al., 1997), *Tilletia* spp. (Trione et al., 1988), *Penicillium expansum* (Saftner et al., 1997), *Gaeumannomyces graminis* (West and Walters, 1989), and powdery mildew (*Erysiphe graminis* f. sp *hordei;* Mackintosh and Walters, 1998). In our study, treatment of plants with the two inhibitors of PA biosynthesis, DFMO and CHA, eliminated enhanced susceptibility of transgenic leaves to *B. cinerea*, while treatment with exogenous Spd increased the susceptibility of wild-type leaves, lending support to the hypothesis that Spd modulates host responses to infection. The application of PA biosynthesis inhibitors did not cause any significant alteration in lesion diameter in the wild-type leaves, suggesting that the concentrations of DFMO and CHA used did not influence PA levels enough to directly affect growth and/or survival of *B. cinerea*. However, the altered PA homeostasis in transgenic leaves treated with PA biosynthesis inhibitors was enough to influence host physiology and reduce lesions due to infection.

Spd-mediated enhanced susceptibility of transgenic tomato leaves suggests an impaired host-signaling network that leads to weakened immune responses against *B. cinerea* infection. Depending on the plant-pathogen interaction, treatment with ET may enhance resistance (Esquerré-Tugayé et al., 1979; El-Kazzaz et al., 1983b; Marte et al., 1993), induce susceptibility, or have no effect (El-Kazzaz et al., 1983a; Brown and Lee, 1993; Thomma et al., 1999). Our data indicate a crosstalk between PA and ET in modulating responses to *B. cinerea*. *SlACS* expression was induced in wild-type leaves upon *B. cinerea* infection but attenuated in transgenic plants. Consistent with this observation was the reversion of host susceptibility by ACC, an

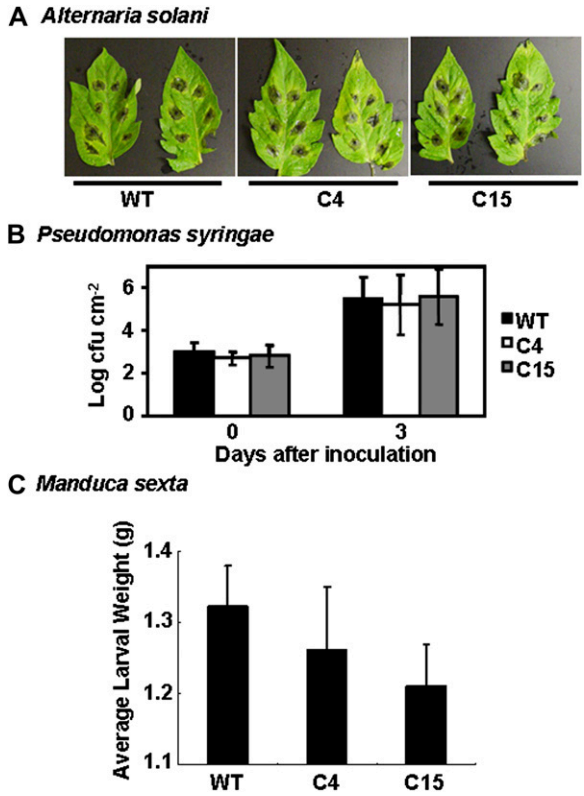

Figure 6. Responses of *ySpdSyn*-expressing tomato transgenic plants to *A. solani*, *P. syringae* pv *tomato* DC3000, and *M. sexta*. A, Wild-type and T2 homozygous transgenic C4 and C15 leaves were drop inoculated with 300 mg/mL *A. Solani* cultures, and lesion diameter was determined 7 d after inoculation and photographed. B, Wild-type, C4, and C15 leaves were infiltrated with *P. syringae* suspension (OD_{600} = 0.001). Bacterial colony-forming units (cfu) were determined 0 and 3 d after infiltration. Data represent average values ± SE from (n = 3). C, Four newly hatched tobacco hornworm larvae weighing 9 to 11 mg each were placed on each of six 8-week-old wild-type, C4, and C15 plants growing in a greenhouse. The larval weight was determined 14 d after the start of the feeding experiment. Data represent average values ± SE from (n = 24). [See online article for color version of this figure.]

ET biosynthesis precursor. Taken together, these results support the interpretation that Spd-induced susceptibility to *B. cinerea* is due to its interference with ET biosynthesis (*SlACS*) and response (*SlERF1B*) pathways.

