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# The *Colletotrichum orbiculare ssd1* Mutant Enhances *Nicotiana benthamiana* Basal Resistance by Activating a Mitogen-Activated Protein Kinase Pathway [C][W]

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**Plant basal resistance is activated by virulent pathogens in susceptible host plants. A *Colletotrichum orbiculare* fungal mutant defective in the *SSD1* gene, which regulates cell wall composition, is restricted by host basal resistance responses. Here, we identified the *Nicotiana benthamiana* signaling pathway involved in basal resistance by silencing the defense-related genes required for restricting the growth of the *C. orbiculare* mutant. Only silencing of *MAP Kinase Kinase2* or of both *Salicylic Acid Induced Protein Kinase* (*SIPK*) and *Wound Induced Protein Kinase* (*WIPK*), two mitogen-activated protein (MAP) kinases, allowed the mutant to infect and produce necrotic lesions similar to those of the wild type on inoculated leaves. The fungal mutant penetrated host cells to produce infection hyphae at a higher frequency in *SIPK WIPK*-silenced plants than in nonsilenced plants, without inducing host cellular defense responses. Immunocomplex kinase assays revealed that SIPK and WIPK were more active in leaves inoculated with mutant fungus than with the wild type, suggesting that induced resistance correlates with MAP kinase activity. Infiltration of heat-inactivated mutant conidia induced both SIPK and WIPK more strongly than did those of the wild type, while conidial exudates of the wild type did not suppress MAP kinase induction by mutant conidia. Therefore, activation of a specific MAP kinase pathway by fungal cell surface components determines the effective level of basal plant resistance.**

## INTRODUCTION

Plants have evolved multiple strategies to defend themselves against infection by microbial pathogens. Plant disease resistance has been studied most thoroughly in cases that depend on the presence of specific resistance genes (*R* genes) that confer immunity to particular races of pathogens carrying a cognate avirulence gene (Laugé and De Wit, 1998; Dangl and Jones, 2001; Chisholm et al., 2006). In addition to *R* gene–mediated resistance, which conditions race cultivar specificity, plants also possess basal or general resistance that confers durable protection against many potential microbial pathogens (Jones and Takemoto, 2004). This type of resistance requires that plants are able to recognize a broad spectrum of microbes, and the currently accepted view is that plants have evolved pathogen-associated molecular pattern (PAMP)-triggered immunity to recognize features that are common to many microbes via cell surface receptors (Thordal-Christensen, 2003; Nürnberger et al., 2004). Several PAMPs have been identified from plant pathogens, including flagellin and elongation factor Tu from Gram-negative bacteria (Felix et al., 1999; Kunze et al., 2004) as well as chitin and β-glucan from fungi and oomycetes (Umemoto et al., 1997; Kaku et al., 2006). Although PAMPs may trigger immune responses in susceptible plants, it is considered that adapted pathogens have evolved to overcome or evade basal resistance so that these responses are no longer sufficient to completely restrict pathogen infection (Jones and Dangl, 2006; de Wit, 2007). However, host basal resistance to fungal pathogens is still poorly understood, and so far PAMP-related fungal mutants have not been exploited for studying the molecular basis of PAMP-triggered immunity in vivo.

*Colletotrichum orbiculare* (syn. *Colletotrichum lagenarium*) is an ascomycete fungus causing anthracnose disease in cucumber (*Cucumis sativus*). This fungus develops a specialized infection structure called an appressorium that effects direct penetration into host epidermal cells by breaching the plant cuticle and cell wall layers through a combination of mechanical force and localized enzymatic dissolution (Perfect et al., 1999; Tucker and Talbot, 2001). Therefore, successful appressorial penetration is an essential first step in the infection process of *C. orbiculare*. In a previous forward genetic screen for pathogenicity

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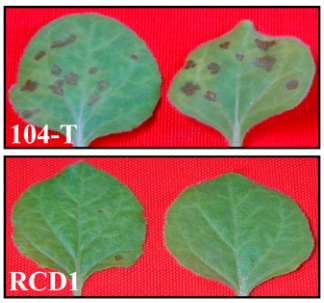


**Figure 1.** Pathogenicity of the *C. orbiculare ssd1* Mutant on *N. benthamiana*.

Pathogenicity test of *C. orbiculare* wild-type strain 104-T and *ssd1* mutant RCD1. Conidial suspension ($1 \times 10^6$ conidia/mL) was sprayed onto the upper leaves of 3-week-old plants. Leaves inoculated with 104-T showed obvious necrotic lesions at 5 DAI, but those inoculated with RCD1 did not.
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mutants, we identified the *SSD1* gene of *C. orbiculare* (Tanaka et al., 2007). This gene is a functional ortholog of *Saccharomyces cerevisiae SSD1*, a putative regulator of fungal cell wall composition (Wheeler et al., 2003). Appressorial penetration by *ssd1* disruption mutants is restricted by the deposition of callose papillae by host cells at sites of attempted penetration, resulting in strongly attenuated pathogenicity on host plants, although the mutants retained wild-type penetration ability on artificial substrata. The induction of host papilla formation by *ssd1* mutants was faster and more frequent than by the wild type and was associated with the complete inhibition of infection. Manipulation of host physiology confirmed that the impaired penetration ability of the *ssd1* mutants resulted from their induction of host basal resistance responses (Tanaka et al., 2007). Based on these findings, we concluded that host basal resistance can completely block infection by an adapted pathogen when induced to a sufficiently high level.

In this study, we attempted to dissect the molecular basis of plant basal resistance using virus-induced gene silencing (VIGS) in *Nicotiana benthamiana* to compromise resistance to the *C. orbiculare ssd1* mutant. Together with evidence from immunocomplex kinase assays, we show that the success or failure of infection by *C. orbiculare* may be determined by the level of mitogen-activated protein (MAP) kinase activity induced in host plants.

## RESULTS

### *SSD1* Is Required for Fungal Pathogenicity on *N. benthamiana*

The *SSD1* gene of *C. orbiculare* is essential for successful penetration by appressoria into epidermal cells of susceptible cucumber plants, and the failed penetration attempts by *ssd1* mutants were associated with deposition of callose papillae by host cells (Tanaka et al., 2007). *N. benthamiana* is also a susceptible host plant for *C. orbiculare* (Shen et al., 2001). First, we examined whether the *SSD1* gene is essential for infection of *N. benthamiana*. When conidial suspensions of the wild-type strain 104-T were inoculated onto leaves, typical necrotic lesions could be observed 5 d after inoculation (DAI). By contrast, leaves inoculated with the *ssd1* mutant RCD1 did not show visible disease symptoms (Figure 1).

