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Molecular Dissection of Early Defense Signaling Underlying Volatile-Mediated Defense Regulation and Herbivore Resistance in Rice[OPEN]

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Herbivore-induced plant volatiles prime plant defenses and resistance, but how they are integrated into early defense signaling and whether a causal relationship exists between volatile defense priming and herbivore resistance is unclear. Here, we investigated the impact of indole, a common herbivore-induced plant volatile and modulator of many physiological processes in plants, bacteria, and animals, on early defense signaling and herbivore resistance in rice (*Oryza sativa*). Rice plants infested by fall armyworm (*Spodoptera frugiperda*) caterpillars release indole at a rate of up to 25 ng*h^{-1}. Exposure to equal doses of exogenous indole enhances rice resistance to *S. frugiperda*. Screening of early signaling components revealed that indole pre-exposure directly enhances the expression of the leucine-rich repeat-receptor-like kinase *OsLRR-RLK1*. Pre-exposure to indole followed by simulated herbivory increases (i.e. primes) the transcription, accumulation, and activation of the mitogen-activated protein kinase OsMPK3 and the expression of the downstream WRKY transcription factor gene *OsWRKY70* as well as several jasmonate biosynthesis genes, resulting in higher jasmonic acid (JA) accumulation. Analysis of transgenic plants defective in early signaling showed that *OsMPK3* is required and that *OsMPK6* and *OsWRKY70* contribute to indole-mediated defense priming of JA-dependent herbivore resistance. Therefore, herbivore-induced plant volatiles increase plant resistance to herbivores by positively regulating early defense signaling components.

INTRODUCTION

Plants that are under attack by insect herbivores emit specific blends of herbivore-induced plant volatiles (HIPVs). HIPVs can prompt intact plant tissues to respond more quickly and/or strongly to subsequent herbivore attack, a phenomenon referred to as “defense priming” (Ton et al., 2007; Kim and Felton, 2013; Balmer et al., 2015; Erb et al., 2015; Mauch-Mani et al., 2017). HIPVs may thus act as within-plant defense signals that overcome vascular constraints (Frost et al., 2007; Heil and Silva Bueno, 2007).

Defense priming by HIPVs often includes the regulation of jasmonate defense hormones. Maize (*Zea mays*) HIPVs, such as indole, prime jasmonic acid (JA) accumulation, and the transcription of jasmonate-responsive genes (Ton et al., 2007; Erb et al., 2015). Similarly, green leaf volatiles (GLVs), such as (*Z*)-3-hexenyl acetate, prime JA production in maize (Engelberth et al., 2004) and hybrid poplar (*Populus deltoides* × *nigra*; Frost et al., 2008). Indole and (*Z*)-3-hexenyl acetate can furthermore interact to increase JA signaling (Hu et al., 2019). As jasmonates are important regulators of plant defense and herbivore resistance (Howe and Jander, 2008), and several HIPVs prime jasmonate accumulation, it is generally assumed that HIPVs increase plant resistance by priming the jasmonate pathway (Engelberth et al., 2004; Ameye et al., 2015). However, this connection has not been tested directly. Recent work shows that some HIPVs can also increase plant resistance directly by being absorbed and transformed into toxins (Sugimoto et al., 2014). Thus, the relative importance of HIPV-mediated defense priming for herbivore resistance remains unclear.

HIPVs may regulate JA signaling by modulating early defense signaling components (Shulaev et al., 1997; Engelberth et al., 2013; Erb et al., 2015). In maize, (*Z*)-3-hexenol increases the expression of the transcription factor gene *ZmWRKY12* and the mitogen-activated protein kinase gene *ZmMAPK6,* which are likely involved in transcriptional defense regulation. (*Z*)-3-hexenol also activates putative JA biosynthesis genes such as the allene oxide synthase *ZmAOS* and the lipoxygenase *ZmLOX5* (Engelberth et al., 2013). In Arabidopsis (*Arabidopsis thaliana*), (*E*)-2-hexenal induces the expression of *AtWRKY40* and *AtWRKY6* (Mirabella et al., 2015). *AtWRKY40* and *AtWRKY6* regulate γ-amino butyric acid metabolism, which mediates GLV-induced root growth suppression in a JA-independent manner (Mirabella et al., 2008). Despite these promising results, how HIPVs are integrated into early defense signaling to regulate JA-dependent defenses remains unclear.