The effect of Spd on downstream ET signaling was investigated to understand Spd-mediated alteration of ET-dependent resistance to *B. cinerea*. ET is sensed and bound by a family of membrane receptor proteins that activate *CTR1*, a negative regulator of the ET signaling cascade. Downstream, this signaling cascade is facilitated by the transcription factors EIN2 and EIN3, paralogs, and the EIL proteins (Solano et al., 1998; Stepanova and Ecker, 2000; Guo and Ecker, 2004). Three functionally redundant homologs of *Arabidopsis EIN3*, *SlEIL1* to *SlEIL3*, have been identified in tomato (Tieman et al., 2001). *ERF1* is an early response gene that has been implicated in several necrotrophic pathogen responses (Lorenzo et al., 2003). In line with Spd and ET interaction being competitive in *B. cinerea*-tomato interactions, we found that ET-responsive pathway genes, such as *SlERF1B*, but not *SlEIL1* and *SlEIL2*, displayed reduced expression in both the transgenic lines compared to the wild type (Fig. 4).

Genetic data in Arabidopsis and tomato have established the role of signaling molecules, such as SA and JA, in addition to ET, and their interactions in mediating responses to pathogen infection (Thomma et al., 1998, 1999; Alonso et al., 2003; Ferrari et al., 2003). *ERF1* integrates ET/JA-dependent responses during pathogen infection in Arabidopsis (Lorenzo et al., 2003), while JA-ET-mediated induction of PR genes was shown to play a role in *B. cinerea* infection (Thomma et al., 1999). However, in tomato, JA acts independently of ET in inducing resistance to *B. cinerea* (Díaz et al., 2002). The influence of JA on *SlERF1B* expression in *ySpdSyn* overexpressing lines remains to be established.

The role of SA-dependent signaling pathway against biotrophs is well documented (Glazebrook, 2005). Although the role of SA in Arabidopsis-*B.cinerea* interac-

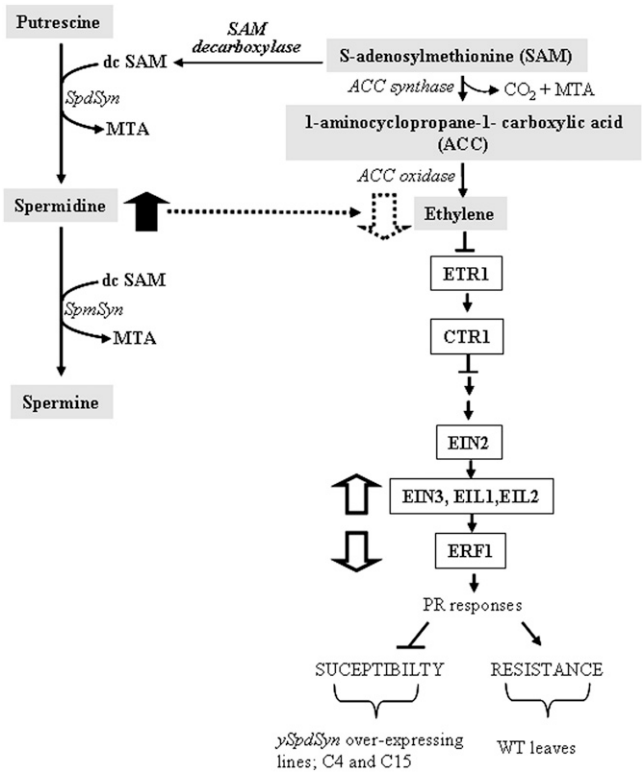

Figure 7. A model depicting interaction between PAs and ET biosynthesis and signaling during *B. cinerea* infection. The black upward arrow indicates increased Spd levels in C4 and C15 transgenic plants that attenuate ET biosynthesis gene *ACS* and may affect ET levels (dotted downward arrow). When induced, the ET response gene *ERF1* enhances resistance in wild-type leaves or leads to susceptibility in transgenic C4 and C15 leaves when impaired.

tion is controversial (Thomma et al., 1998; Zimmerli et al., 2001), SA has been reported to enhance tolerance to *B. cinerea* in tomato (Audenaert et al., 2002), tobacco (Murphy et al., 2000), and French beans (*Phaseolus vulgaris;* De Meyer and Höfte, 1997). *PR1* is induced by SA during defense responses (Thomma et al., 1998; Glazebrook, 2005; Abuqamar et al., 2009). We observed an increase in the expression of SA marker *SlPR1* in C4 and C15 transgenic (Fig. 4B). Increased expression of *PR1* was also observed in *BOTRYTIS-INDUCED KINASE1* (*BIK1*) Arabidopsis mutant plants that showed enhanced susceptibility to *B. cinerea* (Veronese et al., 2006). The role of *BIK1* in SA signaling is still unclear, and the increased growth of the fungus in the *bik1* mutant may have caused the enhanced expression of *PR1*.