Microscopy analysis showed that the *ssd1* mutant formed appressoria that were indistinguishable from those of the wild-type strain 104-T on the *N. benthamiana* (Figure 2A). However, the formation of intracellular infection hyphae in plant epidermal cells by RCD1 was not observed, in contrast with 104-T (Figure 2A). To observe the responses of *N. benthamiana* cells to attempted penetration by appressoria of the mutant, inoculated leaves were stained with aniline blue to detect callose papillae by epifluorescence microscopy (Figure 2B). At attempted penetration sites of the *ssd1* mutant, ~50% of appressoria were accompanied by callose papillae, and intracellular infection hyphae were rarely observed inside host cells (Figure 2C). By contrast, the frequency of papilla formation under appressoria of

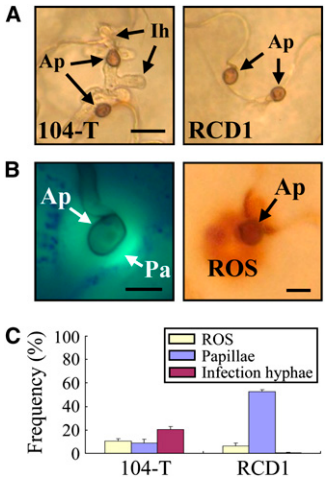


**Figure 2.** Infection Phenotypes of *C. orbiculare ssd1* Mutant and Defense Responses on *N. benthamiana*.

**(A)** Cytology of appressorial penetration by the wild-type 104-T and *ssd1* mutant RCD1. The upper leaves were droplet-inoculated with conidial suspension ($5 \times 10^5$ conidia/mL) and observed at 72 HAI. Ap, appressorium; Ih, intracellular infection hyphae. Bar = 10 μm.
**(B)** Left panel: Callose papilla (Pa) formed under appressoria (Ap). The leaf was inoculated with 104-T, stained with aniline blue, and observed with epifluorescence microscopy at 72 HAI. Callose was detectable by its green fluorescence. Bar = 5 μm. Right panel: ROS accumulation under appressoria. The leaf inoculated with 104-T was stained with DAB and observed with light microscopy at 72 HAI. ROS was detectable as dark-brown staining. Bar = 5 μm.
**(C)** Quantification of papilla formation and ROS accumulation at sites of attempted penetration by *C. orbiculare* appressoria. At 72 HAI, leaves inoculated with the wild-type 104-T and *ssd1* mutant RCD1 were stained with aniline blue to detect callose or with DAB to detect ROS accumulation. At least 200 appressoria were counted for each fungal strain, and standard errors were calculated from three replicate experiments.
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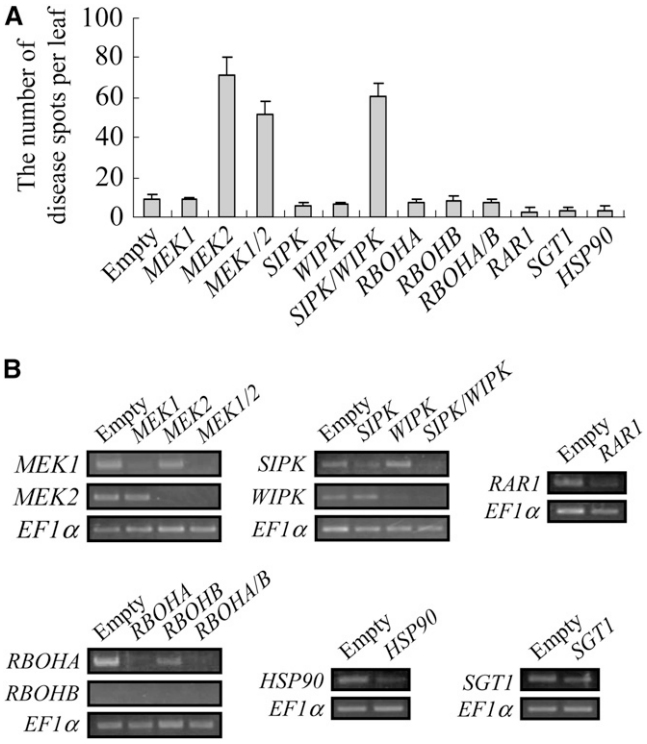


**Figure 3.** Effect of Silencing Plant Defense-Related Genes on Pathogenicity of the *ssd1* Mutant.

**(A)** Lesion formation on gene-silenced plants inoculated with the *ssd1* mutant, RCD1. Conidial suspension ($1 \times 10^6$ conidia/mL) was sprayed onto the upper leaves of plants in which the following genes were silenced: *MEK1*, *MEK2*, *MEK1* and *MEK2* (*MEK1/2*), *SIPK*, *WIPK*, *SIPK* and *WIPK* (*SIPK/WIPK*), *RBOHA*, *RBOHB*, *RBOHA* and *RBOHB* (*RBOHA/B*), *RAR1*, *SGT1*, and *HSP90*. Lesions were counted at 5 DAI, and results are based on three replicate experiments.
**(B)** RT-PCR evaluation of gene silencing. Total RNAs were extracted from the leaves used for the pathogenicity tests shown in **(A),** and the effect of VIGS was evaluated by RT-PCR. PCR was performed with 35 cycles of amplification and evaluated by three biological replicates

104-T was only 10%, and infection hyphae were seen in <20% of appressoria. When inoculated leaves were stained with 3,3′-diaminobenzidine (DAB) to detect accumulation of reactive oxygen species (ROS), positive staining was rarely detected under appressoria (Figure 2B), and the frequency of staining was ~10% in both 104-T and RCD1 (Figure 2C). These responses of *N. benthamiana* to challenge by the *ssd1* mutant were similar to those of *C. sativus* (Tanaka et al., 2007).

