Involvement of Salicylate and Jasmonate Signaling Pathways in *Arabidopsis* Interaction with *Fusarium graminearum*

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Fusarium graminearum is the principal causative agent of *Fusarium* head blight (FHB), a devastating disease of wheat and barley. This fungus can also colonize *Arabidopsis thaliana*. Disease resistance was enhanced in transgenic wheat and *Arabidopsis* plants that constitutively overexpress the *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*) gene, which regulates salicylic acid (SA) signaling and modulates the activation of jasmonic acid (JA)-dependent defenses. Here, we provide several lines of evidence that reveal an important role for SA and JA signaling in *Arabidopsis* defense against *F. graminearum*. SA level was elevated in fungus-inoculated leaves, and SA application and biologically activated systemic acquired resistance enhanced resistance. Furthermore, the disruption of SA accumulation and signaling in the *sid2* mutant and NahG transgenic plant, and the *npr1* and *wrky18* mutants, respectively, resulted in heightened susceptibility to this fungus in leaves and inflorescence. JA signaling was activated in parallel with SA signaling in the fungus-challenged plants. However, the hyperresistance of the JA pathway mutants *opr3*, *coi1*, and *jar1* indicates that this pathway contributes to susceptibility. Genetic and biochemical experiments indicate that the JA pathway promotes disease by attenuating the activation of SA signaling in fungus-inoculated plants. However, the hypersusceptibility of the *jar1 npr1* double mutant compared with the *npr1* mutant suggests that *JAR1* also contributes to defense, signifying a dichotomous role of JA and a *JAR1*-dependent mechanism in this interaction.

Plants have evolved a multitude of defense mechanisms to control diseases. These include preformed mechanisms, such as the physical barrier provided by the cell wall and cuticle, as well as inducible mechanisms. Pathogen attack induces expression of numerous defense-related genes; for example, the pathogenesis-related (*PR*) genes, some of which encode antimicrobial proteins (van Loon et al. 2006). Expression of these genes in pathogen-challenged plants is regulated by a variety of mechanisms, including those dependent on the sig-

naling molecules salicylic acid (SA) and jasmonic acid (JA). However, the overall effectiveness of one or more of these responses in controlling disease depends on the lifestyle of the pathogen (Glazebrook 2005; Wasternack 2007). Necrotrophic pathogens derive their nutrients from dead host cells while biotrophs derive nutrients from live host cells. Some pathogens (hemibiotrophs) display both biotrophic and necrotrophic phases of growth.

JA is an important regulator of plant defenses against pathogens that display necrotrophy. The role of JA in plant defense has been extensively reviewed (Glazebrook 2005; Wasternack 2007). Exogenously applied methyl-JA (MJ) enhanced *Arabidopsis thaliana* resistance to several necrotrophic fungi (Thomma et al. 2000). In contrast, the *Arabidopsis fad3 fad7 fad8* triple mutant, which is deficient in JA, exhibits enhanced susceptibility to *Pythium irregulare* (Staswick et al. 1998). Similarly, compared with the wild-type (WT) plant, resistance against *P. irregulare* and the soft-rot-causing fungus *Botrytis cinerea* was compromised in the *Arabidopsis jasmonate resistant 1* (*jar1*) mutant (Ferrari et al. 2003; Staswick et al. 1998), which is insensitive to JA due to its inability to synthesize JA-isoleucine (Ile) (Staswick and Tiryaki 2004). Resistance to *B. cinerea* was also compromised in the JA-insensitive *coronatine insensitive 1* (*coi1*) mutant (Thomma et al. 1998). JA signaling is also critical for controlling black spot disease caused by *Alternaria brassicicola* (Thomma et al. 1998).

SA signaling primarily impacts plant defense against pathogens that exhibit a biotrophic phase in their life cycle (Glazebrook 2005). The role of SA in plant defense has been widely studied in *Arabidopsis* (Durrant and Dong 2004; Chaturvedi and Shah 2007). The *NONEXPRESSOR OF PR GENES 1* (*NPR1*) gene is a key regulator of SA signaling in *Arabidopsis*. Loss of *NPR1* activity in *npr1* mutant plants is accompanied by the attenuation of SA-dependent defenses, resulting in heightened susceptibility to the oomycete pathogen *Hyaloperonospora arabidopsidis*, the fungal pathogen *Erysiphe orontii*, and the hemibiotrophic bacterial pathogen *Pseudomonas syringae* (Cao et al. 1994; Delaney et al. 1995; Shah et al. 1997; Reuber et al. 1998). In contrast, overexpression of *NPR1* enhances sensitivity to SA and resistance against these pathogens. The *NPR1* protein regulates the activity of a family of b-ZIP transcription factors, resulting in the expression of defense genes. *NPR1* also modulates the expression of genes involved in secretion of defense proteins to the apoplast (Wang et al. 2005). Gene expression studies indicated that the *WRKY18*-encoded transcription factor is required for a subset of *NPR1*-

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directed functions (Chen and Chen 2002; Wang et al. 2006; Xu et al. 2006).

In addition to basal defenses, SA and *NPR1* also are required for the manifestation of systemic acquired resistance (SAR), an inducible defense mechanism that confers resistance against a broad spectrum of pathogens (Métraux et al. 2002; Durrant and Dong 2004; Chaturvedi and Shah 2007). SAR is activated in the uninfected parts of a plant in response to an infection elsewhere in the plant. Another inducible defense mechanism that involves *NPR1* is induced systemic resistance (ISR), which is stimulated by root colonizing rhizobacteria (Pieterse and van Loon 2004). In *Arabidopsis*, ISR confers heightened resistance to *Fusarium oxysporum* and *P. syringae*. Activation of ISR requires JA signaling. However, SA is not required for ISR (Pieterse and van Loon 2004). Although JA and *NPR1* cooperate, leading to the activation of ISR, JA can also antagonize signaling through *NPR1* under other conditions (Koornneef and Pieterse 2008). For example, activation of JA signaling marginalizes SA accumulation and downstream signaling through *NPR1* in *P. syringae*-infected *Arabidopsis* leaves (Kloek et al. 2001; Spoel et al. 2003).

