

The *WRKY45*-Dependent Signaling Pathway Is Required For Resistance against *Striga hermonthica* Parasitism^{1[OPEN]}

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The root hemiparasite witchweed (*Striga* spp.) is a devastating agricultural pest that causes losses of up to \$1 billion US annually in sub-Saharan Africa. Development of resistant crops is one of the cost-effective ways to address this problem. However, the molecular mechanisms underlying resistance are not well understood. To understand molecular events upon *Striga* spp. infection, we conducted genome-scale RNA sequencing expression analysis using *Striga hermonthica*-infected rice (*Oryza sativa*) roots. We found that transcripts grouped under the Gene Ontology term defense response were significantly enriched in up-regulated differentially expressed genes. In particular, we found that both jasmonic acid (JA) and salicylic acid (SA) pathways were induced, but the induction of the JA pathway preceded that of the SA pathway. Foliar application of JA resulted in higher resistance. The *hebiba* mutant plants, which lack the JA biosynthesis gene *ALLENE OXIDE CYCLASE*, exhibited severe *S. hermonthica* susceptibility. The resistant phenotype was recovered by application of JA. By contrast, the SA-deficient *NahG* rice plants were resistant against *S. hermonthica*, indicating that endogenous SA is not required for resistance. However, knocking down *WRKY45*, a regulator of the SA/benzothiadiazole pathway, resulted in enhanced susceptibility. Interestingly, *NahG* plants induced the JA pathway, which was down-regulated in *WRKY45*-knockdown plants, linking the resistant and susceptible phenotypes to the JA pathway. Consistently, the susceptibility phenotype in the *WRKY45*-knockdown plants was recovered by foliar JA application. These results point to a model in which *WRKY45* modulates a cross talk in resistance against *S. hermonthica* by positively regulating both SA/benzothiadiazole and JA pathways.

Striga spp., such as *Striga hermonthica* and *Striga asiatica*, are among the most economically important parasitic plants (Riches and Parker, 1995). *Striga* spp., which belong to the Orobanchaceae family, are obligate root hemiparasites well adapted to their parasitic lifestyle. They produce several hundred thousand tiny seeds that can survive in soil for decades, germinating in response to host root-derived germination stimulants such as strigolactones (Cook et al., 1966). After germination, the parasite uses a multicellular structure called the haustorium to invade the host after the perception of host-derived haustorium-inducing factors (Albrecht et al., 1999). Within a few days, the parasite establishes a

connection with the host vasculature to abstract water, nutrients, and organic solutes (Yoshida and Shirasu, 2009, 2012). Although *Striga* spp. have chlorophyll, the parasites are largely dependent on their hosts (Riches and Parker, 1995). Visible symptoms of parasitic infection in host plants are desiccation, necrosis, and severe stunting, resulting in severe effects on host plant growth and development. *Striga* spp. infect agriculturally important cereal and legume crops, causing severe yield losses, particularly in the dry tropics and subtropics (Riches and Parker, 1995). Especially, *S. hermonthica* and *S. asiatica*, which infect important crops such as maize (*Zea mays*), sorghum (*Sorghum bicolor*), and rice (*Oryza sativa*), affect an estimated 40% of the cereal-producing areas of sub-Saharan Africa, where farmers lose 20% to 80% of their yield (Riches and Parker, 1995). This affects the livelihood of approximately 100 million farmers, resulting in losses of up to \$1 billion US annually (Spallek et al., 2013). In addition, sorghum yield losses of up to 20% occur in *S. asiatica*-infested areas in India (Musselman and Press, 1995).

The fundamental strategies to control *Striga* spp. are to prevent their reproduction, to destroy their soil seed bank, and to limit their spread to new uninfested areas (Riches and Parker, 1995). Several control methods, including cultural and mechanical (crop rotation and hand pulling), biological (using *Fusarium oxysporum*; Yonli

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et al., 2005), or chemical (using ethylene [ET] gas; Iverson et al., 2011), have been tried, but their effectiveness is limited by the size of the infested areas, inefficient delivery systems, and costs. For example, over \$250 million US was spent to eradicate *S. asiatica* in South and North Carolina by suicidal germination using ET gas (Iverson et al., 2011).

One of the most efficient and cost-effective ways to control *Striga* spp. infestation would be to develop resistance or tolerance in host species. Toward this end, cultivars and wild relatives of several crop species, including sorghum, which show resistance to *Striga* spp., have been identified. For example, Hess et al. (1992) reported a *Striga* spp.-resistant sorghum with low germination stimulant production. In the case of rice, Gurney et al. (2006) found that cv Nipponbare, a *japonica* cultivar, is resistant against *S. hermonthica*, whereas other cultivars, including cv Kasalath and cv Koshihikari, are susceptible. Significantly, a phenotypic analysis of New Rice for Africa cultivars, which have gained popularity in many areas of Africa, revealed that several lines exhibit a level of resistance against *S. hermonthica* (Cissoko et al., 2011). However, the mechanisms by which monocots resist *Striga* spp. parasitism are unknown.

Inducible defense responses against most pathogens in plants are regulated mainly by three signaling molecules, salicylic acid (SA), jasmonic acid (JA), and ET (Tsuda et al., 2009). The interactions of these signaling pathways are complicated, but plants are able to activate them depending on the type of pathogen encountered (Kenton et al., 1999; De Vos et al., 2005; Balbi and Devoto, 2008; Robert-Seilantantz et al., 2011). For instance, SA promotes resistance against pathogens with a biotrophic lifestyle, whereas JA/ET-dependent defenses are more effective against necrotrophic pathogens (Glazebrook, 2005). For hemibiotrophic pathogens, such as *Magnaporthe oryzae*, both SA and JA are important for resistance (Shimono et al., 2007; Riemann et al., 2013). In the case of resistance against root knot nematodes, foliar JA application induces systemic defense responses (Nahar et al., 2011), but an intact JA signaling pathway is, in fact, required for susceptibility to root knot nematodes (Bhattarai et al., 2008).

Benzothiadiazole (BTH), a functional SA analog that activates downstream of the SA pathway, has been used as an inducer of plant defense against various microbial diseases (Lawton et al., 1996). For example, BTH is effective in the induction of rice blast resistance, resulting in the reduction of susceptible-type lesions, and its inducible defense response is mediated by *WRKY45*, which constitutes one of the two independent SA/BTH defense pathways (Shimono et al., 2007). In addition, the application of SA or BTH, but not methyl jasmonate (Me-JA), induces resistance against the root parasitic plant *Orobanche minor* in red clover (*Trifolium pratense*; Kusumoto et al., 2007). Similarly, the application of BTH on seeds protects sunflower (*Helianthus annuus*) against another *Orobanche* spp., *Orobanche cumana* (Sauerborn et al., 2002). Interestingly, the stem-connecting parasitic plant *Cuscuta pentagona* grew larger on hosts deficient in SA or insensitive to JA, suggesting that both SA and JA

affect defense against the parasite (Runyon et al., 2010). Root treatments with either SA or JA also induce resistance in a sorghum cultivar infected with *S. hermonthica* (Hiraoka and Sugimoto, 2008). Thus, SA or JA application is often able to induce resistance, but the roles of endogenous SA and JA in immunity against parasitic plants are poorly understood.

In this report, we characterized the roles of SA and JA in resistance against *S. hermonthica* in rice. Genome-wide transcriptome analysis shows that SA and JA pathway marker genes are induced after *S. hermonthica* infection. Analysis of mutants and transgenic plants demonstrates that activation of the JA pathway enhances resistance to *S. hermonthica*, whereas suppression of endogenous SA accumulation results in enhanced resistance. Interestingly, however, knockdown of *WRKY45* by RNA silencing led to reduced resistance against *S. hermonthica* parasitism. Our results support a model in which resistance against *S. hermonthica* parasitism is mediated by cross talk between the JA- and *WRKY45*-dependent SA/BTH defense pathways.

