Defense Against *Sclerotinia sclerotiorum* in *Arabidopsis* Is Dependent on Jasmonic Acid, Salicylic Acid, and Ethylene Signaling

Xiaomei Guo and Henrik U. Stotz

Department of Horticulture, Oregon State University, Corvallis, Oregon 97330, U.S.A.

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Genotypic differences in susceptibility of *Arabidopsis thaliana* to *Sclerotinia sclerotiorum* have not been reported due to the extreme susceptibility of this cruciferous plant. To overcome this limitation, we have established inoculation conditions that enable evaluation of differences in susceptibility to *S. sclerotiorum* among *Arabidopsis* mutants and ecotypes. Two *coi1* mutant alleles conferred hypersusceptibility to *S. sclerotiorum*. The plant defensin gene *PDF1.2* was no longer induced after challenging the *coi1-2* mutant with *S. sclerotiorum*. Hypersusceptibility of the *coi1-2* mutant to *S. sclerotiorum* was not correlated with oxalate sensitivity. The mutants *npr1* and *ein2* were also hypersusceptible to *S. sclerotiorum*. Induction of *PDF1.2* and the pathogenesis-related gene *PR1* was reduced in *ein2* and *npr1* mutants, respectively. Actigard, a commercial formulation of the systemic acquired resistance inducer benzothiadiazole, reduced susceptibility to *S. sclerotiorum*. Based on histochemical analysis of oxalate-deficient and wild-type strains of *S. sclerotiorum*, oxalate caused a decrease in hydrogen peroxide production but no detectable changes in plant superoxide production or gene expression.

Additional keywords: H₂O₂, SAR.

Protection of plants against pathogens depends on constitutive and induced defense mechanisms (Nürnberger and Lipka 2005). Various phytohormones are crucial for induced and systemic defense signaling (Lorenzo and Solano 2005). Extensive research has shown that salicylic acid (SA) plays a key role in local and systemic acquired resistance (SAR) to biotrophic pathogens (Durrant and Dong 2004; Gaffney et al. 1993). Recently, SA signaling was implicated in local resistance to the necrotrophic fungal pathogen *Botrytis cinerea* (Ferrari et al. 2003), but *B. cinerea* does not trigger SAR (Govrin and Levine 2002). Induced systemic resistance depends on jasmonic acid (JA) and ethylene (ET) signaling (Pieterse et al. 1998). Both of these hormonal signaling pathways have been shown to be particularly important for defense against necrotrophic pathogens such as *B. cinerea* (Thomma et al. 1998, 1999). Nevertheless, it has been acknowledged that the dichotomy of SA and JA/ET signaling for defense against biotrophic and necrotrophic pathogens, respectively, is an oversimplification (Glazebrook 2005; Thomma et al. 2001).

Sclerotinia sclerotiorum is a necrotrophic ascomycete closely related to *B. cinerea*. This destructive pathogen attacks more than 400 plant species, including *Arabidopsis thaliana*

(Boland and Hall 1994). *S. sclerotiorum* is distributed around the world (Purdy 1979) and causes annual crop losses exceeding \$200 million in the United States alone (Bolton et al. 2006). Despite this substantial impact on agricultural production, surprisingly little is known about the molecular mechanisms of host resistance to *S. sclerotiorum*.

Arabidopsis has been proposed as a host model to study interactions with *S. sclerotiorum* (Dickman and Mitra 1992). Like other host species, *Arabidopsis* is resistant to oxalate-deficient *S. sclerotiorum* mutants but susceptible to wild-type fungus (Godoy et al. 1990). However, the extreme susceptibility of *Arabidopsis* to *S. sclerotiorum* has precluded discerning genetic determinants of host resistance or susceptibility (Dai et al. 2006).

Pathological effects of oxalic acid have long been recognized (de Bary 1886). Oxalic acid acidifies infected host tissues. This action assists in destruction of host cell walls, because polygalacturonase and other hydrolytic enzymes have an acidic pH-optimum (Bateman and Beer 1965; Lumsden 1979). Oxalate is also a strong chelator able to remove Ca²⁺ ionically bound to pectin. This further enhances polygalacturonase activity, which is dependent on removal of calcium from its substrate. Oxalic acid has been shown to suppress the oxidative burst of host plants (Cessna et al. 2000), but its causal relationship to virulence remains to be determined. Moreover, deregulation of guard cells by oxalic acid is, at least partially, responsible for wilting symptoms induced by *S. sclerotiorum* (Guimaraes and Stotz 2004).