### The Impaired Pathogenicity of the *ssd1* Mutant Depends on MEK2-SIPK/WIPK–Mediated Basal Resistance

To identify the plant defense signaling pathway involved in restricting infection by the *ssd1* mutant, we applied VIGS in *N. benthamiana*. We generated knockdown plants of known defense-related genes, such as MAP kinase kinases (*MEK1* and *MEK2*), MAP kinases (*Salicylic Acid Induced Protein Kinase* [*SIPK*] and *Wound Induced Protein Kinase* [*WIPK*]), genes involved in ROS production (*Respiratory Burst Oxidase Homolog* [*RBOHA* and *RBOHB*]), and *R* gene–mediated resistance (*Required for MLa12 Resistance* [*RAR1*], *Suppressor of G2 Allele of SKP1* [*SGT1*], and *Heat Shock Protein 90* [*HSP90*]), and inoculated these silenced plants with the *ssd1* mutant, RCD1. At 5 DAI, the number of disease lesions on inoculated leaves was counted (Figure 3A). *MEK2*-silenced, *MEK1 MEK2*–silenced, and *SIPK WIPK*–silenced plants showed 60 to 70 lesions per leaf, whereas control plants (empty vector) showed 10 or less lesions. By contrast, the number of lesions formed on plants in which *MEK1*, *RBOHA*, *RBOHB*, *RAR*, *SGT1*, or *HSP90* had been silenced was similar to that of control plants. Interestingly, plants silenced at either *SIPK* or *WIPK* also did not show significant disease symptoms. When the effect of VIGS was evaluated by RT-PCR, the expression of all target genes appeared to be down-regulated, although some variation in the level of silencing between different genes was detected (Figure 3B). We therefore cannot exclude the possibility that plants in which gene silencing produced no enhanced disease phenotype still retained sufficient transcript levels to mount effective defense responses.

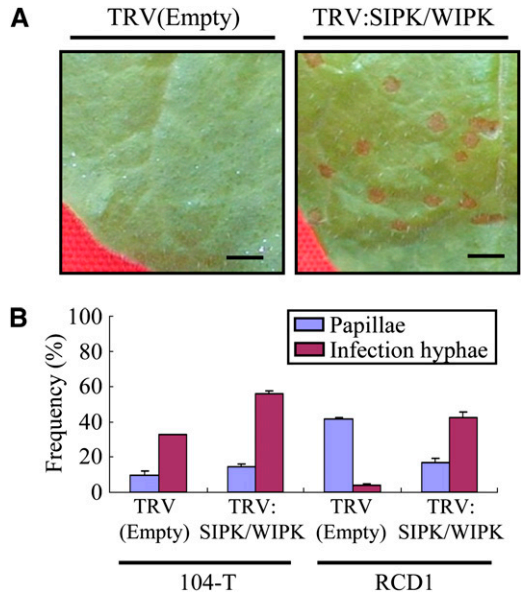


**Figure 4.** Infection Phenotype of the *ssd1* Mutant in *SIPK WIPK*–Silenced Plants.

**(A)** Conidial suspension ($1 \times 10^6$ conidia/mL) of the *ssd1* mutant RCD1 was sprayed onto leaves of control (empty vector) or *SIPK*- and *WIPK*-silenced plants. Typical anthracnose lesions were produced on leaves of *SIPK*- and *WIPK*-silenced plants, while lesions were absent from leaves of control plants at 5 DAI. Bar = 1 cm.
**(B)** Quantification of papilla and infection hypha formation at sites of attempted penetration by *C. orbiculare* appressoria. At 72 HAI, leaves inoculated with the wild-type 104-T and *ssd1* mutant RCD1 were stained with aniline blue to detect callose and infection hyphae. At least 200 appressoria were counted for each fungal strain, and standard errors were calculated from three replicate experiments.
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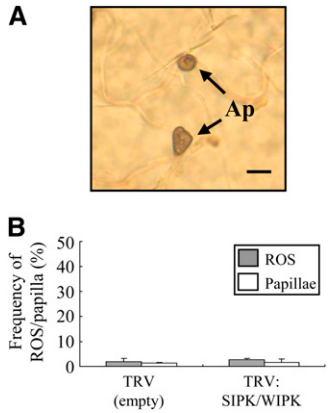


**Figure 5.** Pathogenicity of a Nonadapted Pathogen, *C. graminicola*, on *SIPK WIPK*–Silenced Plants.

**(A)** Microscopy observation of *C. graminicola* on a *SIPK WIPK*–silenced leaf of *N. benthamiana*. Conidia of *C. graminicola* germinate and form appressoria (Ap) on the leaf surface, but infection hyphae do not develop from appressoria inside host epidermal cells. Bar = 10 μm.

**(B)** Quantitative analysis of ROS accumulation and papilla formation under appressoria at 72 HAI. Frequency of appressoria with ROS/papilla was counted. Defense responses were rarely observed under appressoria at 72 HAI.

[See online article for color version of this figure.]

The necrotic lesions formed by the *ssd1* mutant in *SIPK WIPK*–silenced plants were similar in appearance to those formed by the wild-type 104-T (Figures 1 and 4A). Light microscopy showed that the infection process of the *ssd1* mutant on *SIPK WIPK*–silenced plants was also similar to that of 104-T, with typical appressoria and infection hyphae present at 72 h after inoculation (HAI). The frequency with which intracellular infection hyphae were produced by appressoria of the mutant was ~40% in *SIPK WIPK*-silenced plants, whereas the frequency of papilla formation was reduced from ~40 to 20% in *SIPK WIPK*–silenced plants (Figure 4B). In the case of the wild-type 104-T, appressorial penetration frequency was significantly increased, from 30% in nonsilenced control plants to ~50% in *SIPK WIPK*-silenced plants (Figure 4B). Accordingly, the number of necrotic lesions produced by 104-T on *SIPK WIPK*–silenced plants was ~1.5-fold higher than on control plants. Taken together, these findings indicate that a specific MAP kinase pathway is required for the plant resistance responses that restrict appressorial penetration by the *ssd1* mutant.

Next, we investigated whether defense responses mediated by the MEK2-SIPK/WIPK signaling cascade could also be involved in nonhost resistance to *Colletotrichum* (i.e., in the immunity shown by an entire plant species against a nonadapted pathogen species) (Nürnberger and Lipka, 2005). We therefore inoculated *SIPK WIPK*–silenced plants with conidia of the nonadapted maize (*Zea mays*) anthracnose pathogen, *Colletotrichum graminicola*, which is normally unable to cause disease on *N. benthamiana* plants. Although *C. graminicola* formed appressoria on both control plants and *SIPK WIPK*-silenced plants, the formation of intracellular infection hyphae was not observed (Figure 5A), and no disease symptoms were produced. To evaluate the involvement of plant defense responses in restricting appressorial penetration by *C. graminicola*, leaves were stained with aniline blue and DAB to detect callose and ROS accumulation, respectively, at 72 HAI. As shown in Figure 5B, callose deposition and ROS accumulation were rarely detected beneath the appressoria of *C. graminicola* in either control plants