RESULTS

Transcripts of Genes Involved in Response to Biotic Stimuli Are Highly Enriched in Rice Infected with *S. hermonthica*

To characterize the transcriptional profile of host plants during *S. hermonthica* infection, we performed an RNA sequencing (RNA-Seq) analysis using roots of the susceptible rice 'Koshihikari' at 0, 3, and 7 d after infection (dpi). Differential expression (DE) analysis was performed using the RNA-Seq reads mapped to rice reference complementary DNA (cDNA; Supplemental Data Set S1). Using the false discovery rate less than 0.01 as a cutoff to identify differentially regulated genes, 716 up-regulated and 266 down-regulated DE genes were found at 3 dpi, while 398 up-regulated and 106 down-regulated DE genes were detected at 7 dpi compared with 0 dpi (Fig. 1; Supplemental Data Sets S2 and S3). Altogether, we found a total of 787 up-regulated and 315 down-regulated unique DE genes after *S. hermonthica* infection (Fig. 1). To understand the function of these DE genes, we performed Gene Ontology (GO) enrichment analyses on the gene sets. We found that the GO terms response to other organisms, biotic stimulus, as well as defense response were significantly enriched in up-regulated DE genes but not in the down-regulated genes after infection ($P < 0.05$; Supplemental Data Sets S2 and S3). These data suggested that the host rice plants are able to recognize *S. hermonthica* as a pathogen, even though it is a plant, and induce a typical pathogen defense response against the parasite.

JA and SA Defense Pathways Are Induced, and the ET Pathway Is Down-Regulated, after *S. hermonthica* Infection

Studies in *Arabidopsis* (*Arabidopsis thaliana*) have shown that inducible plant defense mechanisms are

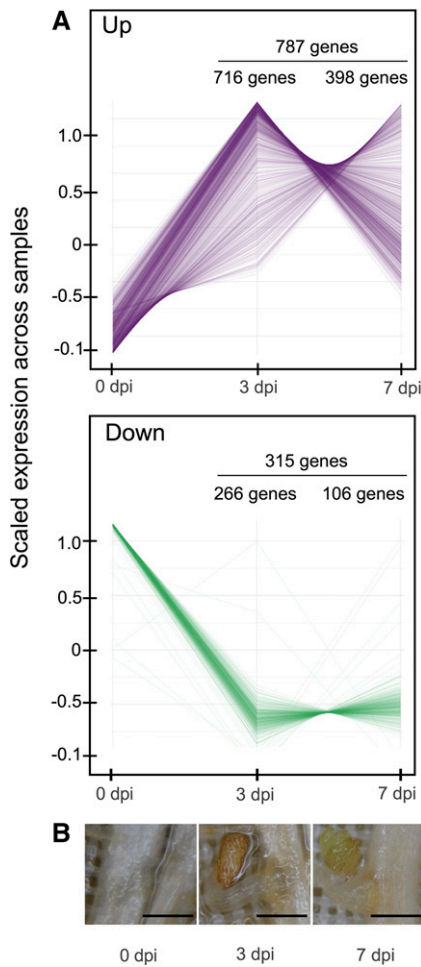


Figure 1. Genes differentially expressed in rice upon *S. hermonthica* infection. A, Genes up- and down-regulated in cv Koshihikari roots infected with *S. hermonthica* at 0, 3, and 7 dpi. B, Rice roots at 0, 3, and 7 dpi. Bars = 0.5 cm.

mostly regulated by the JA, SA, and ET pathways (Tsuda et al., 2009; De Vleeschauwer et al., 2013). These signaling pathways influence each other through antagonistic or synergistic cross talk, allowing plants to adapt their defense responses to the type of pathogen (Koornneef and Pieterse, 2008). Among the 787 up-regulated unique DE genes, we quantified the expression of JA, SA, and ET pathway marker genes in cv Koshihikari and a less susceptible cultivar, cv Nipponbare, by quantitative reverse transcription (qRT)-PCR analysis (Supplemental Table S1). For the JA pathway, we analyzed the expression of the JA biosynthesis genes *ALLENE OXIDE SYNTHASE2* (*OsAOS2*; Nahar et al., 2011) and *ALLENE OXIDE CYCLASE* (*OsAOC*; Riemann et al., 2013). The expression of *OsAOS2* increased after infection by a factor of about 2 at 1 dpi, peaked with a 12-fold increase at 3 dpi, and was reduced at 7 dpi in the roots of both cv Nipponbare and cv Koshihikari (Fig. 2A). The expression of *OsAOC* also peaked at 3 dpi, with a more than 3-fold increase in the *S. hermonthica*-infected roots of cv Nipponbare, while no significant change was observed in cv Koshihikari

(Fig. 2B). Consistently, the high expression of *OsAOC* was accompanied by an increase in the content of *S. hermonthica*-induced JA and JA-Ile in the infected roots of cv Nipponbare; accumulation of JA and JA-Ile also peaked at 3 dpi (819 and 155 ng g⁻¹ dry weight, respectively; Fig. 2, C and D). In contrast, the *S. hermonthica*-infected roots of cv Koshihikari accumulated much lower amounts of JA and JA-Ile, with a peak at 1 dpi (296 and

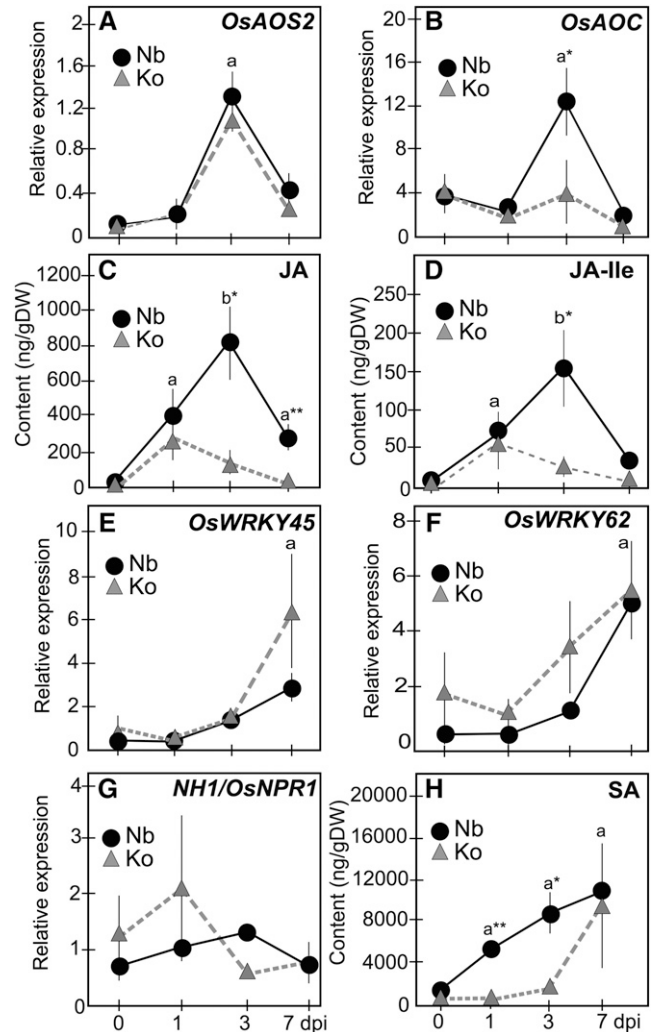


Figure 2. *S. hermonthica* infection induces JA and SA defense pathways. JA defense pathway genes (A and B), endogenous JA content (ng g⁻¹ dry weight [DW]; C), endogenous JA-Ile content (ng g⁻¹ dry weight; D), SA defense pathway genes (E–G), and endogenous SA content (ng g⁻¹ dry weight; H) are shown at 0, 1, 3, and 7 dpi. Gene expression analysis and hormone measurements were conducted using roots of cv Nipponbare (Nb; black circles) and cv Koshihikari (Ko; gray triangles). For gene expression, three biological and three technical replicates each containing a pool of roots from at least five plants were analyzed. For hormone measurement, three biological replicates each containing a pool of roots from at least five plants were analyzed. Values represent means and \pm SE. Gene expression was normalized using *OsCyclophilin*. Different letters indicate statistically significant differences at $P < 0.05$ from 0 dpi. Asterisks indicate statistically significant differences (*, $P < 0.05$; and **, $P < 0.01$) among rice cultivars.

59 ng g⁻¹ dry weight, respectively; Fig. 2, C and D). These data indicate that the JA defense pathway is induced at the early stages of infection starting at 1 dpi, and in the case of the less susceptible cv Nipponbare, much higher accumulation of JA and JA-Ile occurs at 3 dpi in *S. hermonthica*-infected roots.