Herein, we describe an improved protocol of inoculating rosette leaves of *Arabidopsis*, which has made quantification of differences in susceptibility among wild-type plants and mutants with defects in JA, SA, and ET signaling possible. Interest in defense signaling was triggered by the results of a screen for oxalate sensitivity using a library of *Saccharomyces cerevisiae* deletion mutants. A *Arib4* mutant, deleted for the gene encoding lumazine synthase, was found to be one of the 19 most sensitive mutants, failing to grow at oxalic acid concentrations as low as 1 μ M (Cheng et al. 2007). *COS1*, the *Arabidopsis* ortholog of *RIB4*, has been identified as a suppressor of a *coi1* (coronatine insensitive) mutant, and was thus implicated in JA signaling (Xiao et al. 2004). Based on analysis of *coi1* (Xie et al. 1998), *npr1* (Cao et al. 1997), and *ein2* mutants (Alonso et al. 1999), the current study provides evidence for a role of all three defense pathways in defense against *S. sclerotiorum*.

RESULTS

Susceptibility of JA signaling mutants to *S. sclerotiorum*.

A new inoculation procedure was developed to detect genotypic variation in susceptibility of *Arabidopsis* to *S. scler-*

Corresponding author: Henrik U. Stotz; Telephone: +1.541.737.5468; Fax: +1.541.737.3479; E-mail: stotzhe@hort.oregonstate.edu

rotiorum. It proved critical to limit nutrient availability to the fungus by using a minimal medium (Cruickshank 1983) to overcome the inherent susceptibility of *Arabidopsis* (Dai et al. 2006; Dickman and Mitra 1992; Guimaraes and Stotz 2004). Endo-polygalacturonase expression has been shown to be down-regulated when *S. sclerotiorum* was cultured on minimal medium relative to potato dextrose agar (PDA) (Li et al. 2004).

A significant delay in symptom development was evident when wild-type plants were inoculated with fungus grown on

minimal medium relative to PDA (Fig. 1). Consequently, minimal medium was used for all of the inoculation trials with wild-type *S. sclerotiorum* reported in this study. A soft-rotting necrosis occurred as early as 1 day postinoculation. Expansion of the water-soaked lesion led to browning and collapse of the infected leaf tissue. At this stage, fungus grown on PDA consumed approximately 50% of the inoculated leaf. Differences in lesion size were measured as long as inoculated leaves were partially alive. Relative to minimal medium, in vitro cultiva-

Fig. 1. Establishment of conditions to determine quantitative differences in susceptibility of *Arabidopsis* to *Sclerotinia sclerotiorum*. Nutrient-dependent differences in lesion expansion are shown. Wild-type *S. sclerotiorum* was cultivated on minimal medium (left column) or potato dextrose agar (PDA) prior to inoculation of *Arabidopsis* (Col-0). Disease progression is shown from days 0 to 4 after inoculation. Arrows indicate inoculated leaves. The collapsed leaf indicated with the pink arrow has been enlarged to visualize hyphae. The white arrowhead points towards a hypha. The width of the pots is 7.2 cm.

tion of *S. sclerotiorum* on PDA resulted in a 1-day-earlier consumption of inoculated leaves. Once the entire leaf was consumed, mycelia emerged from the infected tissue. As a result, lesion size could no longer be determined. Instead, the percentage of whole-plant decay was estimated.

To evaluate the possible role of JA signaling in defense against *S. sclerotiorum* and in oxalate tolerance, mutations in two different genes were analyzed. Two mutant alleles of *coi1*, a gene encoding an F-box protein and central regulator of JA signaling (Xie et al. 1998), and *jin1*, a mutation in a gene encoding the downstream transcription factor AtMyc2 (Lorenzo et al. 2004), were tested.

The strong recessive *coi1-1* allele causes insensitivity to JA and male sterility (Feys et al. 1994), thus making propagation as heterozygous plants necessary. The *coi1-1* mutant has previously been shown to be hypersusceptible to *B. cinerea* and

other necrotrophic pathogens (Thomma et al. 1998). This mutant was, therefore, a logical choice for testing the contribution of JA signaling to defense against *S. sclerotiorum*.

Progeny of heterozygous *COI1/coi1* plants were grown. The offspring were genotyped using cleaved amplified polymorphic sequence markers (Xie et al. 1998) and were tested for susceptibility to *S. sclerotiorum*. Recessive *coi1-1* mutant plants were more susceptible to *S. sclerotiorum* than wild-type or heterozygous plants (Fig. 2). These differences became apparent when lesion size was measured 2 days postinoculation (Fig. 2A). Thereafter, lesions expanded beyond the inoculated leaf. Entire plants were, therefore, assessed for percentage of decay caused by the fungus to score disease development 3 days postinoculation and beyond (Fig. 2B). In contrast to the *coi1-1* mutant, wild-type plants did not support rapid systemic lesion expansion. The difference in rate of lesion expansion suggests that *COI1*-mediated defenses appear to inhibit or delay the spread of the pathogen. Ultimately, however, the *COI1*-dependent defense pathway is overcome even in wild-type plants.

The oxalate-deficient strain A4 (Godoy et al. 1990) also accelerated lesion expansion in *coi1-1* mutants relative to wild-type plants. However, in plants challenged with this oxalate-deficient mutant strain, disease symptoms were limited to leaves that were inoculated and fungal spread virtually stopped after 5 days of infection (Fig. 2C). These results suggest that the *coi1*-dependent increase in susceptibility is largely independent of oxalic acid.