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We recently identified indole as an herbivore-induced volatile within-plant signal that primes JA and is required for the systemic priming of monoterpenes in maize (Erb et al., 2015). Indole also primes volatiles in cotton (*Gossypium hirsutum*), suggesting that it is active across different plant species (Erb et al., 2015). Indole exposure also directly increases the mortality of early instar cotton bollworm (*Spodoptera littoralis*) caterpillars by ~10%, despite increasing their weight gain (Veyrat et al., 2016) and renders caterpillars more resistant and less attractive to parasitoids (Ye et al., 2018). In Arabidopsis, high doses of indole in the growth medium modulate root growth by interacting with the auxin-signaling machinery (Bailly et al., 2014). Indole can also act as an intracellular signaling molecule in bacteria (Kim and Park, 2015) and suppress regeneration of the planarian worm *Dugesia japonica* (Lee et al., 2018), suggesting that it is a modulator of a wide variety of physiological processes in different organisms.

In this study, to understand if and how indole is integrated into early defense signaling in plants, we studied its role in rice (*Oryza sativa*). Rice is a useful model, as several key players in early defense signaling have been identified in rice, including receptor-like kinases (Ye, 2016; Hu et al., 2018), mitogen-activated protein kinases (MPKs; Wang et al., 2013; Li et al., 2015; Liu et al., 2018), WRKY transcription factors (Wang et al., 2007; Hu et al., 2015, 2016; Li et al., 2015; Huangfu et al., 2016), and jasmonate biosynthesis genes (Zhou et al., 2009; Guo et al., 2014; Hu et al., 2015). By taking advantage of the available knowledge and molecular resources in rice, we investigated how indole is integrated into early defense signaling, and to what extent this integration translates into enhanced herbivore resistance.

RESULTS

Caterpillar-Induced Indole Increases Herbivore Resistance

To determine whether caterpillar attack induces the release of indole in rice, we infested rice plants with fall armyworm (*Spodoptera frugiperda*) caterpillars and measured indole

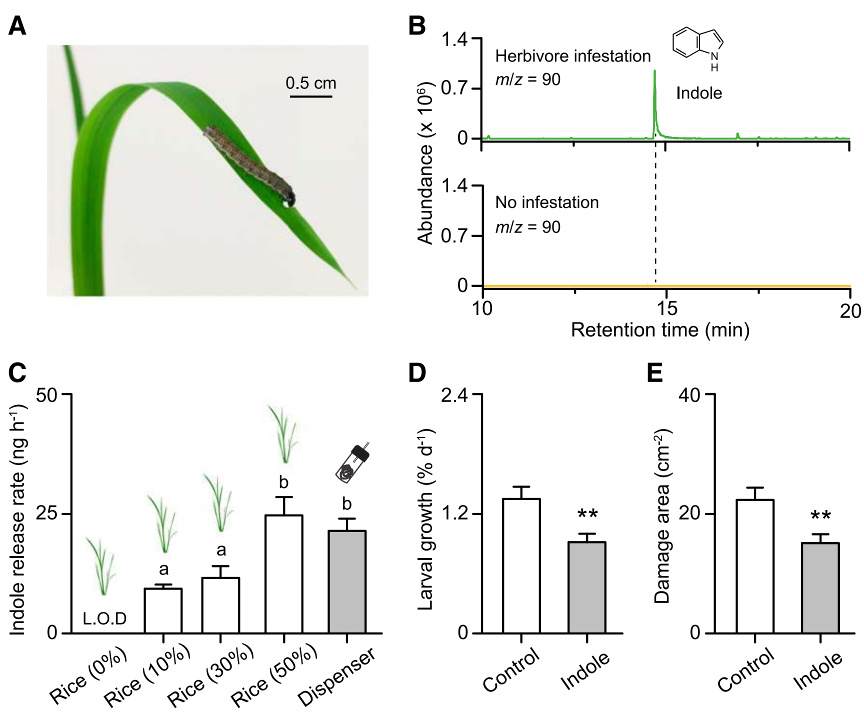

Figure 1. Indole is an HIPV that Increases Rice Resistance to *Spodoptera frugiperda* Larvae at Physiological Doses.