For genes involved in the SA pathway, we chose *OsWRKY45*, *OsWRKY62*, and *Nonexpresser of Pathogenesis-Related Genes1 (OsNPR1)/NPR1 homolog1 (NH1)* as markers. *OsWRKY45* is a key regulator of the SA/BTH signaling pathway and is involved in BTH-induced resistance against *Magnaporthe grisea* (Shimono et al., 2007). *OsWRKY62* is one of the genes downstream of the SA/BTH signaling pathway that is regulated by *OsWRKY45* (Nakayama et al., 2013). *NH1/OsNPR1* is a rice ortholog of Arabidopsis *NPR1*, which regulates an independent SA signaling pathway in rice (Shimono et al., 2007). The induction of *OsWRKY45* and *OsWRKY62* was detected at 3 dpi and was highest at 7 dpi in the *S. hermonthica*-infected roots of both cv Nipponbare and cv Koshihikari (Fig. 2, E and F). In contrast, only a slight increase in the expression of *NH1/OsNPR1* was detected at 3 dpi in the *S. hermonthica*-infected roots of cv Nipponbare (Fig. 2G). In cv Koshihikari, the expression of *NH1/OsNPR1* was slightly reduced from 3 to 7 dpi. To confirm that the increased SA-related gene expression reflects endogenous SA contents, we analyzed SA levels in *S. hermonthica*-infected rice plants (Fig. 2H). In cv Nipponbare, the SA level in roots of plants at 0 dpi was 1,598 ng g⁻¹ dry weight and started to increase at 1 dpi, reaching 10,682 ng g⁻¹ dry weight at 7 dpi (a 6-fold increase). On the other hand, in cv Koshihikari, the SA levels suddenly increased from 1,650 ng g⁻¹ dry weight at 3 dpi to 9,586 ng g⁻¹ dry weight at 7 dpi (an increase of almost 6-fold). Taken together, these data show that the SA defense pathway was also induced in rice in response to *S. hermonthica* infection.

The expression of four of the ET defense pathway genes, *Ethylene-Responsive Element Binding Protein89 (OsEBP89)*, *ETHYLENE RESPONSIVE FACTOR70 (OsERF70)*, *ETHYLENE INSENSITIVE 2a (OsEin2a)*, and *OsEin2b*, was analyzed. *OsEBP89* is a member of the ethylene-responsive element-binding protein (EREBP) group (Mao et al., 2006). *OsERF70*, also known as *OsEREBP1*, is an ET-inducible *ERF* gene that activates *Pathogenesis Related* genes (Cheong et al., 2003) whereas *OsEin2a* is a paralog of *OsEin2b*, a central component of the ET signaling pathway (Nahar et al., 2011). When we examined the expression of these genes in *S. hermonthica*-infected roots of cv Nipponbare, we found that *OsEBP89*, *OsERF70*, and *OsEin2b* did not change significantly at all sampling days (Supplemental Fig. S1, A–C). *OsEin2a* expression was down-regulated in infected plants from 1 to 7 dpi (Supplemental Fig. S1D). In cv Koshihikari roots, *OsEBP89* and *OsEin2a* expression did not change significantly from 1 to 7 dpi. The expression of *OsERF70* and *OsEin2b* was only slightly down-regulated at 7 dpi. In addition, it is noteworthy that the expression of *OsEBP89*, *OsERF70*, and *OsEin2b* was significantly higher in cv Koshihikari roots compared

with those of cv Nipponbare at 0 dpi (Supplemental Fig. S1, A–C).

JA Potently Induces Resistance against *S. hermonthica* Parasitism

To investigate the role of JA in the defense response against *S. hermonthica*, we treated rice leaves with Me-JA or mock solution 24 h before *S. hermonthica* infection on roots. Mock-treated cv Koshihikari plants had over 5-fold more *S. hermonthica* seedlings compared with mock-treated cv Nipponbare plants, confirming the earlier report that cv Koshihikari plants are more susceptible to *S. hermonthica* (Fig. 3A; Gurney et al., 2006; Swarbrick et al., 2008). This also resulted in a significant reduction of cv Koshihikari root biomass (Supplemental Fig. S2A). By contrast, application of Me-JA onto cv Koshihikari leaves resulted in an over 46% reduction in the number of *S. hermonthica* seedlings (Fig. 3A) and in a 39% increase in the root biomass to 44 mg compared with mock treatment (Supplemental Fig. S2A). In the case of cv Nipponbare, we did not detect any *S. hermonthica* seedlings at the six-leaf stage when host plant leaves were treated with Me-JA (Fig. 3A). Consequently, the root biomass of control and Me-JA-treated cv Nipponbare plants did not change significantly (Supplemental Fig. S2A). These results demonstrate that Me-JA is a strong inducer of resistance against *S. hermonthica* parasitism in rice.

The *hebiba* Mutant Is More Susceptible to *S. hermonthica*

As Me-JA application is sufficient to induce resistance, we reasoned that inhibiting JA biosynthesis in rice using a genetic approach may result in increased susceptibility to *S. hermonthica* parasitism. Rice contains a single copy of *AOC*, which the *hebiba* mutant lacks (Riemann et al., 2013). Consistently, the *hebiba* mutant had no detectable *OsAOC* expression, while it was up-regulated in roots of wild-type cv Nihon Masari plants (background of *hebiba*) with a peak at 1 dpi (Fig. 3C). The JA-responsive rice transcription factor gene *OsJAmyb* (Lee et al., 2001) was induced in both the wild type and *hebiba*, but the level of expression was less in *hebiba* (Fig. 3D). The JA levels were much higher in the wild-type than in *hebiba* at 0 dpi but were reduced at 3 dpi (Fig. 3E). In contrast, the levels of JA in *hebiba* were slightly induced at 1 dpi with a peak at 3 dpi (Fig. 3E). Changes in the JA-Ile contents followed similar patterns (Supplemental Fig. S3A). Notably, mock-treated *hebiba* plants had almost 2 times more attached *S. hermonthica* seedlings compared with the wild type (Fig. 3B), resulting in reduced root biomass of mock-treated *hebiba* plants to 21% of that of uninfected plants (Supplemental Fig. S2B). Furthermore, the root biomass of infected *hebiba* plants was only about 35% of that of infected wild-type plants (Supplemental Fig. S2B). Thus, it appears that the racemic JA produced from allene oxide through a nonenzymatic reaction in *hebiba* plants (Riemann et al., 2013) does not affect susceptibility to *S. hermonthica* parasitism. As *hebiba*