In addition to *coi1-1*, the weak *coi1-2* allele was tested. Homozygous *coi1-2* mutants are JA-insensitive but partially fertile (Xu et al. 2002) and, thus, easier to work with. These *coi1-2* mutants were significantly more susceptible to *S. sclerotiorum* than wild-type plants (Fig. 3A). To test JA-dependent gene ex-

Fig. 2. Hypersusceptibility of *coi1-1* mutants to *Sclerotinia sclerotiorum*. Homozygous mutants were compared with heterozygous (Het) or wild-type (WT) plants. Means and standard errors are shown ($n > 10$). **A** and **B**, Four- to six-week-old plants were inoculated with wild-type *S. sclerotiorum* isolate 1980 or **C**, with the oxalate-deficient mutant A4. Expanding lesions (**A**) and whole-plant decay (**B**) were measured. Differences in susceptibility between *coi1-1* and wild-type plants were significant ($P < 0.05$) from days 2 to 4 after inoculation with wild-type *S. sclerotiorum* and from days 2 to 3 after inoculation with oxalate-deficient *S. sclerotiorum*.

Fig. 3. Effect of jasmonic acid (JA) on defense against *Sclerotinia sclerotiorum*. **A**, Susceptibility of wild-type Col-0 ($n = 63$) and *coi1-2* mutant ($n = 42$) *Arabidopsis* to *S. sclerotiorum*. Lesion diameters were measured 48 h postinoculation. Means and standard errors are shown. Letters indicate statistically significant differences ($P < 0.05$). **B**, Reverse transcription-polymerase chain reaction of JA-responsive *PDF1.2* (25 cycles) and salicylic acid-responsive *PR1* (28 cycles) after inoculation of wild-type (WT) and mutant plants with *S. sclerotiorum*; loc. = local (inoculated), sys. = systemic (uninoculated) leaves. The constitutively expressed *ACT2* gene (25 cycles) was used as a loading control. Numbers listed on the top indicate hours postinoculation.

pression, the plant defensin gene *PDF1.2* was monitored by reverse transcription-polymerase chain reaction (RT-PCR) in local and systemic leaves after inoculation of wild-type and *coi1-2* mutant plants with *S. sclerotiorum* (Fig. 3B). In contrast to wild-type plants, *PDF1.2* was not induced in pathogen-challenged *coi1-2* mutants. Conversely, the pathogenesis-related *PR1* gene was induced in both wild-type and mutant plants, illustrating that these SA-induced defense responses were not affected by the *coi1-2* mutation. Collectively, these data strongly suggest that the *COI1*-dependent pathway contributes to defense against *S. sclerotiorum*.

To examine whether other mutations in the JA pathway alter susceptibility to *S. sclerotiorum*, *jin1*, a mutant with enhanced resistance to *Fusarium oxysporum* (Anderson et al. 2004) and to *B. cinerea* but with wild-type susceptibility to *Alternaria brassicicola* (Nickstadt et al. 2004) was tested. Seeds of the

jin1 mutant in the background of Col-*gl* were obtained (Berger et al. 1996). Jasmonate insensitivity was tested by growing *jin1* mutants side-by-side with wild-type seedlings in the presence or absence of 50 μ M methyl-jasmonate (MeJA). Wild-type seedlings were more sensitive to MeJA than the *jin1* mutant (Fig. 4A). The *coi1-2* mutant was used as an additional control and was shown to be less sensitive to MeJA than the *jin1* mutant. For inoculation with *S. sclerotiorum*, seedlings were grown for 4 to 6 weeks in soil. No statistically significant differences in susceptibility to *S. sclerotiorum* were detected between *jin1* mutant and wild-type plants (Fig. 4B). RT-PCR analysis revealed only minor (less than twofold) differences in expression of *PDF1.2* and the vegetative storage protein gene *VSP2* between *jin1* mutants and wild-type plants (Fig. 4C). *PR1* was induced similarly in wild-type and mutant plants, suggesting that the SA-induced defense responses were not

Fig. 4. Disease and defense phenotypes of the *jin1* mutant. **A**, Confirmation of jasmonic acid sensitivity. Seedlings were grown for 1 week in the absence or presence of 50 μ M methyl-jasmonate (MeJA). The size of the squares is 1.35 cm. **B**, Susceptibility of wild-type Col-*gl* ($n = 33$) and the *jin1* mutant ($n = 34$) to *Sclerotinia sclerotiorum*. Means and standard errors are shown. **C**, Reverse transcription-polymerase chain reaction (RT-PCR) of *PDF1.2*, *PR1*, and *VSP2* after inoculation with *S. sclerotiorum*. m = mock-inoculated, s = systemic (uninoculated), l = local (inoculated) leaves. *ACT2* was used as a loading control. *PR1* was analyzed after 30 cycles and the other cDNAs after 25 cycles of RT-PCR. Numbers listed on the top indicated hours postinoculation. Experiments were performed twice with similar results.

altered by the *jin1* mutation. These results demonstrate that the effects of *jin1* and *coil* mutations on susceptibility to *S. sclerotiorum* differ, suggesting that other transcription factors may be more important for *S. sclerotiorum*-related defense signaling than AtMyc2.