(A) An *S. frugiperda* caterpillar feeding on a rice leaf.

(B) Extracted ion chromatograms of GC/MS headspace analyses of control and *S. frugiperda* infested rice leaves. $m/z = 90$ corresponds to a characteristic fragment of indole.

(C) Emission rates of indole from rice plants that are attacked by different densities of *S. frugiperda* caterpillars. The percentage of consumed leaf area relative to total leaf area is indicated on the *x* axis (±SE, $n = 6$ to 8 [individual plants]). The release of synthetic indole by custom-made capillary dispensers is shown for comparison. Letters indicate significant differences between treatments ($P < 0.05$, one-way ANOVA followed by multiple comparisons through FDR-corrected LSMeans). L.O.D., below limit of detection.

(D) Average growth rate of *S. frugiperda* caterpillars feeding on rice plants that were pre-exposed to indole dispensers releasing indole at ~21 ng h^{-1} or control dispensers for 12 h before infestation (±SE, $n = 15$ [individual larvae]).

(E) Average consumed leaf area (±SE, $n = 15$ [individual plants]). Asterisks indicate significant differences between the volatile exposure treatments (Student's *t*-tests, $^{**}P < 0.01$).

release rates 12 to 20 h after the beginning of the attack. Indole emissions increased with the severity of *S. frugiperda* attack and ranged from 9 to 25 ng h^{-1} per plant (Figures 1A to 1C). Based on these results, we calibrated capillary dispensers to release indole at a physiologically relevant rate of 21 ng h^{-1} (Figure 1C) and exposed rice plants to individual dispensers for 12 h. We then removed the dispensers, added *S. frugiperda* larvae to control and indole pre-exposed plants, and measured larval weight gain and plant damage. Indole pre-exposure significantly reduced larval damage and weight gain (Figures 1D and 1E). Thus, physiologically relevant concentrations of indole are sufficient to increase rice resistance against a chewing herbivore.

Indole Pre-Exposure Increases the Expression of Early Defense Signaling Genes

To explore the capacity of indole to regulate early defense signaling, we profiled the expression of known early defense signaling genes (Figure 2), including two receptor-like kinase (Ye, 2016; Hu et al., 2018), two MPK (Wang et al., 2013; Li et al., 2015), seven WRKY transcription-factor (Qiu et al., 2008; Koo et al., 2009; Li, 2012; Han et al., 2013; Hu et al., 2015; Li et al., 2015; Huangfu et al., 2016), and five jasmonate biosynthesis genes (Zhou et al., 2009; Fukumoto et al., 2013; Guo et al., 2014; Hu et al., 2015). Control plants and the plants that were pre-exposed to indole for 12 h were measured 0, 90, and 360 min after simulated herbivore attack to capture the impact of indole pre-exposure alone as well as the impact of indole pre-exposure in combination with simulated herbivory. Higher defense gene expression in indole pre-exposed plants that was not present at 0 min, but became visible upon simulated herbivore attack, was interpreted as evidence for defense priming. Herbivory was simulated by wounding the leaves and adding *S. frugiperda* oral secretions (OS) as described in Erb et al. (2009), Fukumoto et al. (2013), and Chuang et al. (2014). The expression of the *Oryza sativa* leucine-rich repeat receptor-like kinase 1 (*OsLRR-RLK1*), an early responsive receptor-like kinase that localizes to the plasma membrane and regulates herbivore resistance (Hu et al., 2018), was directly induced by indole exposure and expressed at higher levels 90 min after simulated herbivore attack (Figure 2B). The transcription of *OsMPK3*, encoding an MPK that acts downstream of *OsLRR-RLK1* to regulate herbivore-induced defense and resistance (Wang et al., 2013; Hu et al., 2018), was not directly induced by indole but was primed for higher expression 90 min after simulated herbivore attack (Figure 2C). *OsWRKY70*, encoding a positive regulator of herbivore-induced defense that acts downstream of *OsMPK3* (Li et al., 2015), was primed in a similar manner (Figure 2D). Three jasmonate biosynthesis genes, *OsAOS1*, *OsAOC*, and *OsOPR3*, were equally primed by indole 90 min after elicitation (Figure 2E). By contrast, *OsHI-RLK2*, *OsMPK6*, *OsWRKY13*, *OsWRKY24*, *OsWRKY30*, *OsWRKY33*, *OsWRKY45*, *OsWRKY53*, and the JA biosynthesis gene *OsHI-LOX* did not respond to indole pretreatment. The induction of the JA-Ile biosynthesis gene *OsJAR1* decreased upon indole pre-exposure (Figure 2, B to E). Thus, indole increases the expression of a specific subset of early defense signaling genes that function upstream of JA biosynthesis.