SA signaling has been implicated in resistance of hydroponically grown tomatoes to another necrotroph, *A. solani.* Exogenously applied SA induced systemic acquired resistance in these tomatoes, which led to effective resistance against *A. solani* (Spletzer and Enyedi, 1999). Likewise, application of a chemical inducer of systemic acquired resistance, such as benzothiadiazole, conferred resistance to potato against *A. solani* (Bokshi et al., 2003). In this context, it is interesting that the transgenic C4 and C15 tomato plants were similar to the wild type in their response to inoculation with the hemibiotroph *P. syringae* or with *A. solani*. Previously, studies with Arabidopsis suggested that the ET-mediated disease resistance may be pathogen specific based on the type of the necrotroph since *ein2-1* mutants were more susceptible to *B. cinerea* but not *Alternaria brassicicola* (Thomma et al., 1999). Our studies are consistent with these findings. Therefore, observed expression of *PR1* in *ySpdSyn* transgenic plants might be a reflection of increased susceptibility to *B. cinerea* rather than being a marker of enhanced SA response. It is noted here that SA or PAs (Spd or Spm) when applied to tomato slices leads to suppression of *SlACS* transcripts and ET biosynthesis (Li et al., 1992), as depicted for *SlACS* here. Our work also differentiates Spd involvement from that of Spm in *B. cinerea* pathogenesis (Gonzalez et al., 2011) since the *ySpdSyn* transgenic plants are relatively deficient in Spm compared to the wild type and, notably, during *B. cinerea* infection Spm levels actually decreased in the wild type. Collectively, these data suggest a negative effect of increased Spd levels on ET synthesis and/or signaling, which leads to higher susceptibility of tomato leaves to *B. cinerea* (summarized in Fig. 7).

MATERIALS AND METHODS

Plant Growth

Tomato (*Solanum lycopersicum* cv OH 8245) plants were grown in plastic pots containing compost soil mix in a greenhouse with a photoperiod extended to 15 h under fluorescent lights (160 W mol^{-1} m^{-2} s^{-1}) at a temperature of 24°C ± 4°C.

Generation of Transgenic Plants

Transgenic lines expressing the *ySpdSyn* gene driven by the constitutive CaMV35S promoter were generated. The *ySpdSyn* was amplified from a yeast genomic library using the forward primer ScSpe3XhoF (5′-GCCGCTCGAGATGGCACAAGAAATCACTCACCCAA-3′) and reverse primer ScSpe3XbaR (5′-GCCGTCTAGACTAATTTAATTCCTTGGCTGCCCAG-3′) and cloned into the pGEM-T Easy vector system (Promega). The insert was excised using restriction endonucleases, *Xho*I and *Xba*I, and cloned in the sense orientation between a CaMV35S promoter and the 3′ end of a pea (*Pisum sativum*) rbcS-E9 gene in pKYLX71 (Schardl et al., 1987). This construct was introduced into disarmed *Agrobacterium tumefaciens* LBA4404 by chemical transformation. *A. tumefaciens* strains harboring the chimeric constructs were used to transform cotyledons of tomato cv OH 8245 (Tieman et al., 1992). Fifteen independent transgenic plants expressing *ySpdSyn* under the CaMV35S promoter were generated (Nambeesan et al., 2010). Based on the transcript and protein expression analysis of T0 transgenic plants, two independent transgenic plants, C4 and C15, were selected and used for further studies. Seeds from T1 seedlings were analyzed for selecting homozygous plants using PCR. Lines homozygous for the transgenes were selected after PCR analyses of seedlings from T2 seeds and used in studies presented here.

Fungal and Bacterial Disease Assay

The *Botrytis cinerea* strain *BO5-10* was used for disease assays. Fungal culture and preparation of conidial spore suspension were as described previously (AbuQamar et al., 2006). *B. cinerea* disease assays were done on detached leaves by drop inoculation of a conidial suspension on tomato leaves (3×10^5 spores/mL; AbuQamar et al., 2008), and lesion diameter was determined ($n = 60$). Wild-type and transgenic leaves were inoculated with 300 mg/mL *Alternaria solani* cultures, and lesion diameter was calculated 7 d after inoculation. Bacterial disease assays were done essentially as described (Mengiste et al., 2003). Fully expanded leaves of 6-week-old tomato plants were infiltrated with suspensions of the bacterial strain *Pseudomonas syringae* (Optical Density$_{600}$ = 0.001, approximately 5×10^5 colony-forming units/mL in 10 mM $MgCl_2$). Bacterial growth was determined using leaf discs from infected leaves at 0 and 3 d postinfection as described (Abuqamar et al., 2008). Bacterial titer per leaf area was determined in uniform leaf discs using a hole punch. Each experiment was performed in triplicate, and two leaf discs were collected from C4, C15, and wild-type plants for each replicate.

Tobacco Hornworm Feeding Trials

Tobacco hornworm *Manduca sexta* eggs and an artificial diet for the larvae were purchased from Carolina Biological Supply Company. As recommended by the supplier, eggs were hatched by incubation at 26°C. The artificial diet for the hatched larvae was continued for 3 d before transfer to detached leaves or whole tomato plants. For whole-plant assay, four larvae weighing 9 to 11 mg each were placed on each of six 8-week-old wild-type, C4, and C15 plants grown in the greenhouse.