Separation of JA signaling and oxalate sensitivity.

Susceptibility to *S. sclerotiorum* is frequently (Chipps et al. 2005; Kolkman and Kelly 2000) but not always correlated with oxalate sensitivity (Kolkman and Kelly 2003). To test for oxalate sensitivity, an assay was developed by growing seedlings

Fig. 5. Comparison of oxalate sensitivity among wild-type Col-0 plants, *coil-2*, and *coil-2/cos1* mutants. **A**, Images of seedlings grown on Murashige-Skoog (MS) medium in the absence or presence of 3.5 or 4 mM oxalic acid (OA) for 2 weeks. Numbers beneath the images indicate average root length. The size of the squares is 1.35 cm. **B**, Seedlings were grown in the absence (–OA) or presence (+OA) of 4 mM oxalic acid for 3 weeks. Means and standard errors ($n \geq 29$) are shown. Letters indicate statistically significant differences ($P < 0.05$).

in Murashige-Skoog (MS) medium with or without oxalic acid at pH 5.5. Fresh weight or root length of *Arabidopsis* seedlings was measured after 2 to 3 weeks of growth.

Foliar growth and root branching was inhibited when *Arabidopsis* seedlings were grown on MS medium containing oxalic acid concentrations above 3 mM (Fig. 5A). Leaf development was arrested, cotyledons became chlorotic, and root elongation was inhibited when seedlings were grown on medium containing oxalic acid concentrations above 3.5 mM. As a result, oxalic acid caused a reduction of fresh weight of wild-type plants, *coil-2*, and *coil-2/cos1* mutants (Fig. 5B). The double mutant was tested because deletion of *RIB4*, the *Saccharomyces cerevisiae* ortholog of *COS1*, increases sensitivity of budding yeast to oxalic acid (Cheng et al. 2007). The *coil-2/cos1* double mutant grew poorly on MS medium compared with wild-type and *coil-2* mutant plants ($P < 0.0001$) when it was grown in the absence of oxalic acid. A small but significant difference was observed between *coil-2/cos1* and *coil-2* mutants ($P = 0.0298$) when both mutants were grown in the presence of oxalic acid. Nevertheless, growth inhibition of the *coil-2/cos1* mutant relative to the *coil-2* mutant was 28.0 and 27.4% in the presence or absence of oxalic acid, respectively. These data show that, in contrast to yeast, lumazine synthase is likely not involved in oxalate tolerance of *Arabidopsis*.

Additional experiments were conducted to study dose-response relationships by growing wild-type plants, *coil-2*, and *coil-2/cos1* mutant seedlings in MS medium containing 3 to 5 mM oxalic acid at pH 5.5. No significant differences were

observed between wild-type plants and *coil-2* mutants when seedlings were grown in the presence or absence of oxalic acid (data not shown). This result demonstrates that genotype-dependent differences in *coil*-related susceptibility to *S. sclerotiorum* are not caused by changes in oxalate sensitivity.

Effects of oxalic acid on production of reactive oxygen species (ROS) and gene expression.

To analyze the contribution of oxalic acid to pathogenicity, wild-type *Arabidopsis* plants were inoculated with wild-type *S. sclerotiorum* 1980 or oxalate-deficient strain A4. The oxalate-deficient mutant was clearly less virulent than wild-type *S. sclerotiorum* (Fig. 6A), even when the former and latter strains were grown on nutrient-rich PDA and nutrient-poor minimal medium (Cruickshank 1983), respectively, thus supporting previous findings (Dickman and Mitra 1992; Guimaraes and Stotz 2004). Despite this phenotypic difference, RT-PCR analysis revealed no significant differences in temporal and spatial patterns of *PDF1.2* and *PR1* induction when *Arabidopsis* was exposed to wild-type or oxalate-deficient *S. sclerotiorum* (Fig. 6B).

Staining with 3,3'-diaminobenzidine (DAB) indicated higher H_2O_2 accumulation when *Arabidopsis* leaves were inoculated with oxalate-deficient *S. sclerotiorum* than with wild-type fungus (Fig. 7A). This difference in DAB staining was observed when both strains were grown on minimal medium. Whereas wild-type *S. sclerotiorum* generated a spreading lesion, the oxalate-deficient mutant was confined to the area underneath the agar plug used for inoculation. Nitroblue tetrazolium (NBT)