Indole Pre-Exposure Increases OsMPK3 Accumulation and Activation upon Simulated Herbivory

To determine whether the transcriptional response of MPKs to indole pre-exposure and elicitation by simulated herbivory is also reflected in protein abundance, we performed protein gel blot analysis using OsMPK3- and OsMPK6-specific antibodies. Pre-exposure to indole resulted in higher OsMPK3 abundance 90 min after elicitation (Figure 3A). OsMPK6 accumulation was not altered by indole pre-exposure (Figure 3B). To further investigate whether indole pre-exposure increases OsMPK3 activation, we measured OsMPK3 phosphorylation by immunoblot analysis using an anti-phosphoERK1/2 (anti-pTEpY) antibody that interacts with doubly phosphorylated (activated) MPK3 and MPK6 (Seguí-Simarro et al., 2005; Anderson et al., 2011; Schwessinger et al., 2015). Indole pre-exposure increased OsMPK3 activation 90 min after elicitation (Figure 3C). OsMPK6 may also exhibit a slightly higher activation upon indole pretreatment, but the gel blot analysis remains difficult to interpret in this regard (Figure 3C). Thus, indole pre-exposure increases the elicited accumulation and activation of MPKs involved in defense regulation such as OsMPK3. As this effect only occurs upon elicitation by simulated herbivory, indole pre-exposure primes rather than directly induces OsMPK3 accumulation and activation.

Indole Pre-Exposure Induces OPDA and Increases JA Accumulation in Plants Elicited By Simulated Herbivory

To investigate whether the activation of early defense signaling components is associated with higher accumulation of stress-related phytohormones, we quantified 12-oxophytodienoic acid (OPDA), JA and JA-isoleucine (JA-Ile), abscisic acid (ABA), and salicylic acid (SA) in indole-exposed and control plants (Figure 4).

Indole pre-exposure increased the accumulation of OPDA before and after elicitation (Figure 4A). JA concentrations increased in indole-exposed plants 90 and 360 min after elicitation (Figure 4B). The levels of JA-Ile, ABA, and SA were not affected by indole pre-exposure (Figures 4C to 4E). To determine the total dose of indole that is required for the increase in phytohormone concentrations, we exposed rice plants to indole dispensers for 1 to 12 h and measured hormone accumulation 90 min after elicitation by simulated herbivory (Figures 4F to 4I). Exposure to indole dispensers for 1 h (resulting in a total release of 21 ng from the dispensers) was sufficient to increase OPDA and JA levels. Longer exposure did not significantly increase OPDA and JA accumulation (Figures 4F to 4I). Thus, exposure of rice plants to 21 ng of indole over 1 h is sufficient to increase the production of oxylipin defense regulators upon elicitation.