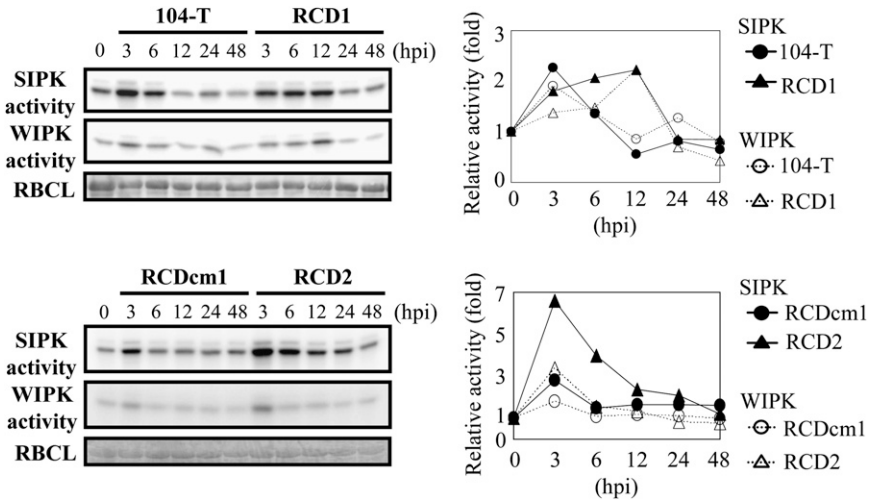


**Figure 6.** Kinase Activity of SIPK and WIPK during Infection.

Conidial suspension ($1 \times 10^6$ conidia/mL) of the wild-type strain 104-T, complemented strain RCDcm1, or *ssd1* mutants RCD1 and RCD2 was sprayed onto *N. benthamiana* leaves. At time points of 0 h (no inoculation) and 3, 6, 12, 24, and 48 HAI, proteins were extracted from the leaves of inoculated plants and subjected to an immunocomplex kinase assay. The graph at the right side of each panel indicates the relative kinase activity calculated by comparison with activity at 0 h. Loading controls represent Coomassie blue staining of the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBCL). Kinase activity of 104-T and RCD1 was determined in three replicated experiments. One set of representative data is shown in this figure. Kinase activity of RCDcm1 and RCD2 was determined in a single experiment.

or *SIPK WIPK*–silenced plants. This result suggests that the nonhost resistance of *N. benthamiana* to *C. graminicola* depends on prepenetration defense responses and that the MEK2-SIPK/WIPK cascade does not play an important role in resistance to this nonadapted pathogen.

### The *ssd1* Mutant Induces Higher SIPK and WIPK Activity Than the Wild-Type Strain during Host Infection

In a previous study, we found that the *ssd1* mutant induced papilla formation more rapidly than the wild type on onion epidermal cells (Tanaka et al., 2007). To examine whether the mutant induces higher MAP kinase activity in host tissue, we performed an immunocomplex kinase assay using SIPK- and WIPK-specific antibodies. Leaves were sprayed with conidial suspensions, and proteins extracted at 3, 6, 12, 24, and 48 HAI were used for immunocomplex kinase assays. In leaves inoculated with the wild-type strain (104-T) and an *ssd1* mutant complemented with a wild-type copy of *SSD1* (RCDcm1), both SIPK and WIPK activities increased up to 3 HAI but declined at 6 HAI and remained at a basal level from 12 to 48 HAI (Figure 6). By contrast, in leaves inoculated with two independent *ssd1* knockout mutants (RCD1 and RCD2) (Tanaka et al., 2007), SIPK and WIPK activity increased at 3 HAI, but the activity of both kinases remained at higher levels than in leaves inoculated with the wild-type fungus from 6 to 12 HAI (Figure 6). This result indicates that the *ssd1* mutant induces activation of SIPK and WIPK more strongly than does the wild type during host infection.

We also evaluated SIPK/WIPK activity in silenced plants inoculated with the wild type and *ssd1* mutant RCD1. Interestingly, in *SIPK*-silenced plants inoculated with the *ssd1* mutant, WIPK activity showed a large increase (Figure 7A) compared with nonsilenced plants (Figure 6), and the activity was higher when plants were inoculated with the mutant than with the wild-type fungus. By contrast, SIPK activity showed a less marked increase in *WIPK*-silenced plants (Figure 7B) than in nonsilenced plants (Figure 6). In plants in which both *SIPK* and *WIPK* were silenced, the activation of SIPK and WIPK was not detected at any time points (Figure 7C). Taken together, these results suggest that SIPK and WIPK have complementary roles in plant basal resistance to *C. orbiculare*.

### Cell Surface Components of the *ssd1* Mutant Induce SIPK and WIPK Activity

In yeast, *SSD1* regulates cell wall composition (Wheeler et al., 2003). Using immunocytochemistry, we also found that mutation of *SSD1* affects cell wall composition in *C. orbiculare*. Thus,