Fig. 6. Effect of oxalate on fungal virulence and plant gene expression. **A**, Differences in susceptibility of *Arabidopsis* (Col-0) to wild-type *Sclerotinia sclerotiorum* 1980 ($n = 17$) and oxalate-deficient mutant A4 ($n = 21$). Means and standard errors are shown. **B**, Reverse transcription-polymerase chain reaction of *PDF1.2* (23 cycles) and *PR1* (26 cycles) after inoculation with *S. sclerotiorum*. loc. = local (inoculated), sys. = systemic (uninoculated) leaves, m = mock-inoculation. *ACT2* (25 cycles) was used as a loading control. Numbers listed on the top indicate hours postinoculation. Wild-type and oxalate-deficient strains were grown on minimal medium and potato dextrose agar, respectively.

staining revealed superoxide accumulation in wild-type but not in oxalate-deficient *S. sclerotiorum* (Fig. 7B). Superoxide accumulation was minimal in *Arabidopsis* leaves. Quantitative analysis suggested that DAB staining in *Arabidopsis* was 2.5-fold higher after inoculation with oxalate-deficient *S. sclerotiorum* relative to the wild-type strain (Fig. 7C). A 179-fold increase in NBT staining was observed in wild-type *S. sclerotiorum* relative to the oxalate-deficient fungus after inoculation of *Arabidopsis*. Collectively, these data suggest that oxalate enhances $O_2^{\cdot-}$ production in the pathogen and suppresses H_2O_2 production in the host but has little influence on *PR1* and *PDF1.2* gene expression.

Effects of SA and ET signaling on defense against *S. sclerotiorum*.

To test whether pathways other than JA signaling are involved in resistance to *S. sclerotiorum*, susceptibilities of *npr1* and *ein2*

mutants were compared with wild-type *Arabidopsis*. *NPR1* encodes an ankyrin-repeat-containing protein that is a central regulator of SAR (Cao et al. 1994, 1997). *EIN2* encodes a protein with similarities to Nrap metal-ion transporters that is a central regulator of ET signaling (Alonso et al. 1999). Comparison of *ein2*, *coil-2*, and *npr1* mutants with wild-type *Arabidopsis* demonstrated that all three mutants are hypersusceptible to wild-type *S. sclerotiorum* (Fig. 8A and B). Mutations in these three pathways appear to reduce defense against *S. sclerotiorum* approximately equally. RT-PCR analysis of *PDF1.2* and *PR1* expression demonstrated that induction of these two genes by *S. sclerotiorum* was delayed and suppressed in *ein2* and *npr1* mutants, respectively (Fig. 8C). Thus, *S. sclerotiorum* triggers SA and ET signaling in addition to JA signaling.

Similarly to the results with the JA signaling mutants, *ein2* and *npr1* mutant seedlings grew as well as wild-type *Arabidopsis* when exposed to oxalate-containing medium (Fig. 8D).

Fig. 7. Effect of oxalate on reactive oxygen species (ROS) production. **A**, 3,3'-Diaminobenzidine (DAB) staining shows larger accumulation of H_2O_2 in *Arabidopsis* leaves inoculated with oxalate-deficient mutant *Sclerotinia sclerotiorum* than in leaves inoculated with wild-type *S. sclerotiorum*. **B**, Nitroblue tetrazolium (NBT) staining shows an increase in $O_2^{\cdot-}$ production in wild-type strain but no major differences in ROS accumulation of *Arabidopsis* leaves inoculated with oxalate-deficient mutant or wild-type *S. sclerotiorum*. Mock-inoculated leaves were stained with neither DAB nor NBT. Leaf tissues were stained 48 h after inoculation with fungal strains grown on minimal media. The same magnification was used for all images. Enlarged portions of leaves are shown. Bar = 1 mm. **C**, Quantification of DAB ($n = 4$ per strain) and NBT ($n = 3$ per strain) staining using image analysis. Differences between wild-type (WT) and oxalate-deficient (OA-) strains are shown. Means and standard errors are shown.

Fig. 8. Susceptibility of wild-type Col-0 plants ($n = 32$) and *npr1* ($n = 51$), *ein2* ($n = 10$), and *coi1-2* ($n = 24$) mutants to wild-type *Sclerotinia sclerotiorum*. **A**, Photograph of inoculated plants 5 days postinoculation with the *S. sclerotiorum* isolate 1980. **B**, Quantitative analysis of whole plant decay. Means and standard errors are shown. Letters indicate statistically significant differences ($P < 0.05$). **C**, Reverse transcription-polymerase chain reaction of *PDF1.2* (25 cycles) and *PR1* (28 cycles) after inoculation with *S. sclerotiorum*. m = mock-inoculated, s = systemic (uninoculated), l = local (inoculated) leaves. *ACT2* (25 cycles) was used as a loading control. Numbers listed on the top indicate hours postinoculation. **D**, Seedlings were grown in the absence (–OA) or presence (+OA) of 3.5 mM oxalic acid for 2 weeks. Means and standard errors ($n \geq 22$) are shown. Letters indicate statistically significant differences ($P < 0.05$).

However, a significant reduction of growth was observed when *npr1* mutants were grown in the absence of oxalic acid. These results suggest that none of these three signaling pathways are influenced by oxalic acid.