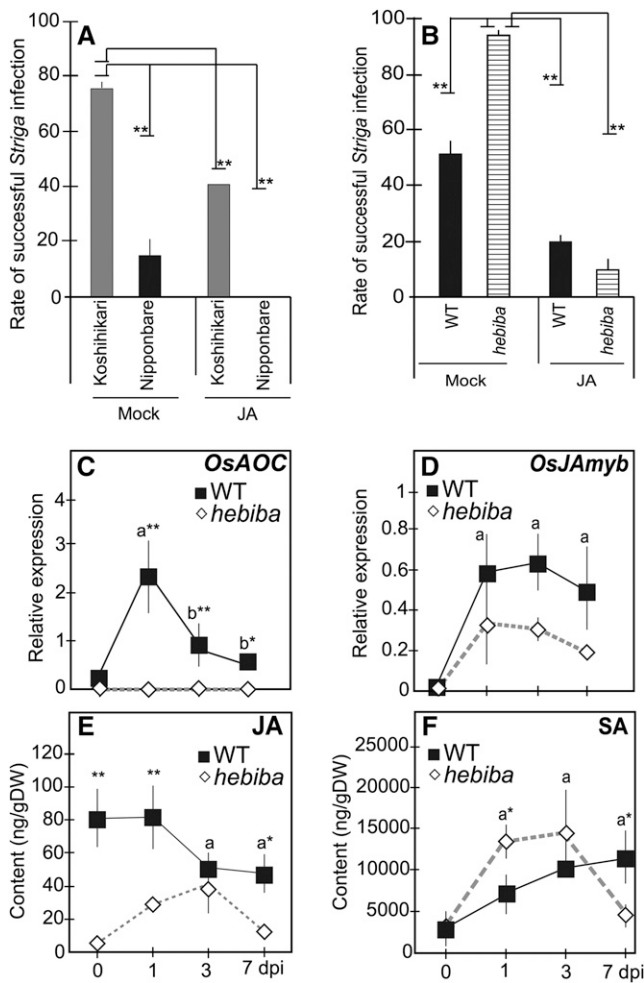


Figure 3. JA induces resistance. A and B, Effects of JA-induced resistance against *S. hermonthica* in cv Koshihikari, cv Nipponbare, cv Nihon Masari, and JA biosynthesis-defective *hebiba*. Three-week-old plants were sprayed until runoff with Me-JA (100 μ M) or the mock solution as described in “Materials and Methods.” Values represent means and \pm SE of the rate of successful *S. hermonthica* infection determined as described in “Materials and Methods.” C and D, *S. hermonthica*-induced expression of *OsAOC* and *OsJAmyb*. E and F, Changes in the content of endogenous JA and SA (ng g⁻¹ dry weight [DW]) in roots of wild-type cv Nihon Masari plants (WT; black squares) and JA biosynthesis defective *hebiba* plants (white diamonds) at 0, 1, 3, and 7 dpi. For gene expression, three biological and three technical replicates each containing a pool of roots from at least five plants were analyzed. Gene expression was normalized using *OsCyclophilin*. For hormone measurements, three biological replicates each containing a pool of roots from at least five plants were analyzed. Different letters indicate statistically significant differences at $P < 0.05$ from 0 dpi. Asterisks indicate statistically significant differences (*, $P < 0.05$; and **, $P < 0.01$) among rice cultivars.

contains a large genomic deletion comprising not only AOC but also 26 other genes (Riemann et al., 2013), we tested whether foliar application of Me-JA complements its enhanced susceptibility phenotype. We found that Me-JA treatment of wild-type and *hebiba* plants resulted in similar levels of resistance (Fig. 3B) and root biomass (Supplemental Fig. S2B). Taken together, these data

demonstrate that activation of the JA pathway leads to resistance against *S. hermonthica* parasitism.

Low SA Contents Induce Resistance against *S. hermonthica*

Next, we investigated the role of SA using rice plants that do not accumulate SA by introducing *NahG*, a bacterial SA hydroxylase. The effect of *NahG* was confirmed by the low SA content and low *OsPR1a* expression upon *S. hermonthica* infection (Supplemental Fig. S4, A and B). Two independent SA-deficient *NahG* rice plants were more resistant against *S. hermonthica* infection compared with wild-type cv Nipponbare (Fig. 4A). As *NahG* plants are known to accumulate catechol (van Wees and Glazebrook, 2003), we tested whether catechol affects *S. hermonthica* infection. No differences were detected in the number of *S. hermonthica* seedlings and the root biomass of mock- and catechol-treated cv Nipponbare or cv Koshihikari plants (Fig. 4B; Supplemental Fig. S2C).

Reduction of SA Activates the JA Pathway

As antagonistic interactions between SA and JA are known in many plant species (Lee et al., 2004; De Vleeschauwer et al., 2013), we reasoned that the resistance of SA-deficient *NahG* rice against *S. hermonthica* may be linked to the induction of the JA defense pathway. Indeed, we found that the *S. hermonthica*-induced expression of *OsAOS2* was much higher at 3 dpi in the *NahG* plants compared with the wild type (Fig. 4C). Similarly, *OsJAmyb* was also highly up-regulated in the *NahG* plants compared with the wild type (Fig. 4D). The high expression of *OsAOS2* was accompanied by a concomitant accumulation of JA and JA-Ile. At 1 dpi, JA content in *S. hermonthica*-infected roots of *NahG* plants was 1,156 ng g⁻¹ dry weight, which was 3-fold higher than that of the wild type. In addition, JA-Ile content was 182 ng g⁻¹ dry weight at 1 dpi, which was around 3-fold higher than that of the wild type (Fig. 4, E and F). Consistently, the application of JA significantly enhanced resistance against *S. hermonthica* in *NahG* plants (Supplemental Fig. S5). These results indicate that the JA defense pathway is strongly up-regulated in the SA-deficient *NahG* rice upon *S. hermonthica* infection.

Foliar BTH Application Triggers Systemic Resistance against *S. hermonthica*

As *NahG* plants exhibited enhanced resistance to *S. hermonthica*, we next tested whether activation of the SA pathway affects the *S. hermonthica* susceptibility. Since rice plants contain significantly higher amounts of SA compared with other plant species, such as *Arabidopsis* (Silverman et al., 1995), exogenous application of low amounts of SA would not show any physiological changes in wild-type rice. On the other hand, the application of high amounts of SA would induce other side

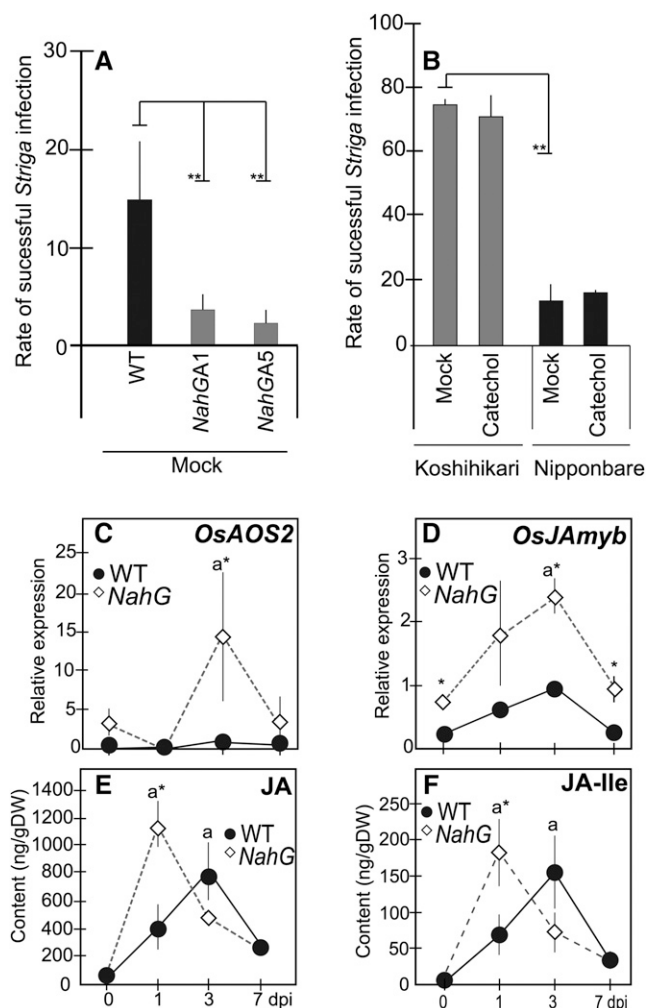


Figure 4. Low SA contents induce resistance. A, Rate of successful *S. hermonthica* infection in wild-type (WT) cv Nipponbare and transgenic *NahG* plants. B, Effects of foliar application of catechol on cv Nipponbare and cv Koshihikari plants. Plants were sprayed until runoff with catechol (100 μ M) or the mock solution as described in “Materials and Methods.” Values represent means and se of the rate of successful *S. hermonthica* infection determined as described in “Materials and Methods.” C and D, Expression of *OsAOS2* (C) and *OsJAmyb* (D). E and F, Changes in the levels of endogenous JA (E) and JA-Ile (F) content (ng g⁻¹ dry weight [DW]). Gene expression analysis and hormone measurements were conducted using roots of wild-type cv Nipponbare plants (black circles) and *NahG* plants (white diamonds) at 0, 1, 3, and 7 dpi. For gene expression, three biological and three technical replicates each containing a pool of roots from at least five plants were analyzed. Gene expression was normalized using *OsCyclophilin*. For hormone measurements, three biological replicates each containing a pool of roots from at least five plants were analyzed. Different letters indicate statistically significant differences at $P < 0.05$ from 0 dpi. Asterisks indicate statistically significant differences (*, $P < 0.05$; and **, $P < 0.01$) among rice cultivars.

effects (Ganesan and Thomas, 2001; Yang et al., 2013). BTH is a functional analog of SA that activates the SA downstream pathway (Lawton et al., 1996) and is one of the plant activators that protects them from a wide range of pathogens (Görlach et al., 1996; Shimono et al., 2007). Thus, we investigated the effects of foliar BTH on

the interaction between rice root and *S. hermonthica*. Surprisingly, foliar application of BTH onto rice 24 h before infection resulted in a 46% reduction in the number of *S. hermonthica* seedlings in roots of cv Koshihikari (Fig. 5A). We also found out that the application of BTH onto wild-type cv Nihon Masari and *hebiba* plants enhanced resistance against *S. hermonthica*, although the resistance level was lower compared with that induced by JA (Supplemental Fig. S3B). Interestingly, the *NahG* rice response to BTH application was similar to that of wild-type cv Nipponbare (Supplemental Fig. S5). This indicates that BTH is not able to complement the loss of SA in *NahG* plants, consistent with the fact that BTH itself does not significantly change the content of SA in the roots of wild-type cv Nipponbare (Supplemental Fig. S5C). These data suggest that BTH and endogenous SA may have different effects on *S. hermonthica* resistance in rice.