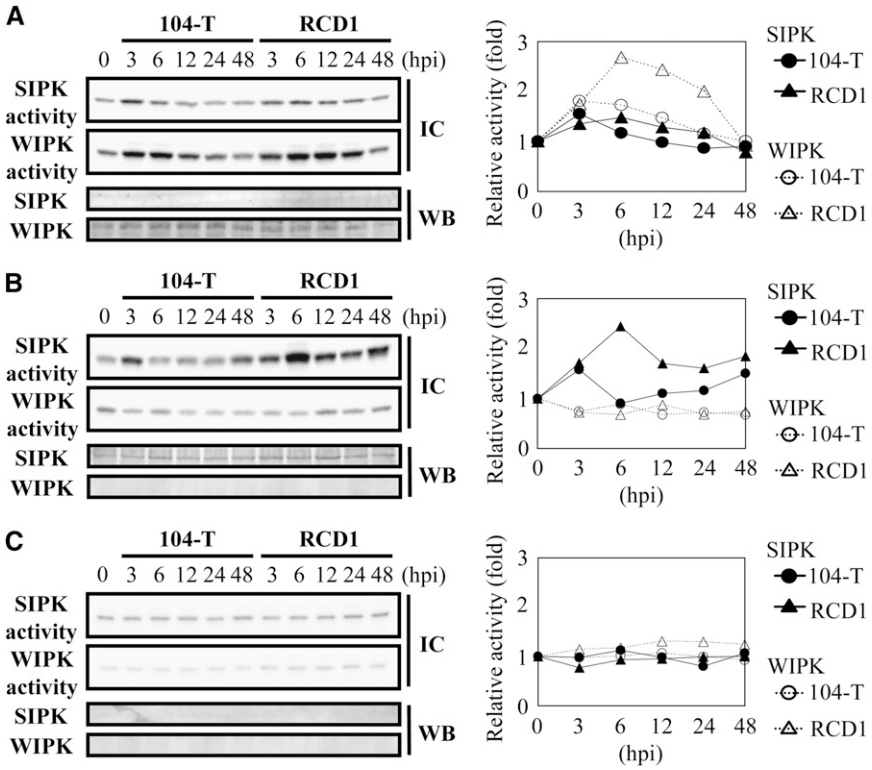


**Figure 7.** Kinase Activity of SIPK and WIPK during Infection of Silenced Plants.

A conidial suspension ($1 \times 10^6$ conidia/mL) of the wild-type 104-T or *ssd1* mutant RCD1 was sprayed onto leaves of *SIPK*-silenced **(A)**, *WIPK*-silenced **(B)**, and *SIPK*- and *WIPK*-silenced **(C)** plants. At time points of 0 h (no inoculation) and 3, 6, 12, 24, and 48 HAI, proteins were extracted from the leaves of inoculated plants and subjected to an immunocomplex kinase assay (IC; top panels). Gene silencing was evaluated by immunoblot analysis (WB; bottom panels). The graph to the right of each panel indicates the relative kinase activity calculated by comparison with activity at 0 h. Kinase activity was determined in silenced plants in two biological replicates. One set of representative data is shown in this figure.

extracellular matrix glycoproteins recognized by monoclonal antibody UB20 (Pain et al., 1992; Hughes et al., 1999) were significantly more abundant on the surface of conidia of *ssd1* mutants RCD1 and RCD2 than on that of the wild type (Figure 8). To test the hypothesis that fungal cell surface components induce SIPK and WIPK, we examined kinase activity in leaves infiltrated with conidia of the wild type and *ssd1* mutant. When heat-inactivated conidia were infiltrated into leaves, higher SIPK and WIPK activity was detected in leaves infiltrated with the *ssd1* mutant RCD1 than with 104-T (Figure 9A; see Supplemental Figure 1A online). This result suggests that inert surface component(s) of the mutant conidia induce SIPK/WIPK activity. To examine whether the factor activating SIPK and WIPK is proteinaceous, we infiltrated protease-treated conidia into leaves. The level of SIPK and WIPK activity induced by *ssd1* mutant conidia treated with protease was similar to that induced by conidia treated with control protease buffer, and in the case of the wild type, the level was slightly increased (Figure 9B; see Supplemental Figure 1B online), suggesting that the fungal component activating SIPK and WIPK is not proteinaceous.

An alternative explanation for the observed difference in kinase induction between the wild type and *ssd1* mutant is that wild-type conidia produce diffusible suppressors of SIPK and WIPK. To evaluate this possibility, we tested whether conidial exudates have suppressor activity against SIPK and WIPK. The level of SIPK and WIPK activity in leaves infiltrated with *ssd1* mutant conidia suspended in conidial exudate of the wild type was similar to that of mutant conidia alone (Figure 9C; see Supplemental Figure 1C online). This result suggests that the wild type does not produce diffusible suppressors of SIPK and WIPK on the plant surface during germination. We also tested whether cell-free conidial exudates have inducer activity for SIPK and WIPK. When exudates from either mutant or wild-type conidia were infiltrated alone, the detected kinase activities were low, similar to the kinase activity of the distilled water control (Figure 9D; see Supplemental Figure 1D online). These results suggest that the induction of SIPK and WIPK activities by the *ssd1* mutant is not due to an inability to suppress these host responses but rather to a specific induction of kinase activity by surface components of the mutant.

## DISCUSSION

The existence of basal resistance in plants has been inferred by the identification of mutants that are more susceptible to adapted pathogens than are wild-type plants (Dangl and Jones, 2001). However, analysis of basal resistance is problematic because the level of resistance is usually insufficient to completely restrict pathogen infection, unlike the resistance conferred by *R* genes. In this study, we sought to identify the plant signaling pathways involved in basal resistance.

Several previous studies have evaluated the involvement of the mitogen-activated protein kinase (MAPK) pathway in disease resistance. In tobacco, the MEK2-SIPK/WIPK cascade regulates multiple defense responses, including the hypersensitive cell death response (Yang et al., 2001). SIPK and WIPK are activated by the endogenous signal WAF-1 in tobacco mosaic virus–infected tobacco leaves, and this activation results in enhanced disease resistance (Seo et al., 2003). MEK2-SIPK/NTF6 plays a pivotal role in nitric oxide and ROS generation (Asai et al., 2008). In *Arabidopsis thaliana*, MPK6 and MPK3, orthologs of SIPK and WIPK, respectively, regulate the synthesis of camalexin, the major *Arabidopsis* phytoalexin (Ren et al., 2008). However, there is no information about the role of this MAPK pathway in basal resistance to adapted pathogens. Here, we have demonstrated

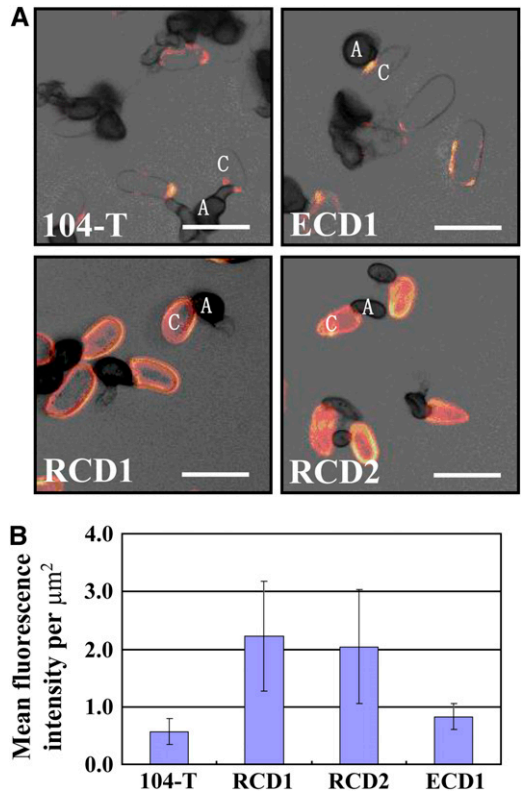


**Figure 8.** Detection of Extracellular Glycoproteins on the Surface of *C. orbiculare* Conidia by Immunofluorescence Labeling with Antibody UB20.

Conidia of two *ssd1* knockout mutants (RCD1 and RCD2), an ectopic mutant (ECD1), and the wild-type strain (104-T) were germinated on glass slides and labeled with mouse monoclonal antibody UB20 followed by goat anti-mouse secondary antibody conjugated with fluorescein isothiocyanate.