Hypersusceptibility of *npr1* mutants to *S. sclerotiorum* suggested that SA is involved in defense against *S. sclerotiorum*. To test whether chemical activators of SAR can be used to enhance resistance against this fungal pathogen, benzothiadiazole (BTH) was used (Lawton et al. 1996; Ward et al. 1991). The ecotypes Col-0 and Ws-0 were sprayed with BTH 4 days prior to inoculation with *S. sclerotiorum*. Resistance of Col-0 and Ws-0 to *S. sclerotiorum* was significantly increased by treatment with BTH (Fig. 9). As expected, BTH did not have an effect on *npr1* mutants because this chemical acts upstream of NPR1 (Shah et al. 1997, 1999).

DISCUSSION

The results of this study clearly implicate JA, SA, and ET in activation of defense responses against *S. sclerotiorum*. The *coil*, *npr1*, and *ein2* mutants were all hypersusceptible to *S. sclerotiorum*. Moreover, signaling through these pathways was demonstrated using the well-characterized marker genes *PDF1.2* and *PR1*. It was previously suggested that the SA pathway protects against biotrophic pathogens, whereas JA and ET signaling enhances resistance to necrotrophic pathogens (Thomma et al. 1998). However, our study clearly shows that all three pathways are involved in defense against *S. sclerotiorum*. Similar to our observation, local resistance to *B. cinerea* is enhanced by the SA pathway, although *npr1* mutants were as susceptible as wild-type plants (Ferrari et al. 2003). The *npr1* mutation only increases susceptibility to *B. cinerea* in the background of the *ein2* mutation (Ferrari et al. 2003). Thus, our study on *S. sclerotiorum* adds to recent conclusions that strict separation of defenses against necrotrophic and biotrophic pathogens via SA and JA/ET signaling, respectively, is an oversimplification (Glazebrook 2005; Thomma et al. 2001). The rapid death of pathogen-infected *npr1*, *coil*, and *ein2* mutants suggests that induced and systemic resistance slows the progression of *S. sclerotiorum* in wild-type plants.

Three lines of evidence suggest that oxalic acid does not influence JA-induced partial resistance to *S. sclerotiorum*. First, *coil-1* mutants are hypersusceptible to both wild-type and oxalate-deficient *S. sclerotiorum*. Second, hypersusceptibility of the *coil-2* mutant to *S. sclerotiorum* does not correlate with changes in oxalate sensitivity. Third, despite differences in virulence between wild-type and oxalate-deficient *S. scler-*

rotiorum, no oxalate-dependent differences in JA-related gene expression were observed. These findings strongly suggest that oxalic acid does not influence JA signaling.

Host resistance mechanisms to *S. sclerotiorum* have not received much attention. Conversely, heterologous overexpression of oxalate oxidase or oxalate decarboxylase genes has previously been used to protect dicots against *S. sclerotiorum* (Donaldson et al. 2001; Kesarwani et al. 2000). Oxalate oxidases and oxalate decarboxylase are derived from cereals (Lane et al. 1993), which are not hosts of *S. sclerotiorum*, and the microbe *Collybia velutipes* (Mehta and Datta 1991), respectively.

In addition to the *coil* mutants, the *jin1* mutant was used to analyze JA signaling. Susceptibilities of *jin1* mutant and wild-type plants to *S. sclerotiorum* were not significantly different. This result suggests that transcription factors other than JIN1, like ERF1, are involved in protecting wild-type plants against *S. sclerotiorum* (Anderson et al. 2004). Overexpression of *ERF1* increases resistance to several necrotrophic fungi (Berrocal-Lobo et al. 2002), whereas JIN1 appears to be a negative regulator of plant defense responses (Lorenzo et al. 2004). These findings imply antagonistic interaction between ERF1 and JIN1. A JA-insensitive mutant would be expected to lower the expression of JA-pathway genes. However, our RT-PCR results showed no significant genotype-dependent changes in *PDF1.2* and *VSP2* expression in *jin1* mutants relative to wild-type plants. *PDF1.2* expression is down-regulated in *jin1* mutants relative to wild-type plants after inoculation with *B. cinerea* (Nickstadt et al. 2004). On the contrary, *PDF1.2* expression is up-regulated in *jin1* mutants exposed to virulent *Pseudomonas syringae* (Laurie-Berry et al. 2006). Thus, regulation of *PDF1.2* expression by JIN1 varies among different plant-pathogen interactions. It is possible that the absence of altered *PDF1.2* and *PR1* expression in *jin1* mutants challenged with *Sclerotinia sclerotiorum* is due to simultaneous activation of JA and SA signaling.