Fungi in the genus *Fusarium* are ubiquitously distributed in terrestrial environments. Several *Fusarium* spp. are important phytopathogens. *F. graminearum* Schwabe (teleomorph *Gibberella zeae* (Schwein.) Petch) causes Fusarium head blight (FHB), seedling blight, and root rot in cereal crops (Bai and Shaner 2004; Goswami and Kistler 2004; Agrios 2005; Walter et al. 2010). Monogenic, gene-for-gene resistance to *F. graminearum* has not been identified. In cultivated wheat, the best source of resistance to FHB, which is controlled by several complex quantitative loci, is the Chinese cv. Sumai 3 (Bai and Shaner 2004). However, Sumai 3-derived resistance is insufficient to control damage under high disease pressure. Other problems associated with using Sumai 3 as a source of resistance include increased shattering and susceptibility to other diseases. *F. graminearum* can enter the host through stomates or directly penetrate the host surface. The fungus secretes an array of hydrolases that could facilitate penetration (Walter et al. 2010). Like most *Fusarium* spp., necrotrophy is accompanied by increase in vigor of colonization by *F. graminearum*. However, there is a brief biotrophic phase during the early stages of infection when *F. graminearum* does not penetrate host cells and exhibits extracellular growth (Goswami and Kistler 2004).

Despite the damaging potential of this fungus, our understanding of plant defense mechanisms against this pathogen is very limited. A recent study demonstrated that FHB resistance in wheat was enhanced by application of MJ (Li and Yen 2008). However, whether plants utilize JA signaling to control *F. graminearum* infection is not known. Relying on transcriptome analysis, the above study also suggested that ethylene signaling likely contributes to FHB resistance. However, genetic studies indicated otherwise. Attenuation of ethylene signaling in wheat resulted in hyperresistance, suggesting that ethylene signaling contributes to susceptibility (Chen et al. 2009). Fungal infection of wheat spikes also resulted in systemic expression of a subset of *PR* genes (Pritsch et al. 2001). However, whether this systemic induction of *PR* genes contributes to controlling spread of the fungus from one spikelet to another is not known. Moreover, benzothiadiazole [benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester] (BTH), a functional analog of SA, when locally applied to wheat heads, did not enhance FHB resistance (Yu and Muehlbauer 2001). Hence, it was suggested that SA signaling is unlikely to contribute to host resistance to FHB (Yu and Muehlbauer 2001; Walter et al. 2010). However, FHB resistance in wheat was enhanced by application of a SAR-inducing activity extracted from *Arabidopsis* (Chaturvedi et al. 2008) and by the constitutive overexpression

(OE) of *NPR1* (Makandar et al. 2006). Furthermore, this enhanced resistance to *F. graminearum* was accompanied by increased sensitivity to BTH, and the faster and stronger expression of *PR1* in the fungus-challenged spikes of *NPR1*(OE) wheat, than in nontransgenic plants (Makandar et al. 2006). These results suggest that SA signaling may have a function in wheat defense against *F. graminearum*. It is equally likely that the *NPR1*(OE)-conferred resistance to *F. graminearum* is due to a mechanism or mechanisms other than that involving SA. As mentioned above, *NPR1* also participates in defenses that are not dependent on SA. Molecular-genetic approaches are needed to further characterize the role of these signaling mechanisms in plant defense and susceptibility to *F. graminearum*. However, these are difficult to conduct in wheat due to the limited availability of molecular-genetic tools.

F. graminearum has been shown to colonize and cause disease in *Arabidopsis*. Inoculation of *Arabidopsis* inflorescence with *F. graminearum* resulted in fungal growth and disease in floral tissues and spread of the fungus to the developing siliques and seed (Urban et al. 2002; Cuzick et al. 2008). In contrast, when fungal spores were placed on the leaf surface, although they germinated and the hyphae branched, penetration of the leaf was poor (Chen et al. 2006). However, wounding of the leaves promoted infection by the fungus and subsequent spread to other organs (Chen et al. 2006). Efficient infection and disease also occurs when the fungus is infiltrated into *Arabidopsis* leaves, thereby avoiding barriers provided by the leaf surface (Makandar et al. 2006) (Fig. 1A to C). Several other studies also have demonstrated that *Arabidopsis* is an amenable host for characterizing host mechanisms that contribute to resistance and susceptibility to *F. graminearum* (Skadsen and Hohn 2004; Van Hemelrijck et al. 2006; Savitch et al. 2007; Chen et al. 2009).

In this study, we have evaluated the involvement of SA and JA signaling in the interaction between *Arabidopsis* and *F. graminearum*. We provide multiple lines of evidence that *Arabidopsis* engages SA signaling through *NPR1* to control disease. In contrast, JA signaling has a dichotomous role in this interaction. On the one hand, JA signaling promotes disease by constraining the activation of SA- and *NPR1*-regulated defenses. On the other hand, JA signaling promotes overall resistance to *F. graminearum* by participating in an *NPR1*-independent mechanism.

RESULTS

Activation of SAR reduces *F. graminearum* disease severity in *Arabidopsis*.

Constitutive expression of *NPR1*, which is a key regulator of SAR, enhances resistance against *F. graminearum* in *Arabidopsis* (Makandar et al. 2006); therefore, we tested whether the biological activation of SAR with an avirulent (Avr) strain of *P. syringae* pv. *tomato* could reduce the severity of disease caused by *F. graminearum*. Fungal disease severity was lower in plants in which SAR was activated (Avr *P. syringae* pv. *tomato*) than in control plants not exhibiting SAR (Mock) (Fig. 2). Activation of SAR requires the translocation of a factor from the Avr pathogen-inoculated leaf to the distal leaves. This SAR-activating factor is present in petiole exudates (PeX) collected from the Avr pathogen-inoculated leaves but not in PeX collected from mock-inoculated leaves (Chaturvedi et al. 2008). The efficacy of SAR in protecting *Arabidopsis* against *F. graminearum* was further evaluated by testing the ability of Avr PeX, when applied to the lower leaves, to protect the upper leaves against *F. graminearum*. Compared with the Mock PeX-treated plants, Avr PeX, when applied to WT plants, enhanced resistance against *F. graminearum* in the upper leaves (Fig. 2). Taken together,

the above results confirm that, in *Arabidopsis*, SAR can reduce the severity of disease caused by *F. graminearum*.