The average larval weight at the beginning of the feeding trial was 7 to 9 mg for detached leaf assay and 9 to 11 mg for whole-plant assay. The insects were left to feed for 1 and 2 weeks for the detached leaf and whole-plant assays, respectively, after which the larval weight was determined (Abuqamar et al., 2008).

RNA-Blot, and Quantitative and Semiquantitative Reverse Transcription-PCR Analyses

Total RNA was extracted from frozen (in liquid nitrogen) tomato leaf tissues as described (Lagrimini et al., 1987). For RNA-blot analyses, RNA was separated on 1.2% formaldehyde agarose gels and blotted onto Hybond N^+ nylon membranes (Amersham Pharmacia Biotech). The probes were labeled with ^{32}P by random priming using a commercial kit (Sigma-Aldrich). Probe hybridization was performed as described (Church and Gilbert, 1984).

For semiquantitative reverse transcription (RT)-PCR analysis, cDNA synthesis was performed using 2 μg of total RNA from control and *B. cinerea*-treated leaves. RT was performed using AMV reverse transcriptase (Promega) and oligo(dT15) primers according to the manufacturer's instructions. PCR was performed using gene-specific primers and 2.5 μL cDNA as a template for

28 cycles. PCR conditions were 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The amplified products were subjected to electrophoresis on 1.5% agarose gels and visualized under UV light after ethidium bromide staining. The primers used are shown in Supplemental Table S1. The tomato translation initiation factor (eIF4A) gene was used as a control for equal cDNA loading between samples.

For real-time PCR analysis, RNA extraction, cDNA synthesis, and quantitative RT-PCR were performed as previously described (Dhawan et al., 2009). Quantitative RT-PCR was performed using gene-specific primers (Supplemental Table S1), with tomato actin used as an endogenous reference for normalization.

Inhibitor Treatments

Wild-type and transgenic tomato plants were grown for 6 weeks in the greenhouse using compost soil mix with a photoperiod of 16 h/8 h (day/night) at a day/night temperature of 23°C/18°C. For growth regulator treatments, leaves were clipped and the petiole was immersed either in 1 mM DFMO (a gift from Dr. Patrick M. Woster, Wayne State University, MI), 1 mM CHA (Sigma-Aldrich), 100 μM ACC, 1 mM Spd (Sigma-Aldrich), 200 μM SAM (Sigma-Aldrich), or water (control). After 24 h of treatment, the leaves were placed on plates and inoculated with *B. cinerea* as described above.

PA Quantification

Tomato leaf tissue (200 mg) infected with *B. cinerea* was sampled at 0, 24, and 48 d postinfection, ground in liquid N_2, and resuspended in 800 μL of 5% (w/v) cold perchloric acid per 0.2 g of ground tissue (Minocha et al., 1994). The samples were mixed and centrifuged at 20,000*g* for 30 min. To each 100 μL of the supernatant, 20 μL of 0.1 mM of an internal standard, 1,7 diaminoheptane, was added. The samples were dansylated at 60°C for 1 h using 100 μL of 20 mg/mL dansyl chloride (in acetone) with 100 μL saturated sodium carbonate. L-Asn (50 μL of 20 mg/mL) was added to terminate the reaction at 60°C for 30 min. The samples were placed in a SpeedVac for 30 min to evaporate acetone, after which 400 μL of toluene was added to the sample, which was mixed and centrifuged. This was followed by removing 200 μL of toluene layer and air-drying it. The sample was dissolved in methanol (1 mL) by mixing for 1 min, and 50 μL of the filtered sample (0.45 μm filter; National Scientific) was injected into a Waters HPLC system consisting of two model 510 pumps and a model 715 WISP autosampler. A reverse-phase Xterra C18 (3.8 × 100mm) column was used to separate dansylated PAs. Detection was performed using a Hewlett Packard model 1046A fluorescence detector (excitation and emission wavelengths were 340 and 510 nm, respectively). A binary gradient composed of solvent A was used, 100% acetonitrile, and solvent B, heptanesulfonate (10 mM, pH 3.4):acetonitrile (90:10), and a 1.1 mL/min flow rate was maintained. A gradient elution was used where initial conditions were set at 50:50 (A:B) followed by a linear gradient as follows: 80:20 (A:B) at 2 min, 100:0 (A:B) at 9 min, 80:20 (A:B) at 12 min, and back to 50:50 (A:B) at 20 min. Authentic standards of PAs (Sigma-Aldrich) were similarly extracted and analyzed as described above. For quantification, the peaks were integrated. Quantification of PA was performed in triplicate with each replicate consisting of at least three leaves.

ET Response Assays

Seeds were surface sterilized with 70% ethanol for 2 min followed by a treatment with 35% commercial bleach (5.25% [w/v] sodium hypochlorite) plus 0.1% Tween 20 for 30 min in solution and finally thoroughly rinsed with water to remove the bleach. For the triple response assay, tomato seeds were cultured on Murashige and Skoog (MS) medium and 0.8% agar with or without ACC and incubated in the dark for 6 d (Abuqamar et al., 2008).