OsMPK3 Is Required for Indole-Dependent Jasmonate Accumulation and Herbivore Resistance

To understand whether the early signaling components that are responsive to indole are required for downstream responses, we measured JA accumulation upon elicitation by simulated herbivory as well as herbivore resistance in control- and indole

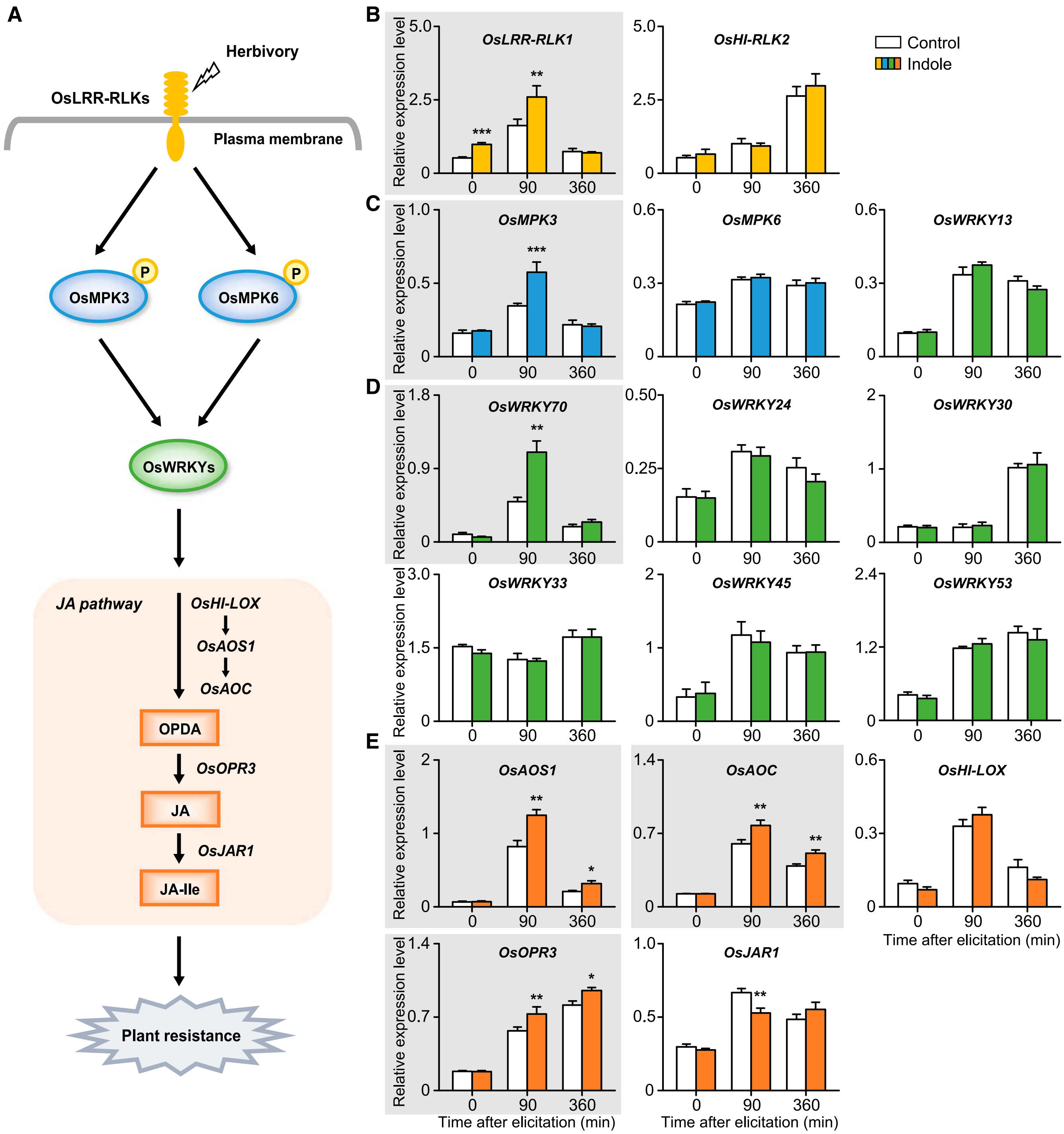

Figure 2. Indole Pre-Exposure Increases the Expression of Early Defense Signaling Genes.