SA signaling is activated in *F. graminearum*-challenged plants.

To determine whether SA-mediated defenses are engaged by *Arabidopsis* when infected with *F. graminearum*, SA content

and expression of the SA-inducible *PR1* gene were monitored in *F. graminearum*-inoculated leaves of WT *Arabidopsis*. Compared with the mock-inoculated leaves, *F. graminearum* inoculation resulted in an increase in SA content and *PR1* transcript level in the WT plant (Fig. 3A and B). Fungal inoculation-induced accumulation of *PR1* transcript was attenuated in the SA biosynthesis mutant *sid2*, which lacks isochorismate synthase activity that is required for SA synthesis, and the *NahG* transgenic, which expresses an SA-degrading salicylate hydroxylase. *PR1* expression was also lower in the leaves of the fungus-inoculated *npr1* mutant than in the corresponding WT plant (Fig. 3B). These results confirm that SA-dependent signaling is activated in *Arabidopsis* leaves inoculated with *F. graminearum*.

Activation of SA signaling reduces *F. graminearum* disease severity in *Arabidopsis* leaves.

SA signaling is constitutively active in the *Arabidopsis cpr5* and *ssi2* mutants. Both of these well-characterized mutants constitutively express the *PR1* gene and accumulate high levels of SA (Fig. 3B) (Bowling et al. 1994; Shah et al. 2001). Disease severity was lower in *F. graminearum*-inoculated leaves of the *Arabidopsis cpr5* and *ssi2* mutants than the WT plant (Fig. 4A). Disease severity was also lower on plants that were pretreated with SA and BTH (Fig. 4B). These results, along with the ability of biologically activated SAR to enhance resistance against *F. graminearum*, suggested that SA may have an important role in controlling the severity of disease caused by *F. graminearum*. However, it should be noted that along with SA, JA signaling is also hyperactive in the *cpr5* mutant (Bowling et al. 1994; Jing et al. 2007) and could potentially influence the interaction between *cpr5* and *F. graminearum*. To further test the involvement of SA signaling in controlling *F. graminearum* infection, disease severity was compared be-

Fig. 1. *Fusarium graminearum* infection of *Arabidopsis* leaves. **A**, Colonization of *Arabidopsis* accession Columbia leaves by *F. graminearum*. Plant cells fluoresce red due to binding of Toluidine blue-O and fungal hyphae fluoresce green due to binding of the chitin specific WGA-Alexa Fluor 488. A mock-inoculated leaf provided the negative control for fluorescence emitted from fungal mass. **B**, Disease symptoms in *F. graminearum*-infected *Arabidopsis* leaves. Diseased leaves are grouped into four classes (<25, 25 to 50, 50 to 75, and >75% chlorosis) based on the percentage of leaf area showing chlorosis relative to the mock-inoculated leaf. **C**, Top panel: polymerase chain reaction (PCR) analysis of *F. graminearum Tri5* gene to monitor fungal growth in *Arabidopsis* leaves exhibiting different levels of chlorosis. PCR analysis for the *Arabidopsis ACT8* gene provided a positive control for DNA quality, DNA extracted from mock (M)-inoculated leaves provided a negative control for *Tri5* amplification, and PCR reactions lacking plant and fungal DNA (-ve) provided a negative control for PCR. PCR conducted with fungal DNA isolated from *F. graminearum* cultivated on synthetic medium was used for determining the relative abundance of fungal DNA in leaf samples. Leaves infiltrated with a suspension lacking fungus provided the mock control. These experiments were conducted two times.

Fig. 2. Systemic acquired resistance (SAR) confers protection against *Fusarium graminearum* in *Arabidopsis*. *F. graminearum* disease severity, 4 days postinoculation, in leaves of wild-type accession Columbia plants in which SAR had been activated by infiltration of three lower leaves with an avirulent strain of *Pseudomonas syringae* pv. *tomato* DC3000 (Avr Pst) or petiole exudates (PeX) collected from Avr *P. syringae* pv. *tomato*-inoculated Col leaves (Avr PeX). Plants that were pretreated with 10 mM MgCl₂ (Mock) or PeX collected from 10 mM MgCl₂-infiltrated Columbia leaves (Mock PeX) provided controls for this experiment. Fractions of inoculated leaves exhibiting necrosis covering <25, 25 to 50, 50 to 75, and >75% of the leaf area were determined for each treatment. Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other. These experiments were repeated three times.

tween *F. graminearum*-inoculated WT and the *sid2* mutant plant. A transgenic line, *NPR1*(OE), that constitutively overexpresses *NPR1* from the *Cauliflower mosaic virus* 35S promoter and exhibits heightened resistance to *F. graminearum* (Makandar et al. 2006), was used as a resistant control. Disease severity was higher in the *sid2* mutant plant than in the WT and *NPR1*(OE) plants (Fig. 4A). Similarly, disease severity was also higher in the NahG transgenic and *npr1* mutant plant than in the corresponding WT plants (Fig. 4A). Also, *NPR1* was required for the SA- and BTH-conferred heightened resistance against *F. graminearum* (Fig. 4B). Similar to the *NPR1*(OE) plants, overexpression of *WRKY18*, which mediates a subset of *NPR1*-directed functions (Chen and Chen 2002; Wang et al. 2006; Xu et al. 2006), enhanced resistance to *F.*

graminearum in the *WRKY18*(OE) plant (Fig. 4A). Conversely, compared with the WT, loss of *WRKY18* function in the *wrky18* mutant resulted in higher severity of infection (Fig. 4A). Together, the above results confirm the importance of SA and *NPR1*-dependent signaling in controlling *F. graminearum* disease severity in *Arabidopsis* leaves.