Hormone Treatments

Seeds prepared as above were plated in vitro on the medium containing MS with 0.8% agar with or without 10 μM MeJA or 2 μM ABA. Seed germination was recorded after 7 d of growth. For ABA transfer assay, seeds were initially germinated on MS media for 3 d and then transferred to MS media supplemented with or without 10 μM ABA, and seedling growth was studied. Solutions of MeJA and ABA used in the experiments reported here were prepared and used as described previously (Anderson et al., 2004).

Supplemental Data

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

Supplemental Figure S1. Expression of *ySpdSyn* does not alter the triple response of wild-type and T2 homozygous C4 and C15 transgenic seedlings in the presence of exogenous ACC.

Supplemental Figure S2. Expression of *ySpdSyn* did not alter germination in the presence of exogenous hydrogen peroxide (H_2O_2), methyl-JA (MeJA), or ABA.

Supplemental Figure S3. Response of wild-type and T2 homozygous C4 and C15 transgenic leaves were similar after treatment with methyl viologen (MV).

Supplemental Table S1. Sequences of forward (F) and reverse (R) primers used for gene expression studies shown in Figures 2C and 4.

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LITERATURE CITED

Abuqamar S, Chai MF, Luo H, Song F, Mengiste T (2008) Tomato protein kinase 1b mediates signaling of plant responses to necrotrophic fungi and insect herbivory. Plant Cell **20:** 1964–1983

AbuQamar S, Chen X, Dhawan R, Bluhm B, Salmeron J, Lam S, Dietrich RA, Mengiste T (2006) Expression profiling and mutant analysis reveals complex regulatory networks involved in *Arabidopsis* response to *Botrytis* infection. Plant J **48:** 28–44

Abuqamar S, Luo H, Laluk K, Mickelbart MV, Mengiste T (2009) Crosstalk between biotic and abiotic stress responses in tomato is mediated by the *AIM1* transcription factor. Plant J **58:** 347–360

Alcázar R, García-Martínez JL, Cuevas JC, Tiburcio AF, Altabella T (2005) Overexpression of ADC2 in *Arabidopsis* induces dwarfism and late-flowering through GA deficiency. Plant J **43:** 425–436

Alonso JM, Stepanova AN, Solano R, Wisman E, Ferrari S, Ausubel FM, Ecker JR (2003) Five components of the ethylene-response pathway identified in a screen for *weak ethylene-insensitive* mutants in *Arabidopsis*. Proc Natl Acad Sci USA **100:** 2992–2997

Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmond OJ, Ehlert C, Maclean DJ, Ebert PR, Kazan K (2004) Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. Plant Cell **16:** 3460–3479

Audenaert K, De Meyer GB, Höfte MM (2002) Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. Plant Physiol **128:** 491–501

Bakanashvili M, Barkai-Golan R, Kopeliovith E, Apelbaum A (1987) Polyamine biosynthesis in *Rhizopus*-infected tomato fruits: possible interaction with ethylene. Physiol Mol Plant Pathol **31:** 41–50

Bari R, Jones JDG (2009) Role of plant hormones in plant defence responses. Plant Mol Biol **69:** 473–488

Berrocal-Lobo M, Molina A, Solano R (2002) Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in *Arabidopsis* confers resistance to several necrotrophic fungi. Plant J **29:** 23–32

Bokshi AI, Morris SC, Deverall BJ (2003) Effects of benzothiadiazole and acetylsalicylic acid on beta-1,3-glucanase activity and disease resistance in potato. Plant Pathol **52:** 22–27

Bouchereau A, Aziz A, Larher F, Martin-Tanguy J (1999) Polyamines and environmental challenges: recent development. Plant Sci **140:** 103–125

Brown GE, Lee HS (1993) Interaction of ethylene with citrus stem-end rot caused by *Diplodia natalensis*. Phytopathology **83:** 1204–1208

Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA **81:** 1991–1995

De Meyer G, Höfte M (1997) Salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 induces resistance to leaf infection by *Botrytis cinerea* in bean. Phytopathology **87:** 588–593

Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature **394:** 585–588

de Torres-Zabala M, Truman W, Bennett MH, Lafforgue G, Mansfield JW, Rodriguez Egea P, Bögre L, Grant M (2007) *Pseudomonas syringae* pv. *tomato* hijacks the *Arabidopsis* abscisic acid signalling pathway to cause disease. EMBO J **26:** 1434–1443

Dhawan R, Luo H, Foerster AM, Abuqamar S, Du HN, Briggs SD, Mittelsten Scheid O, Mengiste T (2009) HISTONE MONOUBIQUITINATION1 interacts with a subunit of the mediator complex and regulates defense against necrotrophic fungal pathogens in *Arabidopsis*. Plant Cell **21:** 1000–1019

Díaz J, ten Have A, van Kan JA (2002) The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. Plant Physiol **129:** 1341–1351