WRKY45 Plays an Important Role in Resistance against *S. hermonthica* Parasitism

The SA/BTH pathway in rice diverges into *WRKY45*- and *NH1/OsNPR1*-dependent pathways (Shimono et al., 2007). In *S. hermonthica*-infected rice roots, we found that *OsWRKY45* expression was highly induced, but *NH1/OsNPR1* expression was not (Fig. 2, E and G). Thus, to elucidate the SA/BTH signaling mechanisms underlying the defense response against *S. hermonthica*, we analyzed rice plants in which *WRKY45* is knocked down by RNA interference (*WRKY45*-kd; Shimono et al., 2007, Supplemental Fig. S6A). The expression of the downstream SA signaling genes *OsWRKY62* and *OsPR1b* was reduced in *WRKY45*-kd plants upon *S. hermonthica* infection (Fig. 5C; Supplemental Fig. S6B), which is consistent with a previous report on rice infected with *Magnaporthe oryzae* (Nakayama et al., 2013). Remarkably, *WRKY45*-kd plants exhibited disease susceptibility, characterized by a large number of *S. hermonthica* seedlings that attached and developed: the number of *S. hermonthica* seedlings attaching to roots of *WRKY45*-kd plants was almost 3-fold higher compared with wild-type cv Nipponbare (Fig. 5B). In addition, the size of successful *S. hermonthica* plants on *WRKY45*-kd was almost double the size of those on wild-type cv Nipponbare (Supplemental Fig. S7). As the reduction of endogenous JA levels resulted in enhanced susceptibility to *S. hermonthica* (Fig. 3, B and F), we analyzed the expression of *OsAOS2*. Compared with the wild type, the expression of *OsAOS2* was significantly reduced (Mann-Whitney test, $P < 0.05$) in the *WRKY45*-kd plants (Fig. 5D). Consistently, the down-regulation of *OsAOS2* was accompanied by a drastic suppression of JA and JA-Ile accumulation in the *WRKY45*-kd plants; *S. hermonthica*-induced JA and JA-Ile contents in *WRKY45*-kd plants at 3 dpi were only 33% and 25%, respectively, those in the wild type (Fig. 5, E and F). The enhanced susceptibility of *WRKY45*-kd is potentially due to the reduction of JA content, as its exogenous application can mostly reverse the phenotype (Fig. 5G). We also noticed that foliar

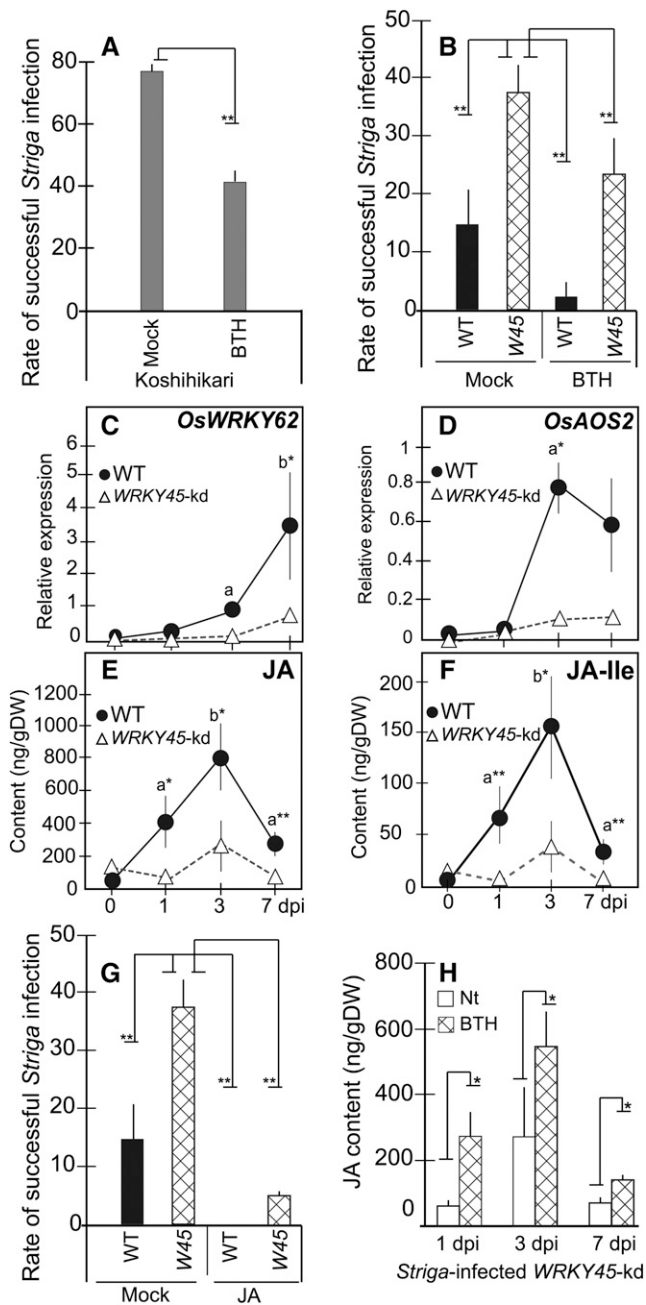


Figure 5. *WRKY45* plays an important role in resistance. A and B, Effects of BTH-induced systemic resistance against *S. hermonthica* in cv Koshihikari (A) and in wild-type (WT) cv Nipponbare and *WRKY45*-kd (B) plants. C and D, Expression of *OsWRKY62* (C) and *OsAOS2* (D). E and F, Changes in the levels of endogenous JA (E) and JA-Ile (F) content (ng g^{-1} dry weight [DW]). G, Effects of JA-induced resistance against *S. hermonthica* in wild-type cv Nipponbare and *WRKY45*-kd (W45) plants. H, Content of JA in nontreated *WRKY45*-kd plants and those whose leaves were treated with BTH. Plants were sprayed until runoff with BTH (250 μM), Me-JA (100 μM), or the mock solution as described in “Materials and Methods.” Values represent means and SE of the rate of successful *S. hermonthica* infection determined as described in “Materials and Methods.” Gene expression analysis and hormone measurements were conducted using roots of wild-type cv Nipponbare plants (black circles) and *WRKY45*-kd plants (white

triangles) at 0, 1, 3, and 7 dpi. For gene expression, three biological and three technical replicates each containing a pool of roots from at least five plants were analyzed. Gene expression was normalized using *Oscyclophilin*. For hormone measurements, three biological replicates each containing a pool of roots from at least five plants were analyzed. Different letters indicate statistically significant differences at $P < 0.05$ from 0 dpi. Asterisks indicate statistically significant differences (*, $P < 0.05$; and **, $P < 0.01$) among rice cultivars.