Fig. 3. Salicylic acid (SA) signaling is activated in *Fusarium graminearum*-inoculated *Arabidopsis* leaves. **A**, Total salicylate content (SA + SA-glucoside) in mock (M)- and *F. graminearum*-inoculated (F) leaves of wild-type (WT) *Arabidopsis* accession Columbia (Col) and the *jar1* and *opr3* mutant plants. Leaves for SA quantification were harvested 24 h postinoculation (hpi). Each bar represents the average SA content $\pm$ standard deviation from five replications per treatment. Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other. This experiment was repeated two times. **B**, *PR1* expression in *F. graminearum*-challenged *Arabidopsis* leaves. *PR1* transcript accumulation was monitored by Northern blot analysis of RNA isolated 24 hpi from mock (M)- and *F. graminearum*-inoculated (F) *Arabidopsis* leaves. A ³²P-labeled *PR1* gene-specific probe was used to monitor *PR1* transcript accumulation and ethidium bromide (EtBr) staining was used to monitor quality of RNA and gel loading. Top panel: *PR1* expression in WT accession Columbia (Col); the *sid2*, *cpr5*, and *npr1* mutants; and *NPR1*(OE) transgenic plant. Bottom panel: *PR1* expression in WT accession Nöthen (Nö), the *ssi2* mutant, and NahG transgenic plant. These experiments were repeated three times. Leaves infiltrated with a suspension lacking fungus provided the mock control.

Fig. 4. Defects in salicylic acid (SA) synthesis and signaling impact *Fusarium graminearum* disease severity in *Arabidopsis* leaves. **A**, Top panel: disease severity in wild-type (WT) *Arabidopsis* accession Columbia (Col) and the *npr1*, *cpr5*, *sid2*, and *NPR1*(OE) transgenic plant at 4 days postinoculation (dpi). Middle panel: disease severity in WT (Col), the *wrky18* mutant, and the *WRKY18*(OE) transgenic plant at 4 dpi. Bottom panel: disease severity in WT *Arabidopsis* accession Nöthen (Nö), the NahG transgenic, and *ssi2* mutant plant at 4 dpi. The three panels are derived from separate experiments. **B**, *F. graminearum* disease severity in leaves of WT (Col) and *npr1* mutant plants that were previously treated with a 200-μM solution of SA and benzothiadiazole [benzo (1,2,3) thiadiazole-7-carboxylic acid *S*-methyl ester] to induce systemic acquired resistance. Plants pretreated with water provided controls for this experiment. Fractions of inoculated leaves exhibiting necrosis covering <25, 25 to 50, 50 to 75, and >75% of the leaf area were determined for each treatment. Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other. Experiments were repeated three times.

SA signaling controls *F. graminearum* disease severity in *Arabidopsis* inflorescence.

Because *F. graminearum* infects floral tissues of wheat and barley, we also compared susceptibility of *Arabidopsis* inflorescence in SA biosynthesis and signaling mutants. Inflorescence infection ratings (*Fusarium*–*Arabidopsis* disease [FAD] score) were determined as previously described (Urban et al. 2002). Compared with the WT plants, the FAD score was higher on inflorescence tissue of the *sid2*, *npr1*, and *wrky18* mutants, suggesting heightened disease susceptibility (Table 1). Disease susceptibility was also elevated in NahG inflorescence. In contrast, FAD scores were lower in the *ssi2*, *cpr5*, *NPR1*(OE), and *WRKY18*(OE) plants, indicating heightened

resistance. Based on these results, we conclude that, similar to the leaf tissue, SA signaling through *NPR1* also is important for limiting severity of disease caused by *F. graminearum* in *Arabidopsis* inflorescence.

JA signaling attenuates SA signaling to promote *F. graminearum* disease severity.

F. graminearum infection resulted in elevated expression of the *PDF1.2* gene (Fig. 5A), which is a molecular marker for the activation of JA signaling. *PDF1.2* expression paralleled the expression of *PR1*. Because JA contributes to plant defense against necrotrophic fungi, we tested whether severity of disease caused by *F. graminearum* was limited by MJ application.

Fig. 5. Jasmonic acid (JA) signaling attenuates the activation of salicylic acid (SA) signaling in *Fusarium graminearum*-challenged plants. **A**, Reverse-transcription polymerase chain reaction (RT-PCR) analysis of *PDF1.2* and *PR1* expression in leaves of wild-type (WT) accession Columbia plants at 6, 12, 24, and 48 h postinoculation (hpi) with *F. graminearum* (F). RNA extracted from mock (M)-inoculated plants provided the negative control. **B**, *F. graminearum* disease severity in leaves of WT accession Col and the *NPR1*(OE) transgenic plant that were exposed to methyl-JA (MJ) vapors 24 h prior to fungal inoculation. Plants that were exposed to ethanol, which was used as a solvent for MJ, and plants that did not receive any chemical treatment provided the controls for this experiment. **C**, Top panel: *F. graminearum* disease severity in WT *Arabidopsis* accession Columbia (Col) and the *opr3* and *jar1* mutant. Lower panel: *F. graminearum* disease severity in WT accession Columbia-*glabra* (Col-gl) and the *coi1* mutant. **D**, *F. graminearum* disease severity in WT accession Columbia (Col) and the *jar1* mutant plants that were infiltrated with a 200-μM solution of JA-isoleucine (JA-Ile) 24 h prior to fungal inoculation. Plants treated with water provided the controls. **E**, RT-PCR analysis of *PR1* expression in mock (M)- and *F. graminearum* (F)-inoculated WT *Arabidopsis* accession Columbia (Col) and the *jar1* and *opr3* mutant plants. Leaves for RT-PCR analysis were harvested 24 hpi. **F**, RT-PCR analysis of *PR1* expression in mock (M)- and *F. graminearum* (F)-inoculated WT (Col) and the *NPR1*(OE) transgenic plants that were exposed to MJ vapors 24 h prior to fungal inoculation. Plants that were exposed to ethanol vapors provided the controls for this experiment. In A, E, and F, *ACT8* expression served as a control for RNA quality. In B, C, and D, disease severity was scored 4 days postinoculation. Fractions of inoculated leaves exhibiting necrosis covering <25, 25 to 50, 50 to 75, and >75% of the leaf area were determined for each treatment. Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other. In A, E, and F, leaves infiltrated with a suspension lacking fungus provided the mock control. Experiments in B, C, and E were repeated three times and those in A, D, and F were repeated two times.