JA-induced but not wound-induced expression of *VSP* is suppressed in leaves of *jin1* mutants relative to wild-type plants (Berger et al. 1996). These results are compatible with our observation that *jin1* mutants do not differ from wild-type plants in terms of *VSP2* induction after inoculation with *S. sclerotiorum*. It was previously suggested that JIN1 differentially regulates two branches of the JA pathway (Lorenzo et al. 2004). Specifically, JIN1 was proposed to activate the wound response while repressing pathogen responses. These conclusions were based on downregulation of *VSP2* expression and upregulation of *PDF1.2* expression in *jin1* mutants relative to wild-type plants after treatment with JA. Collectively, these

Fig. 9. Effect of benzothiadiazole (BTH) on resistance of *Arabidopsis* to wild-type *Sclerotinia sclerotiorum* in ecotypes Col-0 ($n = 18$) and Ws-0 ($n = 18$) but not in *npr1* ($n = 6$) mutants. Means and standard errors are shown. Plants were sprayed with water or 300 μ M BTH 4 days prior to inoculation.

data suggest that JA and pathogens differentially regulate *PDF1.2* and *VSP2* gene expression in *jin1* mutants (Lorenzo et al. 2004; Nickstadt et al. 2004).

We analyzed the relationship between oxalate sensitivity and JA signaling because the *Saccharomyces cerevisiae* mutant *Arib4* was shown to be hypersensitive to oxalic acid (Cheng et al. 2007). However, the *cos1* mutation of the orthologous gene did not increase oxalate sensitivity in *Arabidopsis*. Lumazine synthases encoded by *RIB4* and *COS1* are pentamers and 60-meric icosahedral capsids in fungi and plants, respectively (Persson et al. 1999). In addition, lumazine synthase is targeted to the chloroplast in plants (Jordan et al. 1999), whereas the enzyme in budding yeast has been localized to the cytoplasm and to the nucleus (Huh et al. 2003). Differences in protein structure and cellular localization may therefore be responsible for the different phenotypic effects of fungal and plant lumazine synthases.

We confirmed earlier findings that oxalate-deficient mutants of *S. sclerotiorum* are less virulent than wild-type fungus in *Arabidopsis* (Dickman and Mitra 1992). It was previously shown that oxalic acid suppresses the oxidative burst of host plants (Cessna et al. 2000). Accordingly, DAB staining indicated that H_2O_2 accumulation increased when wild-type *Arabidopsis* plants inoculated with oxalate-deficient *S. sclerotiorum* relative to wild-type fungus. However, we could not discern any oxalate-dependent suppression of NBT staining indicative of $O_2^{\cdot-}$ production in tobacco (data not shown) or *Arabidopsis*. Unlike Cessna and associates (2000), we used the oxalate-deficient mutant strain A4, which could imply strain-specific differences in elicitation of ROS. We also observed no significant changes in *PR1* expression after inoculation of *Arabidopsis* with oxalate-deficient or wild-type *S. sclerotiorum*, although oxalate and H_2O_2 are expected to change SA-dependent gene expression (Hu et al. 2003). Oxalate-dependent differences in DAB staining were only observed after inoculation of *Arabidopsis* with *S. sclerotiorum* precultured on minimal medium. *PR1* expression was not up-regulated after inoculation of the host with oxalate-deficient fungus pregrown on PDA (Fig. 6B) or minimal medium (data not shown). Possibly, the amount of H_2O_2 production triggered by the oxalate-deficient strain was insufficient to significantly increase *PR1* expression. Effects of oxalate on pectin metabolism may, therefore, be as or even more important than the suppression of induced host defenses (Bateman and Beer 1965).

In addition to testing the involvement of SA signaling genetically, we determined the effects of BTH on resistance to *S. sclerotiorum*. Similarly to a previous study in soybean (Dann et al. 1998), we showed that BTH significantly increased resistance of wild-type *Arabidopsis* to *S. sclerotiorum*. Thus, activation of SAR may be used commercially to improve resistance of crop species to *S. sclerotiorum* in the field.

MATERIALS AND METHODS

Biological material and growth conditions.

Homozygous *coil-2* and *coil-2/cos1* mutants were generously provided by D. Xie (National University of Singapore, Republic of Singapore). The *jin1* mutant and Col-*gl* wild-type seeds were donated by S. Berger (Bayerische Julius-Maximilians-Universität, Würzburg, Germany). The *coil-1* mutant seeds came from J. G. Turner (University of East Anglia, Norwich, U.K.). All other lines were obtained from the *Arabidopsis* Biological Resource Center. A randomized complete block design was used to grow plants in 72-cell flats. *Arabidopsis thaliana* seeds were sown on wet Sunshine SB40 soil mix (Sun Gro Horticulture, Bellevue, WA, U.S.A.) and were stratified at 4°C in the dark for up to 5 days. An MB-60B growth chamber

(Percival Scientific, Perry, IA, U.S.A.) was used for plant cultivation. A light intensity of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a photoperiod of 11 h of light and 13 h of darkness were used at 22°C. Fungal strains have previously been obtained from M. Dickman (Guimaraes and Stotz 2004).

Chemical treatment.