**(A)** Confocal microscope images showing overlays of bright-field and fluorescence channels, presented as projections of four optical sections (1 μm thick) with the fluorescence false-colored in orange to improve contrast. Note that conidia (C) of the two *ssd1* mutants label strongly and uniformly with UB20, whereas those of the wild type and ectopic mutant are unlabeled or show patchy labeling. A, appressoria. Bars = 10 μm.

**(B)** Histogram showing the intensity of UB20 immunofluorescence labeling of RCD1 RCD2, ECD1, and 104-T. Data represent mean fluorescence intensities based on analyses of the maximum projections of image stacks from 30 conidia of each fungal strain. Imaging parameters (gain, offset, confocal pinhole, and magnification) were identical for all samples. Error bars indicate SD.

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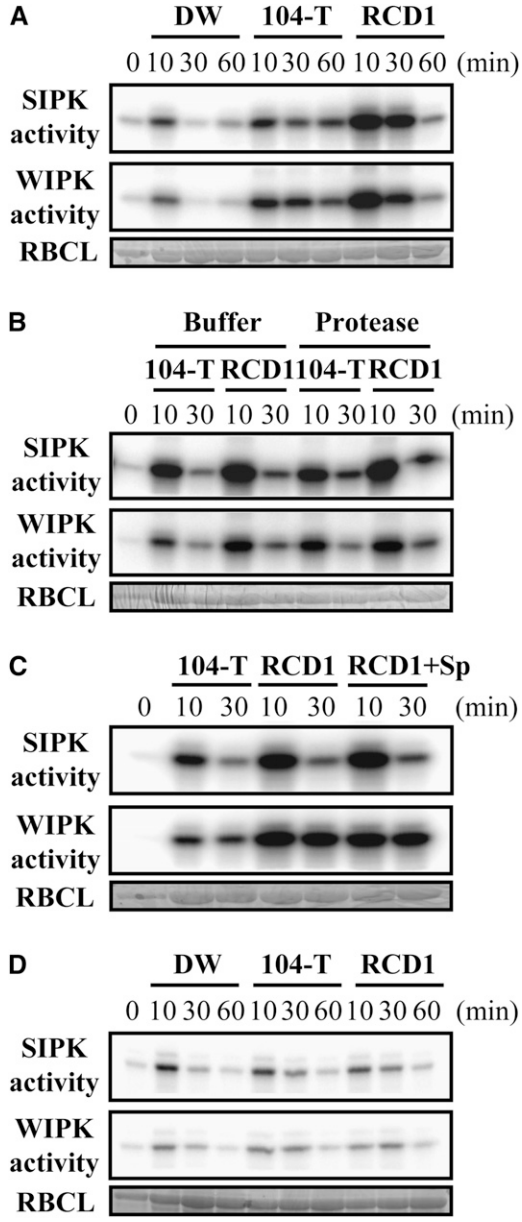


**Figure 9.** Activity of SIPK and WIPK in Leaves Infiltrated with Conidia.

**(A)** SIPK and WIPK activity in leaves infiltrated with conidia. Proteins were extracted at time points of 10, 30, and 60 min after infiltration with distilled water (DW) or a conidial suspension ($1 \times 10^6$ conidia/mL) of the wild-type 104-T or *ssd1* mutant RCD1.

**(B)** SIPK and WIPK activity in leaves infiltrated with conidia treated with protease. Proteins were extracted at 10 and 30 min after infiltration of conidial suspension ($1 \times 10^6$ conidia/mL) of the wild-type 104-T and *ssd1* mutant RCD1 treated with 3 mg/mL actinase E in 10 mM Tris-HCl, pH 7.6, buffer at 37°C for 60 min.

**(C)** SIPK and WIPK activity in leaves infiltrated with conidia suspended in exudate from wild-type conidia. Proteins were extracted at 10 and 30 min after infiltration of conidial suspension ($1 \times 10^6$ conidia/mL) of the wild type (104-T), *ssd1* mutant (RCD1), and *ssd1* mutant suspended in conidial exudate of the wild type (RCD1+Sp).

**(D)** SIPK and WIPK activity in leaves infiltrated with incubated conidial supernatant. Proteins were extracted at time points of 10, 30, and 60 min after infiltration of distilled water (DW) or incubated conidial supernatant of the wild type (104-T) and *ssd1* mutant (RCD1). Kinase activity was determined in two replicate experiments. One set of representative data is shown in this figure. Loading controls are presented as Coomassie blue staining of the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBCL).

that MEK2 and SIPK/WIPK are involved in basal resistance to *C. orbiculare* by gene silencing experiments in *N. benthamiana*, where knockdown of these genes allowed full infection by a fungal mutant that is normally restricted by cell wall–associated defense responses in wild-type plants (Figures 3 and 4). By contrast, infection assays using the nonadapted maize anthracnose pathogen, *C. graminicola*, revealed that the *SIPK*/*WIPK*-mediated signaling pathway does not play a central role in nonhost resistance (Figure 5A). Microscopy observations suggested that plant defense responses to this nonadapted pathogen differed from those mounted against the *C. orbiculare ssd1* mutant (Figure 5B). It is possible that nonhost resistance is expressed at prepenetration stage, involving defense mechanisms that operate independently of the *SIPK*/*WIPK*-mediated signaling pathway.

The *ssd1* mutant of *C. orbiculare* used in this study is deficient in a gene orthologous to *S. cerevisiae SSD1* (Tanaka et al., 2007). The bakers' yeast *S. cerevisiae* is not a human pathogen in healthy individuals but is increasingly being isolated from immunocompromised patients. Deletion of the *SSD1* gene causes yeast to be more virulent in the mouse infection model, and the *ssd1Δ* mutant elicits potent proinflammatory cytokines from macrophages in vitro (Wheeler et al., 2003). These authors presented evidence that quantitative and qualitative changes in cell wall components, such as β-glucan or chitin, in the yeast *ssd1* mutant result in misrecognition by the mammalian innate immune system.

In this study, infiltration of *N. benthamiana* leaves with heat-inactivated conidia of the *C. orbulare ssd1* mutant induced greater SIPK and WIPK activity than infiltration with wild-type conidia (Figure 9A). Although certain extracellular glycoproteins were more abundant on the surface of mutant conidia, these are unlikely to function in host recognition since treatment of the conidia with protease did not affect SIPK and WIPK induction (Figure 9B). These results suggest that polysaccharide components, such as glucans or chitin, on the fungal cell surface may induce plant innate immunity via activation of the SIPK/ WIPK signaling pathway and that the altered surface composition of the *ssd1* mutant provides a stronger inducing signal than does the surface of the wild type.