Quite to the contrary, compared with the untreated and ethanol-treated plants, pretreatment with MJ enhanced disease severity in WT *Arabidopsis* (Fig. 5B). JA's role in *Arabidopsis* interaction with *F. graminearum* was further characterized by evaluating disease severity in the JA biosynthesis mutant, *opr3*, and the JA signaling mutants, *jar1* and *coil*. Compared with the WT plant, disease severity was lower in leaves of the *opr3*, *jar1*, and *coil* mutants (Fig. 5C). Similarly, compared with the WT, disease severity was lower in the inflorescence of the *opr3* and *jar1* mutants (Table 1). Application of JA-Ile, a product of the *JAR1*-encoded enzyme, restored disease severity in the *jar1* mutant (Fig. 5D), thus confirming that signaling through *JAR1* contributes to disease severity.

SA content and *PR1* transcript accumulation were higher in the *F. graminearum*-inoculated leaves of the *opr3* and *jar1* mutants than the WT plant (Figs. 3A and 5E), suggesting that the impact of JA signaling on promoting disease is likely due to the attenuation of SA signaling during *Arabidopsis* interaction with *F. graminearum*. In concurrence with this hypothesis, pretreatment with MJ attenuated the fungal infection-induced accumulation of *PR1* transcript in WT and *NPR1*(OE) plants (Fig. 5F) and the *NPR1*(OE)-conferred heightened resistance to *F. graminearum* (Fig. 5B). To test this hypothesis further, disease severity was evaluated in the *jar1 npr1* double mutant plant. Presence of the *npr1* allele resulted in loss of the *jar1*-conferred heightened resistance to *F. graminearum* in leaves

(Fig. 6). The *jar1*-conferred hyperresistance in inflorescence tissue was also dependent on the presence of the WT *NPR1* allele (Table 2).

JA signaling contributes to resistance during later stages of infection.

Progression of disease in leaves was faster and more severe on the *jar1 npr1* double mutant than the *npr1* mutant (Fig. 6; Supplementary Fig. S1). Most of the *jar1 npr1* plants had completely succumbed to disease by 3 days postinoculation (dpi), which is a few days earlier than the *npr1* mutant. Similarly, disease ratings in inflorescence tissue were higher for the *jar1 npr1* double mutant than the *npr1* single mutant (Table 2). These results suggest that *JAR1* contributes to overall resistance, as well, although its contribution was masked in the presence of *NPR1*. We further tested the involvement of JA signaling in *Arabidopsis* defense against *F. graminearum* by exposing plants inoculated with *F. graminearum* to MJ beginning at 0, 6, 12, and 24 h postinoculation (hpi). Plants exposed to ethanol provided the control. Contrary to the enhanced disease severity observed in plants exposed to MJ at the beginning of the infection (0 hpi) and at 6 hpi, exposure to MJ at 12 and 24 hpi enhanced resistance (Fig. 7). We conclude that, although JA signaling promotes *F. graminearum* disease by attenuating the activation of SA signaling, JA signaling also contributes to *Arabidopsis* defense, presumably during the later stages of infection.

Table 1. *Fusarium graminearum* disease in *Arabidopsis* inflorescence

Genotype ^a	FAD ^b
WT (Col)	5.7 ± 0.7
<i>npr1</i>	12.0 ± 1.2
<i>NPR1</i> (OE)	1.5 ± 0.2
<i>cpr5</i>	1.7 ± 0.2
<i>sid2</i>	9.3 ± 0.8
<i>wrky18</i>	12.7 ± 1.4
<i>WRKY18</i> (OE)	1.5 ± 0.2
<i>opr3</i>	1.2 ± 0.2
<i>jar1</i>	1.8 ± 0.2
WT (Nö)	4.0 ± 0.6
NahG	16.3 ± 2.2
<i>ssi2</i>	1.7 ± 0.3

^a WT = wild type and OE = overexpression.

^b *Fusarium*-*Arabidopsis* disease (FAD) value was evaluated at 7 days postinoculation.

Fig. 6. *NPR1* is required for the *jar1*-conferred enhanced resistance to *Fusarium graminearum*. *F. graminearum* disease severity in leaves of wild-type (WT) *Arabidopsis* accession Columbia (Col) and the *npr1*, *jar1*, and *jar1 npr1* double mutant plants at 4 days postinoculation. Fractions of inoculated leaves exhibiting necrosis covering <25, 25 to 50, 50 to 75, and >75% of the leaf area were determined for each treatment. Different letters next to the bars indicate values that are significantly different ($P < 0.05$) from each other. This experiment was repeated three times.

Table 2. *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*) is required for the *jasmonate resistant 1* (*jar1*)-conferred enhanced disease resistance in inflorescence tissue

Genotype ^a	FAD ^b
WT (Col)	5.7 ± 0.5
<i>npr1</i>	12.9 ± 1.2
<i>jar1</i>	1.4 ± 0.3
<i>jar1 npr1</i>	19.7 ± 1.0

^a WT = wild type.

^b *Fusarium*-*Arabidopsis* disease (FAD) value was evaluated at 7 days postinoculation.

Fig. 7. Methyl-jasmonic acid (MJ) applied later during infection enhances resistance against *Fusarium graminearum*. *F. graminearum* disease severity in leaves of wild-type (WT) *Arabidopsis* Columbia exposed for 24 h to MJ vapors beginning at 0, 6, 12, and 24 h postinoculation (hpi) with *F. graminearum*. Plants exposed to ethanol (EtO) vapors at 0 hpi provided the control. Disease severity was scored 4 days postinoculation. Fractions of inoculated leaves exhibiting necrosis covering <25, 25 to 50, 50 to 75, and >75% of the leaf area were determined for each treatment. Different letters to the right of each treatment indicate disease severities that are significantly different ($P < 0.05$) from each other. This experiment was repeated two times.