(A) Current model of herbivore-induced defense signaling in rice, including LRR-RLKs, MPKs, WRKY transcription factors, jasmonate biosynthesis genes, and oxylipins. P, phosphorylation.

(B) to **(E)** Effect of indole pre-exposure on the expression of genes coding for the different early signaling steps at different time points after elicitation by wounding and application of *S. frugiperda* OS (±SE, n = 4 to 6 [individual plants]). Asterisks indicate significant differences between volatile exposure treatments at different time points (two-way ANOVA followed by pairwise comparisons through FDR-corrected LSMeans; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Genes responding positively to indole are highlighted in gray.

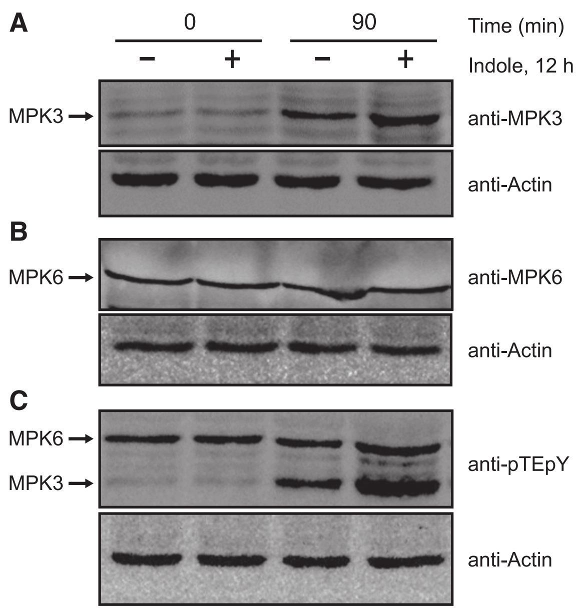

Figure 3. Indole Pre-Exposure Increases OsMPK3 Accumulation and Activation upon Simulated Herbivory.

(A) to **(C)** Protein accumulation and activation of OsMPK3 and OsMPK6 with (+) or without (−) indole pre-exposure before (0 min) and after elicitation by simulated herbivory (90 min). Leaves from six replicate plants were harvested at the indicated times after elicitation. Immunoblotting was performed using **(A)** an anti-MPK3 antibody for OsMPK3, **(B)** an anti-MPK6 antibody for OsMPK6, and **(C)** an anti-pTEpY antibody to detect phosphorylated MPKs, or an actin antibody as a loading control. Actin was measured on a replicate blot. This experiment was repeated two times with comparable results.

pre-exposed wild-type and transgenic plants, including the *OsLRR-RLK1*-silenced line ir-*lrr1* (Hu et al., 2018), the *OsMPK3*- and *OsMPK6*-silenced lines ir-*mpk3* and ir-*mpk6* (Wang et al., 2013; Li et al., 2015), and the *OsWRKY70*-silenced line ir-*wrky70* (Li et al., 2015). *OsLRR-RLK1* silencing did not affect indole-dependent herbivore growth suppression, OPDA induction, or JA accumulation (Figures 5, A, 5F, and 5K). By contrast, silencing *OsMPK3* completely suppressed herbivore growth reduction, indole-dependent OPDA induction, and JA accumulation (Figures 5B, 5G, and 5L). The induction of JA by herbivore elicitation was still clearly visible in *ir-mpk3* plants, demonstrating that the absence of indole-dependent resistance is not due to a complete suppression of JA signaling. Silencing *OsMPK6* reduced herbivore growth suppression and indole-dependent JA accumulation by ~50% and led to an almost complete disappearance of OPDA induction (Figures 5C, 5H, and 5M). Silencing *OsWRKY70* also reduced herbivore growth suppression, indole-dependent OPDA induction, and JA accumulation by ~50% (Figures 5D, 5I, and 5N). To exclude the potential allelic effects, we profiled the responses of two independent *OsMPK3*- and *OsMPK6*-silenced lines in a separate experiment. Consistent with our earlier results, OPDA induction was completely suppressed in *OsMPK3*- and *OsMPK6*-silenced lines. Furthermore, JA accumulation after simulated herbivory was compromised in both *OsMPK6*-silenced lines and completely disappeared in the two *OsMPK3*-silenced lines (Supplemental Figure 1). Thus, *OsMPK3* is required for, and *OsMPK6* and *OsWRKY70* contribute to, defense regulation by the volatile indole.