Edreva A (1997) Tobacco polyamines as affected by stresses induced by different pathogens. Biol Plant **40:** 317–320

El-Kazzaz MK, Sommer NF, Fortlage RJ (1983a) Effects of different atmospheres on postharvest decay and quality of fresh strawberries. Phytopathology **73:** 282–285

El-Kazzaz MK, Sommer NF, Kader AA (1983b) Ethylene effects on in vitro and in vivo growth of certain postharvest fruit-infecting fungi. Phytopathology **73:** 998–1001

El Oirdi M, El Rahman TA, Rigano L, El Hadrami A, Rodriguez MC, Daayf F, Vojnov A, Bouarab K (2011) *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. Plant Cell **23:** 2405–2421

Esquerré-Tugayé MT, Lafitte C, Mazau D, Toppan A, Touzé A (1979) Cell surfaces in plant-microorganism interactions: II. Evidence for the accumulation of hydroxyproline-rich glycoproteins in the cell wall of diseased plants as a defense mechanism. Plant Physiol **64:** 320–326

Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM (2003) *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. Plant J **35:** 193–205

Fluhr R, Mattoo AK (1996) Ethylene-biosynthesis and perception. Crit Rev Plant Sci **15:** 479–523

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol **43:** 205–227

Gonzalez ME, Marco F, Minguet EG, Carrasco-Sorli P, Blázquez MA, Carbonell J, Ruiz OA, Pieckenstain FL (2011) Perturbation of *spermine synthase* gene expression and transcript profiling provide new insights on the role of the tetraamine spermine in Arabidopsis defense against *Pseudomonas viridiflava*. Plant Physiol **156:** 2266–2277

Greenland AJ, Lewis DH (1984) Amines in barley leaves infected with brown rust and their possible relevance to formation of 'green-islands'. New Phytol **96:** 283–291

Groll M, Schellenberg B, Bachmann AS, Archer CR, Huber R, Powell TK, Lindow S, Kaiser M, Dudler R (2008) A plant pathogen virulence factor inhibits the eukaryotic proteasome by a novel mechanism. Nature **452:** 755–758

Guo HW, Ecker JR (2004) The ethylene signaling pathway: new insights. Curr Opin Plant Biol **7:** 40–49

Guzmán P, Ecker JR (1990) Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell **2:** 513–523

Han L, Li GJ, Yang KY, Mao G, Wang R, Liu Y, Zhang S (2010) Mitogen-activated protein kinase 3 and 6 regulate *Botrytis cinerea*-induced ethylene production in *Arabidopsis*. Plant J **64:** 114–127

Handa AK, Mattoo AK (2010) Differential and functional interactions emphasize the multiple roles of polyamines in plants. Plant Physiol Biochem **48:** 540–546

Igarashi K, Kashiwagi K (2006) Polyamine modulon in *Escherichia coli*: genes involved in the stimulation of cell growth by polyamines. J Biochem **139:** 11–16

Kasukabe Y, He L, Nada K, Misawa S, Ihara I, Tachibana S (2004) Overexpression of spermidine synthase enhances tolerance to multiple environmental stresses and up-regulates the expression of various stress-regulated genes in transgenic *Arabidopsis thaliana*. Plant Cell Physiol **45:** 712–722

Kaur-Sawhney R, Tiburcio AF, Altabella T, Galston AW (2003) Polyamines in plants: an overview. J Mol Cell Biol **2:** 1–12

Knoester M, van Loon LC, van den Heuvel J, Hennig J, Bol JF, Linthorst HJ, (1998) Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. Proc Natl Acad Sci USA **95:** 1933–1937

Kumar A, Taylor M, Mad Arif SA, Davies H (1996) Potato plants expressing antisense and sense S adenosylmethionine decarboxylase (SAMDC) transgenes show altered levels of polyamines and ethy1ene: antisense plants display abnormal phenotypes. Plant J **9:** 147–158

Kumar V, Mills DJ, Anderson JD, Mattoo AK (2004) An alternative agriculture system is defined by a distinct expression profile of select gene transcripts and proteins. Proc Natl Acad Sci USA **101:** 10535–10540

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. Curr Opin Plant Biol **5:** 325–331

Lagrimini LM, Burkhart W, Moyer M, Rothstein S (1987) Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. Proc Natl Acad Sci USA **84:** 7542–7546

Laluk K, Luo H, Chai M, Dhawan R, Lai Z, Mengiste T (2011) Biochemical and genetic requirements for function of the immune response regulator BOTRYTIS-INDUCED KINASE1 in plant growth, ethylene signaling, and PAMP-triggered immunity in *Arabidopsis*. Plant Cell **23:** 2831–2849

Li N, Parsons BL, Liu DR, Mattoo AK (1992) Accumulation of wound-inducible ACC synthase transcript in tomato fruit is inhibited by salicylic acid and polyamines. Plant Mol Biol **18:** 477–487