DISCUSSION

Although *F. graminearum* is considered primarily a pathogen of cereal plants, it is also capable of causing disease in the dicotyledonous species *Arabidopsis thaliana*. Previously, Urban and associates (2002) showed that *Arabidopsis* flowers are susceptible to *F. graminearum* infection. Although the leaf surface provides a physical barrier to fungal penetration, *F. graminearum* can grow and cause disease when infiltrated into *Arabidopsis* leaves (Fig. 1A to C). This combination of leaf and inflorescence infection assays in *Arabidopsis* provides a powerful system for the molecular-genetic characterization of factors and mechanisms that govern resistance and susceptibility to *F. graminearum*. Experiments with *Arabidopsis* mutants defective in SA and JA signaling indicate that the genetic requirements for controlling disease severity are similar in the inflorescence tissues and leaves (Tables 1 and 2; Figs. 4A, 5C, and 6). These experiments demonstrate that SA signaling has an important role in controlling severity of *F. graminearum* disease, and JA signaling through *JAR1* has two contrasting roles in this interaction.

The heightened susceptibility of the *npr1* mutant (Fig. 4A and B; Table 1) combined with the inability of SA and BTH to promote resistance against *F. graminearum* in the *npr1* mutant (Fig. 4B) indicates that, in *Arabidopsis*, the effect of SA on controlling *F. graminearum* growth is mediated through an *NPR1*-dependent mechanism. The heightened susceptibility of the *wrky18* mutant and enhanced resistance of the *WRKY18*(OE) plant (Fig. 4A; Table 1) suggests that the *WRKY18* arm of the *NPR1*-dependent mechanism contributes to this resistance. Similar to its interaction with *F. graminearum*, the SA-deficient *sid2* mutant is more susceptible than the WT to wilt disease caused by *F. oxysporum* and inflorescence infection by *F. culmorum* (Diener and Ausubel 2005; Cuzick et al. 2008). Moreover, *NPR1* is required for basal resistance to *F. culmorum* (Cuzick et al. 2008) and, in tomato, constitutive expression of *NPR1* enhanced resistance against *F. oxysporum* (Lin et al. 2004). Thus, SA signaling through *NPR1* is a mechanism that is engaged to control disease caused by a variety of *Fusarium* fungi.

Although necrotrophy coincides with increase in vigor of colonization by *Fusarium* spp., it is now an accepted fact that *F. graminearum* is a hemibiotroph that does not penetrate host cells during early stages of infection (Goswami and Kistler 2004). SA signaling could target this early stage of infection. Indeed, SA signaling was activated early during infection. Expression of *PR1*, which is a molecular marker for the activation of SA signaling, was induced within 12 h of *F. graminearum* infection (Fig. 5A). *PR1* expression was detectable as early as 6 hpi. Furthermore, prior exposure to SA and BTH enhanced disease resistance (Fig. 4B). It is expected that the antifungal factors that target the fungus during this stage of the infection will be secreted outside the plant cell. In pathogen-infected plants, *NPR1* is required for the elevated expression of genes that encode proteins associated with the secretory machinery (Wang et al. 2005). These secretory pathway genes are required for the SAR-conferred enhanced resistance to pathogens, thus implying an important function for the secretory pathway in *NPR1*-orchestrated resistance. Indeed, several of the PR proteins that are synthesized in pathogen-infected leaves accumulate in the extracellular spaces, and many of these proteins possess antifungal activities (Jayaraj et al. 2004). Furthermore, constitutive co-expression of two secreted PR proteins enhanced FHB resistance in transgenic wheat, and apoplastic fluids collected from these plants possessed an activity that was inhibitory to *F. graminearum* growth (Anand et al. 2004). SA signaling could also control disease progression during the

necrotrophic phase of *F. graminearum* life cycle. There is precedence for SA's involvement in promoting resistance against necrotrophic fungi. SA accumulation was required for full resistance to *B. cinerea* and *Plectosphaerella cucumerina* (Thomma et al. 1998, 1999; Zimmerli et al. 2001; Berrocal-Lobo et al. 2002; Ferrari et al. 2003). Furthermore, in the case of *B. cinerea*, *NPR1* was required for the local restriction of pathogen growth by SA (Zimmerli et al. 2001; Ferrari et al. 2003).

Although JA signaling was activated in parallel with SA signaling in *Arabidopsis* leaves inoculated with *F. graminearum* (Fig. 5A), JA signaling promoted disease by constraining the activation of SA signaling through *NPR1* (Figs. 3A, 5E and F, and 6). The complete breakdown of *NPR1*(OE)-conferred heightened resistance when these plants were pretreated with MJ (Fig. 5B) supports this hypothesis. The higher content of SA in *F. graminearum*-inoculated *opr3* and *jar1* leaves compared with the WT leaves (Fig. 3A) suggests that JA most likely attenuates SA-mediated defenses by limiting SA accumulation in the *F. graminearum*-inoculated leaves. SA accumulation is similarly restricted by the activation of JA signaling in *Arabidopsis* and tomato infected with *Pseudomonas syringae* (Kunkel and Brooks 2002; Zhao et al. 2003; Uppalapati et al. 2007). Coronatine, a phytotoxin produced by *P. syringae*, mimics jasmonates and, thus, activates JA signaling in *P. syringae*-infected plants (Brooks et al. 2005; Zhao et al. 2003; Uppalapati et al. 2007). Mycotoxins produced by *Fusarium* spp. also activate expression of the JA-regulated *PDF1.2* gene (Nishiuchi et al. 2006), and a recent study has suggested that *F. oxysporum* hijacks a *COI1*-dependent mechanism to promote disease in *Arabidopsis* (Thatcher et al. 2009). Whether *F. graminearum* mycotoxins hijack host JA machinery for pathogenesis, similar to the function of coronatine in *P. syringae*-infected plants, remains to be determined.