DISCUSSION

In this study, we performed genome-scale RNA-Seq analysis and characterized various JA- and SA-defective rice plants to elucidate the mechanisms of resistance against *S. hermonthica*. We found that both JA and SA defense pathways were induced after *S. hermonthica* infection, although the kinetics of induction was different. The JA pathway genes were induced at earlier time points, starting at 1 dpi with a peak at 3 dpi, before returning to basal levels at 7 dpi, while those of the SA pathway were induced at 3 dpi with a higher expression at 7 dpi (Fig. 2, A, B, E, and F). Consistently, the accumulation of JA preceded that of SA (Fig. 2, C, D, and H). Since JA accumulates in response to wounding in rice (Hiraoka and Sugimoto, 2008; Riemann et al., 2013), perhaps the early induction of the JA pathway is due to the damage caused by *S. hermonthica* on host cells. Host cell wall degradation precedes penetration at 3 dpi (Olivier et al., 1991; Losner-Goshen et al., 1998; Yoshida and Shirasu, 2009). This is followed by vascular connection at 7 dpi, the time at which the levels of JA and JA-Ile are reduced (Fig. 2, C and D). The reason for the sharp decline at 7 dpi may be that the wounding site is sealed by this time point and/or that *S. hermonthica* may produce yet unknown virulent component(s) to suppress the JA pathway. On the other hand, SA accumulates at 3 dpi and increases to higher levels at 7 dpi, suggesting that the host might perceive the parasite as a biotroph, similar to the case with other biotrophic fungal and bacterial pathogens. The host may also have detected a yet-unknown virulent component(s) to trigger the SA pathway.

The late induction of the SA pathway and the sharp decrease in the levels of JA contents may also be due to an antagonistic interaction between JA and SA.

Although there is ample evidence suggesting that SA produced during pathogen infection plays an important role in antagonizing the JA-dependent response (Spoel et al., 2003), little is known about the changes of SA accumulation in the presence or absence of endogenous JA in monocot roots. We showed that the JA-deficient *hebiba* plants accumulated higher levels of endogenous SA content at 1 dpi (Fig. 3F), indicating that endogenous JA suppresses SA accumulation at this time point. It was also apparent that SA antagonistically suppressed the JA pathway at this time point (Fig. 4E). However, at 7 dpi, the antagonistic effect of JA on SA was no longer detected (Fig. 3, F and E). It is possible that the JA-SA antagonistic interaction occurs only at the initial defending stage but not later, when *S. hermonthica* establish vascular connection and take control of the host immune signaling pathways.

Our data clearly show that endogenous SA is not required for resistance against *S. hermonthica* (Figs. 3B and 4A). This is somewhat counterintuitive, as resistance to most obligate biotrophic plant pathogens requires SA but not JA (Spoel et al., 2003; Glazebrook, 2005; Robert-Seilaniantz et al., 2011). In addition, we found that application of the SA analog BTH enhances *S. hermonthica* resistance in rice (Fig. 5A). In fact, the loss of endogenous SA in *NahG* plants leads to enhanced resistance to *S. hermonthica*, and the phenotype is not reversed by BTH application (Supplemental Fig. S5A). This fact indicates that the exogenous BTH functions differently from the endogenous SA in rice. We reasoned that the enhanced resistance in *NahG* plants is likely due to higher JA accumulation and enhanced JA responses early after infection (Fig. 4, C–F). Interestingly, we also showed that the JA pathway is positively regulated by *WRKY45*, a well-studied key regulator of the SA/BTH pathway in rice (Fig. 5, E–G; Shimono et al., 2007). The rice subspecies *japonica* and *indica* have distinct allelic pairs of *WRKY45* termed *WRKY45-1* and *WRKY45-2*, respectively. These alleles have opposing roles in resistance against *Xanthomonas oryzae* pv *oryzae* and pv *oryzicola* and in JA accumulation (Tao et al., 2009). The *WRKY45-1* knockout mutant in the *japonica* cv Dongjin background accumulated higher levels of JA in *X. oryzae* pv *oryzae*-infected leaves compared with the wild type, although *AOS2* gene expression was suppressed (Tao et al., 2009). This differs from our finding that knocking down the *WRKY45-1* allele in cv Nipponbare suppresses *AOS2* gene expression and JA accumulation. Perhaps the *WRKY45*-dependent JA regulation is different between parasitic plants and fungal pathogens, on the one hand, and bacterial pathogens, on the other. We propose a model in which SA has two roles in resistance to *S. hermonthica*: one is to induce the *WRKY45*-dependent pathway to positively control the JA pathway, and the other is to negatively regulate it (Fig. 6). In this model, in the absence of SA, the JA pathway is enhanced. If the SA pathway is intact, SA/BTH-responsive *WRKY45* can drive the JA pathway. Consistently, BTH application induces JA accumulation (Supplemental

Fig. S8) and JA pathway genes such as *LIPOXYGENASE*, *12-Oxophytodieneate Reductase1 (OPR1)*, and *OPR4* (Shimono et al., 2007; Nakayama et al., 2013). However, BTH application can induce resistance even in *WRKY45*-kd plants, suggesting that downstream components in the *WRKY45*-independent SA/BTH pathway are also able to contribute to resistance against *S. hermonthica* in rice. Such a pathway is likely to be the *NH1/OsNPR1* pathway, as shown previously (Shimono et al., 2007; Nakayama et al., 2013). The involvement of JA in the *NH1/OsNPR1* pathway is unlikely, because foliar BTH application onto the *hebiba* mutants is able to induce resistance (Supplemental Fig. S3).

Curiously, cv Nipponbare is more resistant against *S. hermonthica* than cv Koshihikari (Fig. 3A; Supplemental Fig. S9; Gurney et al., 2006). It is possible that this is due to the differences in responsiveness. For example, upon *S. hermonthica* infection, only low levels of JA and JA-Ile accumulate in cv Koshihikari compared with cv Nipponbare (Fig. 2, B and C). In addition, previous studies showed that cv Nipponbare has preexisting and induced physical defense mechanisms that could prevent *S. hermonthica* from making vascular connections, whereas cv Koshihikari

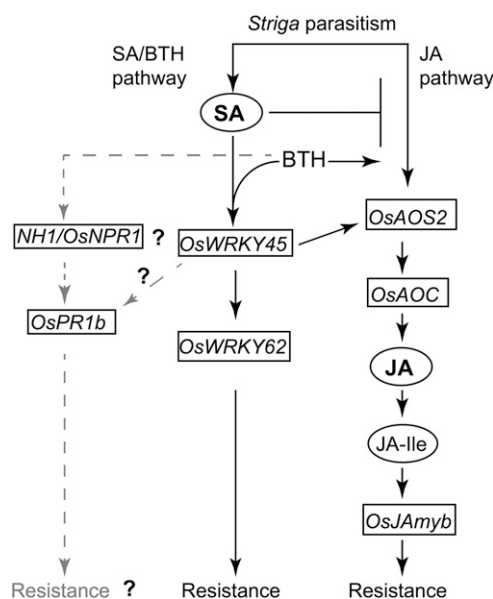


Figure 6. Schematic model of cross talk mediated by the JA and *WRKY45*-dependent SA/BTH defense pathways. Both JA and SA defense pathways are induced during resistance against *S. hermonthica*, but JA induction precedes that of SA. Although inhibiting JA biosynthesis results in susceptibility, inhibiting SA accumulation results in resistance characterized by a strong induction of the JA defense pathway. Also, knocking down *WRKY45* results in susceptibility by suppressing the JA defense pathway as well as downstream SA signaling. The *WRKY45*-kd loss of resistance phenotype is recovered by the application of BTH but strongly recovered by the application of JA. Both BTH and JA result in enhanced accumulation of endogenous JA. Although it appears that *NH1/OsNPR1* does not have a role in resistance against *S. hermonthica*, *OsPR1b* expression is similar to those of other SA pathway genes.

appears to lack such physical defense mechanisms (Supplemental Fig. S10; Gurney et al., 2006; Yoshida and Shirasu, 2009). Quantification of metabolic products such as lignin using NMR fingerprinting, which quantifies the syringyl:guaiacyl:*p*-hydroxyphenyl ratio of lignin (Kim and Ralph, 2010), may help to determine whether these weaknesses in cv Koshihikari are related to specific metabolic products such as lignin or cell wall components, which may be under the control of JA. Isolation of resistance genes from cv Nipponbare rice will further clarify the defense mechanisms that differentiate it from cv Koshihikari.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Plant materials and growth conditions were as reported previously (Yoshida and Shirasu, 2009) with a few changes. Briefly, *Striga hermonthica* seeds collected from a field in Kano, Nigeria, were a gift from Dr. Alpha Kamaro of the International Institute of Tropical Agriculture (IITA) in Kano. Rice seeds (*Oryza sativa japonica* 'Nipponbare' and 'Koshihikari') were obtained from the National Institute of Biological Sciences. The rice 'Nihon Masari' and 'hehiba' seeds were gifts from Peter Nick and Michael Riemann. WRKY45-kd rice plants in the cv Nipponbare background were provided by Hiroshi Takatsuji (National Institute of Agrobiological Sciences). The rice 'Nipponbare' and 'Koshihikari' seeds were dehusked and sterilized with a 10% (v/v) commercial bleach solution for 15 min and washed thoroughly with distilled water. Seeds were placed on filter paper moisturized with sterile water and grown in a 16-h-light/8-h-dark cycle at 26°C for 1 week.