Lloyd AJ, William Allwood J, Winder CL, Dunn WB, Heald JK, Cristescu SM, Sivakumaran A, Harren FJM, Mulema J, Denby K, et al (2011) Metabolomic approaches reveal that cell wall modifications play a major role in ethylene-mediated resistance against *Botrytis cinerea*. Plant J **67:** 852–868

Lorenzo O, Piqueras R, Sánchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15:** 165–178

Lumbreras V, Vilela B, Irar S, Solé M, Capellades M, Valls M, Coca M, Pagès M (2010) MAPK phosphatase MKP2 mediates disease responses in *Arabidopsis* and functionally interacts with MPK3 and MPK6. Plant J **63:** 1017–1030

Mackintosh CA, Walters DR (1998) Effects of cyclohexylamine and norspermidine on powdery mildew infection of spring barley. Int J Pest Man **44:** 181–184

Marte M, Buonaurio R, Dellatorre G (1993) Induction of systemic resistance to tobacco powdery mildew by tobacco mosaic virus, tobacco necrosis virus or ethephon. J Phytopathol **138:** 137–144

Martin-Tanguy J (1997) Conjugated polyamines and reproductive development: biochemical and physiological approaches. Physiol Plant **100:** 675–688

Mattoo AK, Handa AK (2008) Higher polyamines restore and enhance metabolic memory in ripening fruit. Plant Sci **174:** 386–393

Mauch-Mani B, Mauch F (2005) The role of abscisic acid in plant-pathogen interactions. Curr Opin Plant Biol **8:** 409–414

Mehta RA, Cassol T, Li N, Ali N, Handa AK, Mattoo AK (2002) Engineered polyamine accumulation in tomato enhances phytonutrient content, juice quality, and vine life. Nat Biotechnol **20:** 613–618

Mengiste T, Chen X, Salmeron J, Dietrich R (2003) The *BOTRYTIS SUSCEPTIBLE1* gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. Plant Cell **15:** 2551–2565

Minocha R, Shortle W, Long S, Minocha S (1994) A rapid and reliable procedure for extraction of cellular polyamines and inorganic ions from plant tissues. J Plant Growth Regul **13:** 187–193

Mitsuya Y, Takahashi Y, Berberich T, Miyazaki A, Matsumura H, Takahashi H, Terauchi R, Kusano T (2009) Spermine signaling plays a significant role in the defense response of *Arabidopsis thaliana* to cucumber mosaic virus. J Plant Physiol **166:** 626–643

Murphy AM, Holcombe LJ, Carr JP (2000) Characteristics of salicylic acid-induced delay in disease caused by a necrotrophic fungal pathogen in tobacco. Physiol Mol Plant Pathol **57:** 47–54

Nambeesan S, Datsenka T, Ferruzzi MG, Malladi A, Mattoo AK, Handa AK (2010) Overexpression of yeast spermidine synthase impacts ripening, senescence and decay symptoms in tomato. Plant J **63:** 836–847

Nambeesan S, Mattoo AK, Handa AK (2008) Polyamines and regulation of ripening and senescence. *In* G Paliyath, DP Murr, AK Handa, S Lurie,

eds, Postharvest Biology and Technology of Fruits, Vegetables, and Flowers. Wiley-Blackwell Publishing, Ames, IA, pp 319–340

Peremarti A, Bassie L, Christou P, Capell T (2009) Spermine facilitates recovery from drought but does not confer drought tolerance in transgenic rice plants expressing *Datura stramonium* S-adenosylmethionine decarboxylase. Plant Mol Biol **70:** 253–264

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol **5:** 308–316

Rajam MV, Galston AW (1985) The effects of some polyamine biosynthetic inhibitors on growth and morphology of phytopathogenic fungi. Plant Cell Physiol **26:** 683–692

Saftner RA, Conway WS, Sams CE (1997) Effects of some polyamine biosynthesis inhibitors and calcium chloride on *in vitro* growth and decay development in apples caused by *Botrytis cinerea* and *Penicillium expansum*. J Am Soc Hortic Sci **122:** 380–385

Schardl CL, Byrd AD, Benzion G, Altschuler MA, Hildebrand DF, Hunt AG (1987) Design and construction of a versatile system for the expression of foreign genes in plants. Gene **61:** 1–11

Solano R, Stepanova A, Chao Q, Ecker JR (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev **12:** 3703–3714

Spletzer ME, Enyedi AJ (1999) Salicylic acid induces resistance to *Alternaria solani* in hydroponically grown tomato. Phytopathology **89:** 722–727

Spoel SH, Dong X (2008) Making sense of hormone crosstalk during plant immune responses. Cell Host Microbe **3:** 348–351

Srivastava A, Chung SH, Fatima T, Datsenka T, Handa AK, Mattoo AK (2007) Polyamines as anabolic growth regulators revealed by transcriptome analysis and metabolite profiles of tomato fruits engineered to accumulate spermidine and spermine. Plant Biotechnol **24:** 57–70