Jones and Dangl (2006) proposed that the level of plant basal resistance is a summation of PAMP-triggered immunity (PTI) minus the effect of effector-triggered susceptibility (ETS), as illustrated in their zigzag model. Our findings can be interpreted in relation to this model, as shown in Figure 10. In wild-type plants, the recognition of wild-type fungal PAMPs induces PTI through the MEK2-SIPK/WIPK pathway, but PTI is sufficiently suppressed by ETS to allow successful infection. By contrast,

recognition of the modified PAMPs displayed by the *ssd1* mutant induces a higher level of PTI that cannot be suppressed by ETS below the threshold for effective resistance, resulting in attenuated pathogenicity. When PTI is compromised by the silencing of *SIPK*/*WIPK*, ETS by the *ssd1* mutant is again sufficient to reduce the amplitude of plant defense responses below the threshold for effective resistance, resulting in successful infection. Additionally, the wild-type fungus has slightly increased infectivity when PTI is reduced by silencing of *SIPK*/*WIPK*. Thus, the activity of SIPK/WIPK correlates with the level of PTI. In *Arabidopsis*, transcriptional overexpression of *MKK7* (MAPKK) conferred enhanced resistance to compatible pathogens *Pseudomonas syringae* pv *maculicora* and *Hyaloperonospora parasitica* (Zhang et al., 2007). Moreover, transgenic potato (*Solanum tuberosum*) plants that carry a constitutively active form of potato MEK2, orthologous to tobacco MEK2, driven by a pathogen-inducible promoter of potato showed enhanced resistance to the adapted pathogens *Phytophthora infestans* and *Alternaria solani* (Yamamizo et al., 2006). Together with our own findings, these data suggest that infection by adapted pathogens can be restricted by basal resistance, provided that activation of the MAPK signaling pathway is sufficiently strong. In particular, it seems that early activation of the MAPK signaling pathway is important for counteracting penetration by the *C. orbiculare ssd1* mutant, which may in turn regulate the expression of host genes required for cell wall–associated defense responses, such as deposition of callose papillae.

Bacterial pathogens deliver effector proteins into plant cells through the Type III secretion system to suppress basal immune responses (Chisholm et al., 2006). Fungal pathogens, particularly those that form long-term biotrophic relationships with their hosts, may also suppress host defense reactions, although experimental evidence to support this assumption has been scarce (Panstruga, 2003). A specific effector delivery apparatus, like the Type III secretion system of Gram-negative bacteria, has not been discovered for fungal pathogens. However, a characteristic feature of most biotrophic fungi is their ability to form specialized infection structures inside host cells called haustoria (O'Connell and Panstruga, 2006). Fungal effectors may be secreted from haustoria into the extrahaustorial matrix or plant apoplast, from where they are presumed to be transferred into host cells (Chisholm et al., 2006). *C. orbiculare* does not form haustoria; however, during the initial biotrophic phase of infection, it produces intracellular hyphae inside living host cells, which may similarly function in effector delivery (Perfect et al., 1999). It is also possible that *C. orbiculare* conidia produce diffusible suppressors during germination on the plant surface to overcome host resistance to appressorium-mediated penetration. However, this seems unlikely, since exudates from conidia germinating in vitro could not suppress MAPK activity. Immunocomplex kinase assays revealed that MAPK activation occurs from 3 to 6 HAI in leaves inoculated with wild-type *C. orbiculare* (Figure 6). At this early stage of infection, *C. orbiculare* is engaged in conidial germination and appressorium morphogenesis on the plant surface and has not yet penetrated epidermal cells. These morphogenetic events are likely to be tightly coordinated with biosynthesis and remodeling of the fungal cell wall. Thus, different PAMPs may be displayed on the fungal cell surface and recognized by plant PAMP receptors to trigger MAPK-mediated defense responses.

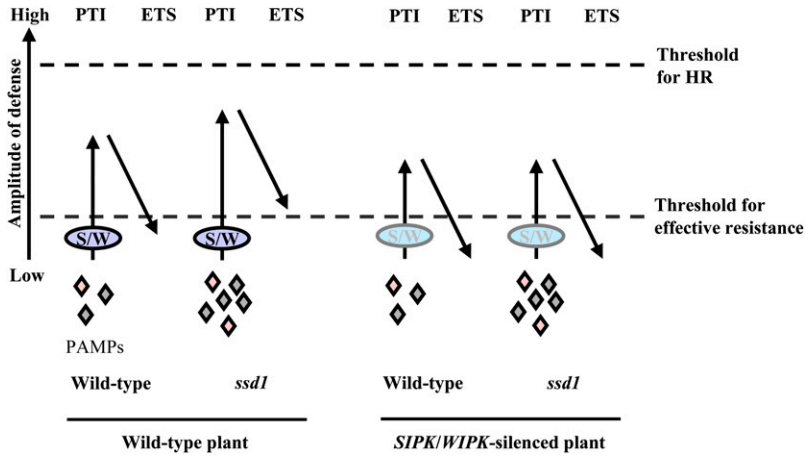


**Figure 10.** Schematic Model for Basal Resistance in Compatible Interactions Based on the Zigzag Model.

This model summarizes the quantitative output of the plant immune system, based on the summation of PTI and ETS in compatible interactions, as proposed in the zigzag model of Jones and Dangl (2006). In the case of a wild-type plant attacked by the wild-type fungus, PTI mediated through SIPK/WIPK (S/W) is suppressed by ETS, resulting in lesion formation. However, PTI by the *ssd1* mutant is induced to higher levels than by the wild-type fungus, and ETS is not sufficient to suppress PTI below the threshold required for effective resistance, resulting in attenuated pathogenicity. In the case of *SIPK WIPK*–silenced plants, the level of PTI induced by the wild-type fungus and *ssd1* mutant is reduced compared with that of wild-type plants. ETS by the *ssd1* mutant suppresses PTI below the threshold for effective resistance, resulting in lesion formation. Thus, the SIPK/WIPK activity induced by the wild-type fungus and *ssd1* mutant correlates with PTI, and the threshold for effective resistance depends on the sum of PTI minus ETS. HR, hypersensitive response.

## METHODS

### Plant Growth Conditions and Fungal Strains

*Nicotiana benthamiana* was grown in a controlled environment chamber (16 h photoperiod, 24°C). *Colletotrichum orbiculare* isolate 104-T (MAFF240422) was the wild-type strain used in this study. The *ssd1* mutants, RCD1, RCD2, and the complemented strain, RCDcm1, were described previously (Tanaka et al., 2007). *Colletotrichum graminicola* isolate MAFF236902 was obtained from the National Institute of Agrobiological Sciences GenBank, Tsukuba, Japan. All fungal cultures were maintained at 24°C on potato dextrose agar medium (Difco).