Transgenic and Mutant Rice Seeds

Transgenic rice lines of 'Nipponbare' expressing *NahG* from *Pseudomonas putida* (*NahG* rice) were generated as described previously (Yang et al., 2004; Tanabe et al., 2014). WRKY45-kd and *NahG* rice seeds were dehusked and sterilized as above before being placed on square petri dishes containing 0.7% (w/v) agar and grown in a 16-h-light/8-h-dark cycle at 28°C/23°C for 1 week. The rice 'Nihon Masari' and 'hehiba' seeds were dehusked and sterilized as above before being placed on square petri dishes containing 0.7% (w/v) agar. They were transferred to a growth chamber and grown under continuous light at 25°C for 3 d. The presence of the *hehiba* mutation was verified by PCR as described by Riemann et al. (2013) using 5'-CCTCATGCCACATAGAAGGATC-3' and 5'-GGCGGGTTAAATCTAGAGG-3'. The presence of the *NahG* gene was verified using 5'-GAGACCGCGGATTATACGA-3' and 5'-TCAGTGTGAGGTCGTGGT-3'.

S. hermonthica Seed Preconditioning and Infection in a Rhizotron System

S. hermonthica seeds were briefly washed with 20% (v/v) commercial bleach solution and sterilized in a renewed bleach solution for 5 min with gentle agitation. The seeds were then washed thoroughly with distilled water and placed on glass microfiber filter paper (Whatman GF/A) moisturized with sterile water. The sterilized seeds were kept in the dark at 26°C for 10 to 14 d for preconditioning. Infection and subsequent observation were performed in the modified rhizotron system as described previously (Yoshida and Shirasu, 2009). The rhizotrons (10- × 14-cm square petri dish) were filled with rockwool (Nichiasu) onto which a 100- μ m nylon mesh was placed. The tops and bottoms of the petri dishes were perforated to allow shoot growth and draining. One-week-old rice seedlings were transferred to the rhizotrons and fertilized with one-half-strength Murashige and Skoog medium. Then, the rhizotrons were kept in growth chambers at a temperature cycle of 28°C/23°C for a 16-h-light/8-h-dark cycle. Two weeks after rice plants were transferred to the rhizotron, inoculation with *S. hermonthica* seeds was conducted. Preconditioned *S. hermonthica* seeds were treated with 10 nM strigol (a gift from Dr. K. Mori; Hirayama and Mori, 1999) for 2 to 6 h to synchronously induce germination and carefully placed next to roots of each rice plant. Strigol was

not washed prior to infection, as our previous experiments reported similar infection rates with or without rinsed seeds (Umehara et al., 2008; Yoshida and Shirasu, 2009). After inoculation, the rhizotron chambers were incubated under the same conditions as described above.

Chemical Treatments

For chemical treatment of plants, leaves of 3-week-old rice seedlings were sprayed until runoff with Me-JA (100 μ M), BTH (250 μ M), or catechol (100 μ M) 24 h before *S. hermonthica* inoculation. Distilled water containing 0.02% (v/v) Tween 20 was used as a control treatment. Me-JA and catechol were purchased from Sigma Japan. Me-JA, BTH, and catechol solutions were prepared in water containing 0.02% (v/v) Tween 20.

Sampling

Sampling was done at 0 (uninfected control), 1, 3, and 7 dpi. The 0-d (uninfected) samples were obtained at the same time as the 7-dpi samples. Rice roots were cut and immediately transferred into chilled 2-mL tubes with a steel top (BMS) and dipped in liquid nitrogen before storage at -80°C. Roots used for the determination of hormone content were obtained the same way, but the parasite seedlings were carefully removed using a stereomicroscope (Zeiss), weighed, and transferred into 2-mL tubes. Care was taken to avoid root parts that did not have contact with the parasite by first observing the roots using a stereomicroscope (Zeiss). Also, care was taken to minimize sample handling time to avoid loss of quality. Phenotypic data were obtained at 30 dpi.

RNA-Seq Analysis

S. hermonthica-infected cv Koshihikari roots were sampled as described above in three independent experiments. In addition, *S. hermonthica* seeds and seedlings germinated without hosts were sampled as controls. Total RNA was extracted as reported previously (Yoshida et al., 2010). For quality control of total RNAs, the 260:280 ratio (over 1.8) and the RNA integrity number (over 7) were measured by NanoDrop 1000 (Thermo Scientific) and 2100 Bioanalyzer (Agilent Technologies), respectively. Four micrograms each of total RNA was used for library preparation. The paired-end Illumina sequence libraries (insert size, 180 bp) were prepared using the TruSeq RNA Sample Prep Kit according to the manufacturer's instructions. Paired 101-bp sequences were obtained by sequencing with the Illumina HiSeq2000 sequencer for approximately 5 Gbp for each library. The sequence reads were divided to *S. hermonthica* and rice sequences by subsequent read mapping of rice ('Nipponbare') cDNAs and genome (Michigan State University Rice Genome Annotation Project version 7) using the CLC Genomics Workbench (version 5). The sequences unmapped to rice cDNA and genome were considered *S. hermonthica* sequences, and these unmapped sequences were de novo assembled using the CLC Genomics Workbench and the CAP3 program, resulting in 83,490 contigs. The de novo assembly of *S. hermonthica* will be described elsewhere. The resultant *S. hermonthica* assembly and rice cDNAs were concatenated and used for a reference sequence for mapping the sequences of both organisms. The Illumina sequence reads were mapped on the concatenated sequences using Bowtie2 with default setting (Langmead and Salzberg, 2012). The rice cDNAs having total mapped reads of more than 10 counts from sequences obtained from *S. hermonthica* seedlings and seed samples (without rice tissues) were eliminated from the subsequent analysis to avoid cross mapping. The cDNAs with fewer than 20 total mapped reads across uninfected and infected rice samples were also removed as lowly expressed genes. The reads mapped to the rice reference sequence were normalized using the trimmed mean (M-value) method (Robinson and Oshlack, 2010), and fragments per kilobase of exon per million fragments mapped values were calculated using the RSEM program (Li and Dewey, 2011).

Differential Expression and GO Enrichment Analysis

Differential expression calls in the normalized fragments per kilobase of exon per million fragments mapped data of rice were made using edgeR version 3.4.2 (Robinson and Smyth, 2007, 2008; Robinson et al., 2010; Robinson and Oshlack, 2010; McCarthy et al., 2012). Genes whose adjusted *P* values (Benjamini and Hochberg, 1995) were less than 0.01 were considered differentially expressed. GO enrichment analysis was performed using the Plant GeneSet Enrichment Analysis Toolkit

(<http://structuralbiology.cau.edu.cn/PlantGSEA/analysis.php>). Data visualization was performed using the ggplot2 package (Wickham, 2009).

Quantification of Postattachment Resistance and the Effect of *S. hermonthica* on Host Biomass

Postattachment resistance was quantified at 30 dpi as described by Cissoko et al. (2011) with some modifications. Before harvest, the root system of each rhizotron was photographed using the Canon EOS Kiss X6i digital camera. The rate of successful *S. hermonthica* infection was determined as a percentage of the total number of *S. hermonthica* seedlings later than the six-leaf stage against the total number of attached *S. hermonthica* seedlings. Attachment was a prerequisite because we noticed that some germinated *S. hermonthica* died before attaching to the host roots. This was done to avoid overestimation that might result from using the total number of *S. hermonthica* seeds instead of the total number of attached *S. hermonthica* seeds. *S. hermonthica* seedlings growing on the roots of each infected plant were then harvested, and the fresh weight and length were determined before drying at 42°C for 2 d. In order to assess the effect of *S. hermonthica* on the growth and partitioning of host biomass, the dry weight of rice roots after detachment of *S. hermonthica* seedlings was determined at 30 dpi. The effect of *S. hermonthica* on the host was quantified by comparing the root biomass of infected plants with that of the uninfected control plants.