Stepanova AN, Ecker JR (2000) Ethylene signaling: from mutants to molecules. Curr Opin Plant Biol **3:** 353–360

Tabor CW (1981) Mutants of *Saccharomyces cerevisiae* deficient in polyamine biosynthesis: studies on the regulation of ornithine decarboxylase. Med Biol **59:** 272–278

Thaler JS, Bostock RM (2004) Interactions between abscisic acid-mediated responses and plant resistance to pathogens and insects. Ecology **85:** 48–58

Thomma BPHJ, Eggermont K, Penninckx IA, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci USA **95:** 15107–15111

Thomma BPHJ, Eggermont K, Tierens KFMJ, Broekaert WF (1999) Requirement of functional *ethylene-insensitive 2* gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiol **121:** 1093–1102

Tieman DM, Ciardi JA, Taylor MG, Klee HJ (2001) Members of the tomato LeEIL (EIN3-like) gene family are functionally redundant and regulate ethylene responses throughout plant development. Plant J **26:** 47–58

Tieman DM, Harriman RW, Ramamohan G, Handa AK (1992) An antisense pectin methylesterase gene alters pectin chemistry and soluble solids in tomato fruit. Plant Cell **4:** 667–679

Torget R, Lapi L, Cohen SS (1979) Synthesis and accumulation of polyamines and S-adenosylmethionine in Chinese cabbage infected by turnip yellow mosaic virus. Biochem Biophys Res Commun **87:** 1132–1139

Trione EJ, Stockwell VO, Austin HA (1988) The effects of polyamines on the growth and development of the wheat bunt fungi. Bot Gaz **149:** 173–178

Tun NN, Santa-Catarina C, Begum T, Silveira V, Handro W, Floh EIS, Scherer GFE (2006) Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. Plant Cell Physiol **47:** 346–354

Uehara Y, Takahashi Y, Berberich T, Miyazaki A, Takahashi H, Matsui K, Ohme-Takagi M, Saitoh H, Terauchi R, Kusano T (2005) Tobacco ZFT1, a transcriptional repressor with a Cys_2/His_2 type zinc finger motif that functions in spermine-signaling pathway. Plant Mol Biol **59:** 435–448

Veress I, Haghighi S, Pulkka A, Pajunen A (2000) Changes in gene expression in response to polyamine depletion indicates selective stabilization of mRNAs. Biochem J **346:** 185–191

Veronese P, Nakagami H, Bluhm B, Abuqamar S, Chen X, Salmeron J, Dietrich RA, Hirt H, Mengiste T (2006) The membrane-anchored *BOTRYTIS-INDUCED KINASE1* plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. Plant Cell **18:** 257–273

Waie B, Rajam MV (2003) Effect of increased polyamine biosynthesis on stress response in transgenic tobacco by introduction of human S-adenosylmethionine gene. Plant Sci **164:** 727–734

Walters DR (1995) Inhibition of polyamine biosynthesis in fungi. Mycol Res **2:** 129–139

Walters DR (2003a) Polyamines and plant disease. Phytochemistry **64:** 97–107

Walters DR (2003b) Resistance to plant pathogens: possible roles for free polyamines and polyamine catabolism. New Phytol **159:** 109–115

Walters DR, Meurer-Grimes B, Rovira I (2001) Antifungal activity of three spermidine conjugates. FEMS Microbiol Lett **201:** 255–258

Walters DR, Wilson PWF, Shuttleton MA (1985) Relative changes in levels of polyamines and activities of biosynthetic enzymes in barley infected-with the powdery mildew fungus, *Erysiphe graminis* D.C. ex Merat f.sp. *hordei* Marchal. New Phytol **101:** 695–705

Wen XP, Pang XM, Matsuda N, Kita M, Inoue H, Hao YJ, Honda C, Moriguchi T (2008) Over-expression of the apple spermidine synthase gene in pear confers multiple abiotic stress tolerance by altering polyamine titers. Transgenic Res **17:** 251–263

West HM, Walters DR (1989) Effects of polyamine biosynthesis inhibitors on growth of *Pyrenophora teres, Gaeumannomyces graminis, Fusarium culmorum* and *Septoria nodorum*. Mycol Res **92:** 453–457

Yoshida M, Kashiwagi K, Shigemasa A, Taniguchi S, Yamamoto K, Makinoshima H, Ishihama A, Igarashi K (2004) A unifying model for the role of polyamines in bacterial cell growth, the polyamine modulon. J Biol Chem **279:** 46008–46013

Zheng L, Campbell M, Murphy J, Lam S, Xu JR (2000) The *BMP1* gene is essential for pathogenicity in the gray mold fungus *Botrytis cinerea*. Mol Plant Microbe Interact **13:** 724–732

Zimmerli L, Métraux JP, Mauch-Mani B (2001) β-Aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea*. Plant Physiol **126:** 517–523