We have repeatedly observed that disease was more severe in the inflorescence and leaves of the *jar1 npr1* double mutant than in the *npr1* single mutant (Table 2; Fig. 6). Although disease severity measurements were conducted at 4 dpi, most of *jar1 npr1* double mutant plants had succumbed to infection by 3 dpi, which is a few days earlier than the *npr1* mutant. These results suggest that *JAR1* contributes to overall resistance as well, although its contribution was masked in the presence of *NPR1*. In *Arabidopsis*, similarly, *JAR1* is required for an *NPR1*-independent mechanism that contributes to basal resistance against the bacterial pathogen *P. syringae* (Clarke et al. 2000). Thus, although JA signaling through *JAR1* suppresses SA- and *NPR1*-mediated defenses, a *JAR1*-dependent mechanism also contributes to the control of fungal growth, presumably during later stages of infection. The ability of MJ to promote resistance when applied 12 to 24 h after fungal inoculation (Fig. 7) supports this hypothesis. Together, SA- and JA-dependent defenses could provide maximal protection against this fungus.

Although previous studies in which wheat heads were sprayed with BTH did not result in enhanced FHB resistance (Yu and Muehlbauer 2001), we have observed that irrigating wheat plants with SA and BTH effectively enhanced FHB resistance (R. Makandar and J. Shah, *unpublished*). Furthermore, FHB resistance was enhanced in transgenic wheat that accumulated elevated SA levels and constitutively expressed *PR* genes (Anand et al. 2003a and b). These observations, combined with the enhanced FHB resistance observed in wheat plants constitutively expressing *Arabidopsis NPR1* and the genetic evidence presented here confirming the importance of SA signaling in defense against *F. graminearum* in *Arabidopsis* inflorescence and leaves, advocates that the role of SA in wheat defense against *F. graminearum* needs to be reevaluated. Similarly, although MJ application was reported to con-

trol severity of FHB in wheat (Li and Yen 2008), the role of JA signaling in wheat interaction with *F. graminearum* has not been genetically validated. Future experiments designed to discern the role of JA signaling in wheat interaction with *F. graminearum* should consider the results presented here on the dichotomous role of JA signaling in *Arabidopsis* interaction with *F. graminearum*, and the involvement of a JA-dependent mechanism as a susceptibility factor in *Arabidopsis* interaction with two other *Fusarium* fungi, *F. oxysporum* and *F. culmorum* (Cuzick et al. 2008; Thatcher et al. 2009).

MATERIALS AND METHODS

Arabidopsis genotypes.

The *ssi2* mutant and the NahG transgenic (Shah et al. 1999, 2001) are in the *A. thaliana* accession Nössen and the *sid2-2* (Wildermuth et al. 2001), *cpr5* (Bowling et al. 1994), *npr1-1* (Cao et al. 1994), *jar1-1* (Staswick et al. 1998), *wrky18* (Xu et al. 2006), and *opr3* mutants; and the *NPR1*(OE) and *WRKY18*(OE) transgenic lines (Cao et al. 1998; Xu et al. 2006) are in the *Arabidopsis* accession Columbia. *Arabidopsis* plants were cultivated at 22°C in a growth chamber programmed for a cycle of 14 h of light (100 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 10 h of darkness. Approximately 4-week-old *Arabidopsis* plants were used for all experiments.

Fungal cultivation and plant infections.

F. graminearum isolate Z-3639 was cultured for 7 days on half-strength potato dextrose agar (Difco Laboratories, Detroit) at 22°C. The fungus mass was then washed off the plate using a brush in 10 ml of sterile distilled water and filtered through four layers of cheesecloth. The fungal suspension containing very short mycelial fragments (30,000 ml^{-1}) was infiltrated into the abaxial surface of *Arabidopsis* leaves with a needleless syringe. Four leaves per plant were infiltrated. Similarly processed washings collected from uninoculated plates were used for mock treatments. Infiltrated plants were covered with a clear plastic dome and disease severity recorded 4 dpi. The fraction of inoculated leaves exhibiting chlorosis covering <25% (category 1), 25 to 50% (category 2), 50 to 75% (category 3), and >75% (category 4) of leaf area were determined for each genotype. A minimum of 60 leaves from 15 plants of each genotype was analyzed for each experiment. Single-factor analysis of variance ($P < 0.05$) (v5.1; SAS, Cary, NC, U.S.A.) was used to compare disease severity among different genotypes and treatments.

For bioassays on *Arabidopsis* inflorescence, a hand atomizer was used to spray inoculate *F. graminearum* on plants that had bolts with open flowers on the terminal inflorescence and few developing siliques. Fungal suspension was applied until droplet run-off. Control plants were spray inoculated with water, and 15 to 20 plants from each genotype were inoculated with the fungus. After inoculation, plants were irrigated and covered individually with plastic separators to maintain high relative humidity. FAD value, which represents the combined disease severity on flowers, new siliques, and old siliques, was evaluated at 7 dpi, as previously described (Urban et al. 2002).

Microscopic analysis of *F. graminearum* in *Arabidopsis* leaves.

Fungus-inoculated leaves were cut at the base and cleared with isopropanol. Cleared leaves were vacuum infiltrated (2 h at 25 mm Hg) with a solution containing WGA-Alexa Fluor 488 (Invitrogen, Carlsbad, CA, U.S.A.) and Toluidine Blue-O supplemented with bovine serum albumin (New England Biolabs, Ipswich, MA, U.S.A.) in phosphate-buffered saline (PBS, pH 7.4), each at 10 $\mu\text{g/ml}$. The leaves were then washed with

PBS and mounted using 30% glycerol. The leaves were visualized under UV light with a Nikon Eclipse E600 (Lewisville, TX, U.S.A.) epifluorescent microscope equipped with a Sony (Park Ridge, NJ, U.S.A.) DSC-F707 camera and a triple excitation filter (excitation filter: 395 to 410, 490 to 505, and 560 to 580; emission filter: 450 to 470, 515 to 545, and 600 to 650). The excitation wavelengths used for WGA-Alexa Fluor 488 and Toluidine Blue-O were between 395 to 410 and 490 to 505 nm, respectively, with capture between 450 to 470 and 515 to 545 nm, respectively.

Induction of SAR.