RNA Extraction, cDNA Synthesis, and qRT-PCR

Root RNA extraction and DNaseI treatment were done using the RNeasy Plant Mini Kit (Qiagen) and Qiagen DNaseI solution following the manufacturer's instructions. RNA concentration and purity were measured using the NanoDrop spectrophotometer (NanoDrop Technologies). First strand cDNA synthesis was done using the ReverTra Ace qPCR Reverse Transcriptase (RT) kit (Toyobo). 5X RT buffer, RT enzyme mix, and primer mix were added into 35 ng of total RNA and incubated at 37°C for 15 min followed by 98°C for 5 min using the Bio-Rad C1000 Thermal Cycler. After incubation, the mixture was diluted 10 times and stored at -20°C until use. qRT-PCR was performed using Thunderbird SYBR qPCR mix (Toyobo). The reaction mixture contained 2 μL of template cDNA, 10 μL of SYBR qPCR mix, 0.04 μL of 50X ROX Reference Dye (Thermo Fisher), 0.6 μL each of the forward and reverse primers, and 6.76 μL of distilled autoclaved water. All qRT-PCRs were performed in three technical replicates, and three independent biological replicates were analyzed. Each biological replicate contained a pool of two to five plants, from which at least four *S. hermonthica*-infected roots were obtained. qRT-PCR was performed in the Stratagene MX3000P and analyzed using MXPro QPCR Software version 4.10d (Stratagene). qRT-PCR was performed in three segments. Segment 1 consisted of 15 min at 95°C for one cycle, segment 2 consisted of 15 s at 95°C and 30 s at 60°C for 40 cycles, and segment 3 consisted of 1 min at 95°C, 30 s at 55°C, and 30 s at 95°C for one cycle. Data were obtained and transferred to Microsoft Excel for further handling. Gene expression was normalized using *OsCyclophilin* (Gutjahr et al., 2008). Statistically significant induction was determined by comparing gene expression at 1, 3, and 7 dpi with that at 0 dpi (uninfected control). Supplemental Table S1 shows the description of target genes used in this study and the primer pairs used for qRT-PCR.

Sample Preparation and Quantitative Analysis of Plant Hormones

Samples were freeze dried and used for hormone extraction and purification as described previously (Jikumaru et al., 2013) with some modifications. After grinding with 5-mm zirconia beads, 1 mL of acetonitrile:acetic acid:water (80:1:19) solution containing D₅-12-oxo-phytodienoic acid (OPDA; 2 ng; Olchemim), D₂-JA (200 pg; Kanto Chemicals), [¹³C₆]JA-Ile (20 pg; Jikumaru et al., 2004), D₂-indole-3-acetic acid (IAA; 200 pg; Olchemim), D₆-abscisic acid (ABA; 200 pg; Olchemim), and D₆-SA (2 ng; Olchemim) was added to approximately 2 mg dry weight of rice root samples. Supernatants were collected after centrifugation, and the residue was extracted again with 1 mL of acetonitrile:acetic acid:water (80:1:19) solution. The supernatant was collected after centrifugation and combined with supernatant collected previously. Acetonitrile was removed from the combined supernatant with a SpeedVac (Thermo Scientific), and acidic water extract was loaded onto an Oasis HLB column cartridge (30 mg, 1 mL; Waters). After washing with 1 mL of water:

acetic acid (99:1) solution, the target compounds (JA, JA-Ile, OPDA, IAA, ABA, and SA) were extracted 2 times with 1 mL of acetonitrile:acetic acid:water (80:1:19) solution. Acetonitrile in the eluted extract was removed by evaporation using a SpeedVac system (Thermo Scientific), and acidic water extract was loaded onto an Oasis MCX column cartridge (30 mg, 1 mL; Waters). After washing with 1 mL of water:acetic acid (99:1) solution, the target compounds (JA, JA-Ile, OPDA, IAA, ABA, and SA) were extracted 2 times with 1 mL of acetonitrile:acetic acid:water (80:1:19) solution. Eluted extract (200 μL) was collected, vacuum dried, dissolved with 30 μL of water:acetic acid (99:1) solution, and subjected to SA measurement. Acetonitrile in the remaining extract (approximately 1.8 mL) was removed by evaporation in a SpeedVac system, and acidic water extract was loaded onto an Oasis WAX column cartridge (30 mg, 1 mL; Waters). After washing with 1 mL of water:acetic acid (99:1) solution and 1 mL of acetonitrile:water (80:20) solution, the target compounds (JA, JA-Ile, OPDA, IAA, and ABA) were extracted 2 times with 1 mL of acetonitrile:acetic acid:water (80:1:19) solution. The eluted extract was vacuum dried using a SpeedVac system, dissolved with 1 mL of chloroform:ethyl acetate:acetic acid (49.5:49.5:1) solution, and loaded onto a Sep-Pac Vac Silica Cartridge (100 mg, 1 mL; Waters). After the flow-through fraction was collected, the target compounds (JA, JA-Ile, OPDA, IAA, and ABA) were extracted 2 times with 1 mL of chloroform:ethyl acetate:acetic acid (49.5:49.5:1) solution. The collected flow-through and eluted fractions were combined together and dried using a SpeedVac system, and the remaining extract was dissolved with 30 μL of water:acetic acid (99:1) solution and subjected to hormone measurement. The purified fractions of SA and the other hormones were subjected to liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS), comprising a Triple Quad LC/MS 6410 (Agilent) with a liquid chromatographer 1200 (Agilent) equipped with a ZORBAX Eclipse XDB-C18 (2.1 mm × 50 mm, 1.8 μm; Agilent) column. Then, the contents of the target hormones were quantified as described previously (Jikumaru et al., 2013). Data contained in Supplemental Figures S5C and S8 were obtained by subjecting JA and SA to LC-ESI-MS/MS, comprising an AB SCIEX Triple TOF 5600 with a chromatographer, Nexera (Shimadzu), equipped with a ZORBAX Eclipse XDB-C18 (2.1 mm × 50 mm, 1.8 μm; Agilent) column. Conditions for liquid chromatography and parameters of tandem mass spectrometry are shown in Supplemental Table S2.

Data Analysis

Statistical analysis (Mann-Whitney test) was done using GraphPad Prism version 6.0 (GraphPad Software; www.graphpad.com).

Sequence data from this article can be found in the GenBank/EMBL/DNA Data Bank of Japan data libraries under accession number DRA003608 (BioProject ID: PRJDB3897).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. The ethylene pathway is not induced upon *S. hermonthica* infection.

Supplemental Figure S2. Effects of *S. hermonthica* infection on rice root weight.

Supplemental Figure S3. BTH application on *hebiba* induces some resistance.

Supplemental Figure S4. *OsPR1a* is not expressed in *NahG* rice.

Supplemental Figure S5. *NahG* rice response to BTH application is similar to that of the wild type.

Supplemental Figure S6. *OsPR1b* is expressed in *WRKY45*-kd plants.

Supplemental Figure S7. *S. hermonthica* seedlings are bigger in *WRKY45*-kd plants.

Supplemental Figure S8. BTH application induces JA accumulation.

Supplemental Figure S9. cv *Nipponbare* is more resistant to *S. hermonthica* infection than cv *Koshihikari*.

Supplemental Figure S10. Invasion of *S. hermonthica* is blocked at the endodermis.

Supplemental Table S1. Description of target genes used in this study and the primer pairs used for qRT-PCR.

Supplemental Table S2. Conditions for LC-ESI-MS/MS and parameters for the Nexera (Shimadzu) Triple TOF 5600 (AB SCIEX) system.

Supplemental Data Set S1. Transcript levels in *S. hermonthica*-infected rice roots.

Supplemental Data Set S2. Transcripts of genes up-regulated in *S. hermonthica*-infected rice roots.

Supplemental Data Set S3. Transcripts of genes down-regulated in *S. hermonthica*-infected rice roots.

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