### Pathogen Inoculation and Cytological Assays

For inoculation with fungal pathogens, the upper leaves of 3-week-old *N. benthamiana* plants were sprayed with 2 mL of conidial suspension ($1 \times 10^6$ conidia/mL) and incubated in a humid plastic box at 24°C. For pathogenicity tests, inoculated leaves were observed for symptoms at 5 DAI. In the case of gene-silenced plants, the upper leaves were inoculated with conidia 3 to 4 weeks after inoculation with *Agrobacterium tumefaciens* carrying the VIGS constructs. For evaluation of kinase activity, the inoculated leaves were collected at 3, 6, 12, 24, and 48 HAI. Cytological observations and the detection of callose depositions (papillae) were performed as previously described (Tanaka et al., 2007). ROS accumulation was detected by staining with DAB (Yoshioka et al., 2003). Extracellular matrix glycoproteins were labeled on conidia germinating on glass microscope slides using monoclonal antibody UB20 for immunofluorescence microscopy (Pain et al., 1992; Hughes et al., 1999). Fluorescence was imaged with a Leica TCS SP2 confocal microscope and the 488-nm line of an argon laser. Images were analyzed and processed with LCS Lite software (Leica Microsystems) and Adobe Photoshop 8.0.

### VIGS

VIGS was performed as previously described (Ratcliff et al., 2001). The following primers were used to amplify cDNA fragments of Nb *HSP90*, Nb *RAR1*, and Nb *SGT1* from an *N. benthamiana* cDNA library (Yoshioka et al., 2003). Restriction sites (underlined) were added to the 5′-ends of the following forward and reverse primers for cloning into a tobacco rattle virus vector pTV00 (RNA2): NbHSP90-F-ClaI (5′-CCATCGATACCCTGACTATCATTGATAGTGGTATTGGT-3′) and NbHSP90-R-BamHI (5′-CGGGATCCTAATTTTTGTCCCCCTCCAAAGGTTC-3′) (restriction sites are underlined), which produced a 301-bp fragment; NbRAR1-F-ClaI (5′-CCATCGATAGGAAAGCACACAACAGAAA-3′) and NbRAR1-R-BamHI (5′-CGGGATCCAGCATTTCCATCCTCTCATC-3′), which produced a 395-bp fragment; and NbSGT1-F-ClaI (5′-CCATCGATTCGCCGTTGACCTGTACACTCAAGC-3′) and NbSGT1-R-BamHI (5′-CGGGATCCGCAGGTGTTATCTTGCCAAACAACCTAGG-3′), which produced a 633-bp fragment. The cDNA fragments of Nb *MEK1*, Nb *MEK2*, Nb *SIPK*, Nb *WIPK*, Nb *RBOHA*, and Nb *RBOHB* were prepared from an *N. benthamiana* cDNA library (Asai et al., 2008). *N. benthamiana* was infected by viruses via *Agrobacterium*-mediated transient expression of infectious constructs. The vectors pBINTRA6 and pTV00, containing the inserts RNA1 and RNA2, respectively, were transformed separately by electroporation into *Agrobacterium* strain GV3101, which includes the transformation helper plasmid pSoup (Hellens et al., 2000). A mixture of equal parts of *Agrobacterium* suspensions containing RNA1 and RNA2 was inoculated into 2- to 3-week-old *N. benthamiana* seedlings. The effect of VIGS was confirmed by RT-PCR. Total RNA (2 μg) was extracted from leaves inoculated with the *ssd1* mutant at 5 DAI using TRIzol reagent (Invitrogen) following the manufacturer's protocol. RT-PCR was performed using the ReverTra Dash RT-PCR kit (Toyobo) following the manufacturer's protocol. The primers used for RT-PCR are listed in Supplemental Table 1 online.

### Infiltration of Conidia into Leaves

Conidia of the wild type (104-T) and *ssd1* mutant (RCD1) were harvested from 6-d-old cultures and washed twice in 5 mL of sterilized distilled water, and the concentration of the conidia was then adjusted to $1 \times 10^6$ conidia/mL. Approximately 200 μL of conidial suspension was gently pressure-infiltrated onto the abaxial surface of leaves using a 1-mL syringe. For heat-inactivation, conidial suspension was boiled at 100°C for 5 min. For protease treatment, a pellet of cells ($1 \times 10^6$ conidia) was diluted with 3 mg/mL Actinase E (Kaken Pharmaceutical) in 10 mM Tris-HCl, pH 7.6, and incubated at 37°C for 60 min. After incubation, the conidial suspension was boiled at 100°C for 5 min to inactivate Actinase E and washed twice with sterilized distilled water. Cell-free exudates were collected from germinating conidia as follows. One milliliter of conidial suspension ($1 \times 10^5$ conidia/mL) was incubated at 24°C for 6 h on a Petri dish (3-cm diameter), and the supernatant was collected with a micropipette and centrifuged (14,000 rpm, 2 min).

### Immunocomplex Kinase Assay and Immunoblot Analysis

Immunocomplex kinase assays and immunoblot analysis were performed as previously described, with some modifications (Katou et al., 2003; Asai et al., 2008). The kinase reaction was performed at 25°C for 30 min in a volume of 25 μL reaction buffer containing 0.5 mg/mL myelin basic protein (Sigma-Aldrich), 20 μM ATP, and 2.5 μCi [$\gamma$-$^{32}$P]ATP. The phosphorylated myelin basic protein was detected using a BAS-1800II imaging system (Fuji LifeScience). The kinase activity was evaluated using MultiGauge software. SIPK protein was detected with 50 μg of total protein. WIPK protein was immunoprecipitated with 200 μg of total protein and anti-WIPK antibody and subjected to immunoblot analysis. The signals were detected with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

### Accession Numbers

Sequence data from this article can be found in GenBank/EMBL databases under the following accession numbers: AB245428 (Co *SSD1*), AB360635 (Nb *MEK1*), AB360636 (Nb *MEK2*), AB373025 (Nb *SIPK*), AB098729 (Nb *WIPK*), AB079498 (Nb *RBOHA*), AB079499 (Nb *RBOHB*), AF480487 (Nb *RAR1*), AF494083 (Nb *SGT1*), and AY368904 (Nb *HSP90*).

### Supplemental Data

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

**Supplemental Figure 1.** Quantitative Analysis of Kinase Activity Shown in Figure 9.

**Supplemental Table 1.** Primers Used for RT-PCR.

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