Biological activation of SAR was initiated by infiltrating three fully expanded *Arabidopsis* leaves with a suspension of the avirulent pathogen *P. syringae* pv. *tomato* DC3000 *avrRpt2*, as previously described (Chaturvedi et al. 2008). Plants infiltrated with 10 mM MgCl_2 provided the mock controls. SAR was also activated by infiltrating three fully expanded *Arabidopsis* leaves with PeX collected from avirulent pathogen-challenged leaves. PeX collected from 10 mM MgCl_2 -infiltrated leaves provided the mock controls. PeX was collected as previously described by Chaturvedi and associates (2008). Three days after infiltration with avirulent bacteria or Avr PeX to activate SAR, three upper leaves of each plant were challenged with *F. graminearum* and disease rating evaluated 4 dpi, as described above.

Chemical treatment of plants.

SA and BTH treatments were carried out by subirrigating and spraying *Arabidopsis* plants to run-off with 200 μM solutions of the chemicals or with water as a control. MJ treatment was accomplished by placing plants in a plexiglass chamber (48-liter volume) along with a cotton ball soaked with 1 ml of 200 μM MJ solution in 0.1% ethanol. Plants treated with ethanol (0.1%), which was used to dissolve MJ, were used as a control for experiments with MJ.

DNA extraction and polymerase chain reaction analysis.

To extract DNA from fungus-infected leaves, *Arabidopsis* leaves showing different classes of disease symptoms were collected 6 dpi. Ten leaves per disease class were collected from four biological replicates and ground in liquid nitrogen using a mortar and pestle. Total DNA was extracted using the QIAGEN DNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cetyltrimethylammonium bromide (Leslie and Summerell 2006) method was used for extraction of fungal DNA from *F. graminearum* mycelia grown in potato dextrose broth. Hoechst dye 33258 (Sigma-Aldrich, St. Louis) was used for quantification of double-stranded DNA.

Genomic polymerase chain reaction (PCR) was used to detect the presence of fungus in plant tissue. Equal amounts of DNA (25 ng) from fungal-infected leaves were used for PCR analysis. The primers FgTri5-F (5'-AGCGACTACAGGCTTC CCTC-3') and FgTri5-R (5'-AAACCATCCAGTTCTCCATCT G-3'), designed to amplify the fungal Tri5-cluster (GenBank accession number AB060689), were used as a molecular probe to detect the presence of *F. graminearum*. These primers amplify a 486-bp fragment from the *F. graminearum* genome. The primers ACT8-1 (5'-ATGAAGATTAAGGTCGTGGCA-3') and ACT8-2 (5'-TCCGAGTTTGAAGAGGCTAC-3'), derived from the *Arabidopsis* ACT8 (Atlg49240) gene, were used as a control for the genomic PCR. These primers amplify a 450-bp fragment. PCR was performed using the following conditions: 95°C for 5 min; followed by 40 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were resolved on 2%

agarose gel, stained with ethidium bromide, and visualized with a BioDoc-It system (UVP).

RNA analysis.

RNA from *Arabidopsis* leaves was extracted by a guanidine-phenol method (Chomczynski and Sacchi 1987). For reverse-transcription (RT)-PCR analysis, oligo(dT) primer (Promega Corp., Madison, WI, U.S.A.) was used for the RT reaction. RT reactions were carried out at 50°C for 30 min in 20-µl reactions with 100 ng of total RNA as template. This cDNA (1 µl) was utilized for the subsequent PCR step. The gene-specific primers PR1-F (5'-GCTCTTGTAGGTGCTCTTGTTTC-3') and PR1-R (5'-CAGCTCTTATTTGTATTATTG-3'), PDF1.2a-F (5'-TCATGGCTAAGTTGCTTCC-3') and PDF1.2a-R (5'-AATACACACGATTTAGCACC-3'), and ACT8-1 (5'-ATGAAGATTAAGGTCGTGGCA-3') and ACT8-2 (5'-TCCGAGTTTG AAGAGGCT-3') were used for PCR amplification of the *PR1* (At2g14610), *PDF1.2a* (At5g44420), and *ACT8* (At1g49240) cDNAs, respectively. The PCR amplifications were carried at 95°C for 5 min; followed by 25 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 1 min; with a final extension step of 72°C for 5 min. For Northern blot analysis, 10 µg of RNA was denatured and loaded on formaldehyde-agarose gels. RNA was transferred to Hybrid-N+ Nylon membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.) and RNA blots were probed with the *Arabidopsis* *PR1* cDNA.

SA quantification.

A gas chromatography-mass spectrometry method, described by Schmelz and associates (2004), was adapted to quantify SA content in *Arabidopsis* leaves. Leaves (100 to 200 mg fresh weight) were harvested and frozen immediately in liquid nitrogen. The frozen samples were ground with a pestle. Then, 300 µl of a 1-propanol/water/HCl (200:100:2) mix were added to the ground tissue, followed by vortexing. Next, 1 ml of dichloromethane was added and, after mixing, the tubes were spun in a centrifuge to promote the separation of phases. The bottom phase containing extracted SA was transferred into a 4-ml glass vial and SA content was quantified as previously described (Schmelz et al. 2004). To determine any SA-glucoside (SAG) that remained in the upper phase, the upper phase was transferred to a 4-ml glass vial that contained 1 ml of dichloromethane. Dichloromethane was added to bring the volume to 2 ml. To hydrolyze the SAG to SA, 100 µl of concentrated HCl was added and the tubes. After vortexing, the tubes were heated at 96°C for 60 min. The hydrolyzed samples were centrifuged and the bottom organic phase pipetted into a fresh vial. The upper phase was reextracted a second time with 1 ml of dichloromethane. The lower organic phases (containing SA released from SAG) obtained from two rounds of extraction were combined, and the solvent was evaporated under N₂ gas. High-performance liquid chromatography-grade methanol (200 µl) was added and the contents were filtered through 0.22-µm syringe-driven filters. Derivatization and quantification of SA was done as previously described (Schmelz et al. 2004). Total SA content in the samples was expressed as the sum of SA and SAG content.

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