Each soil-grown plant was sprayed with approximately 0.6 ml of 300 μM BTH in the form of Actigard 50WG (Syngenta, Greensboro, NC, U.S.A.) or water. Plants were dried under ambient laboratory conditions for 2 h and, then, were moved back to the growth chamber. Plants were inoculated with *S. sclerotiorum* 4 days later.

Plant inoculation.

Oxalate-deficient *S. sclerotiorum* A4 (Godoy et al. 1990) was cultured on PDA or on minimal medium (1 g of NaOH, 3 g of DL-malic acid, 2 g of NH_4NO_3 , 0.1 g of $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, and 39 g of Bacto-agar per liter) to reduce the aggressiveness of the fungus (Cruickshank 1983). Wild-type *S. sclerotiorum* isolate 1980 was cultured exclusively on minimal medium prior to inoculation. An agar plug (2.5 mm in diameter) containing the advancing edge of *S. sclerotiorum* mycelia was removed to inoculate *Arabidopsis* leaves. A single rosette leaf of 4- to 6-week-old *Arabidopsis* plants was inoculated for gene expression data. Two rosette leaves per plant were inoculated for phenotypic experiments. Lengths and widths of lesions were measured with a caliper before disease symptoms expanded beyond inoculated leaves. Decay percentage of individual plants was scored after lesions expanded beyond the inoculated leaf to assess disease development.

RT-PCR analysis.

Inoculated and uninoculated leaves were collected at various stages after infection with *S. sclerotiorum*. Total RNA was extracted from *Arabidopsis* leaves using the phenol-LiCl method (Sambrook et al. 1989). cDNA was synthesized using 2 μg of RNA from each treatment and a first-strand synthesis kit (GE Healthcare, Piscataway, NJ, U.S.A.). PCR was run from 23 to 30 cycles with primers for *PDF1.2*, *VSP2*, *PR1*, and *actin2* at annealing temperatures of 67, 57, 51, and 55°C, respectively. Primers for amplification of *PDF1.2* were 5'-AATGAGCTCTCATGGCTAAGTTTGCTTCC-3' and 5'-AATCCATGGAATACACGATTTAGCACC-3'. Primers for amplification of *VSP2* were 5'-GTACTGGTTGTGGTTAGGGAC-3' and 5'-GAAGGTACGTAGTAGAGTGG-3'. Primers for amplification of *PR1* were 5'-CTCAAGATAGCCCACAAGA-3' and 5'-TAGTATGGCTTCTCGTTCAC-3'. Primers for amplification of *actin2* were 5'-CAGAGCGGGAAATTGTAAGAGAC-3' and 5'-ACAAAAAGGGAAATGAAACAAACA-3'.

Oxalate sensitivity assay.

Square petri dishes (100 $\times$ 15 mm) were used to grow plants vertically. MS basal medium with sucrose and agar (Sigma, St. Louis) was used as a control. To measure oxalate sensitivity, MS medium was supplemented with 3 to 5 mM oxalic acid at pH 5.5. Seeds were surface-sterilized, and 10 seeds of each genotype (Col-0, *coil-2*, and *coil-2/cos1*) were placed side-by-side on each plate. Growth was determined by measuring root length or fresh weight of individual seedlings after 2 to 3 weeks of growth.

NBT staining.

Leaves were detached 24 and 48 h postinoculation and were vacuum-infiltrated in 2 ml of 10 mM potassium phosphate buffer, pH 7.5, containing 10 mM NaN_3 and 0.1% NBT (Sigma) for 30 min at room temperature (Sang et al. 2001).

Leaves were boiled in 96% ethanol for 10 min before visualizing blue precipitates. ImageJ software (National Institutes of Health, Bethesda, MD, U.S.A.) was used to quantify the intensity of staining. A threshold of 0 to 150 was used, the minimal particle size was set to 50, and the same region of interest was used for all images.

H₂O₂ detection by DAB staining.

Leaves were detached 48 h postinoculation. Individual leaf petioles were dipped into 1 mg of DAB per milliliter (Sigma), pH 3.8, and were incubated for 8 h prior to the end of the experiment in the dark at room temperature. Thereafter, leaves were removed, placed into acetic acid/glycerol/ethanol (1:1:1, vol/vol/vol) and were boiled for 5 min in water bath. Subsequently, the solution was exchanged and leaves were maintained in 60% glycerol. H₂O₂ was detected as a brown precipitate in inoculated leaves, using a microscope (Thordal-Christensen et al. 1997). ImageJ software (National Institutes of Health) was used to quantify the intensity of staining. A threshold of 0 to 175 was used, the minimal particle size was set to 50, and the same region of interest was used for all images.

Statistical analysis.

The SAS program package (Cary, NC, U.S.A.) was used for statistical analysis. Specifically, the PROC general linear model was used to analyze disease or oxalate sensitivity data. LSMEANS and PDIF were used to separate significant differences among means that were explained by genotype, treatment, or genotype-by-treatment interaction. Levene's test was used to ascertain homogeneity of variances.

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