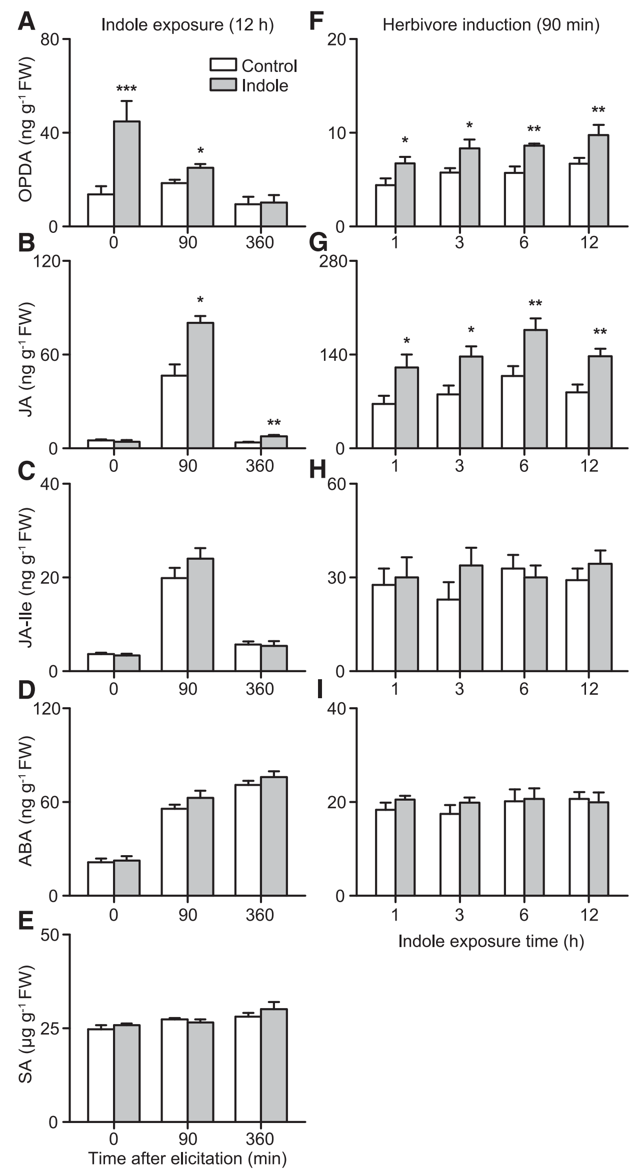

Figure 4. Indole Pre-Exposure Induces OPDA and Increases JA Accumulation in Plants Elicited by Simulated Herbivory.

(A) to **(E)** Average concentrations of **(A)** OPDA, **(B)** JA, **(C)** JA-Ile, **(D)** ABA, and **(E)** SA in indole pre-exposed and control plants at different time points after elicitation (+SE, $n = 5$ to 6 [individual plants]). Rice plants were pre-exposed to indole for 12 h before elicitation.

(F) to **(I)** Average concentrations of OPDA, JA, JA-Ile, and ABA in rice plants that were exposed to indole for 1 h, 3 h, 6 h, or 12 h, or control dispensers 90 min after elicitation (±SE, $n = 5$ to 6 [individual plants]). SA levels were not measured in this experiment. Asterisks indicate significant differences between treatments (two-way ANOVA followed by pairwise comparisons through FDR-corrected LSMeans; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

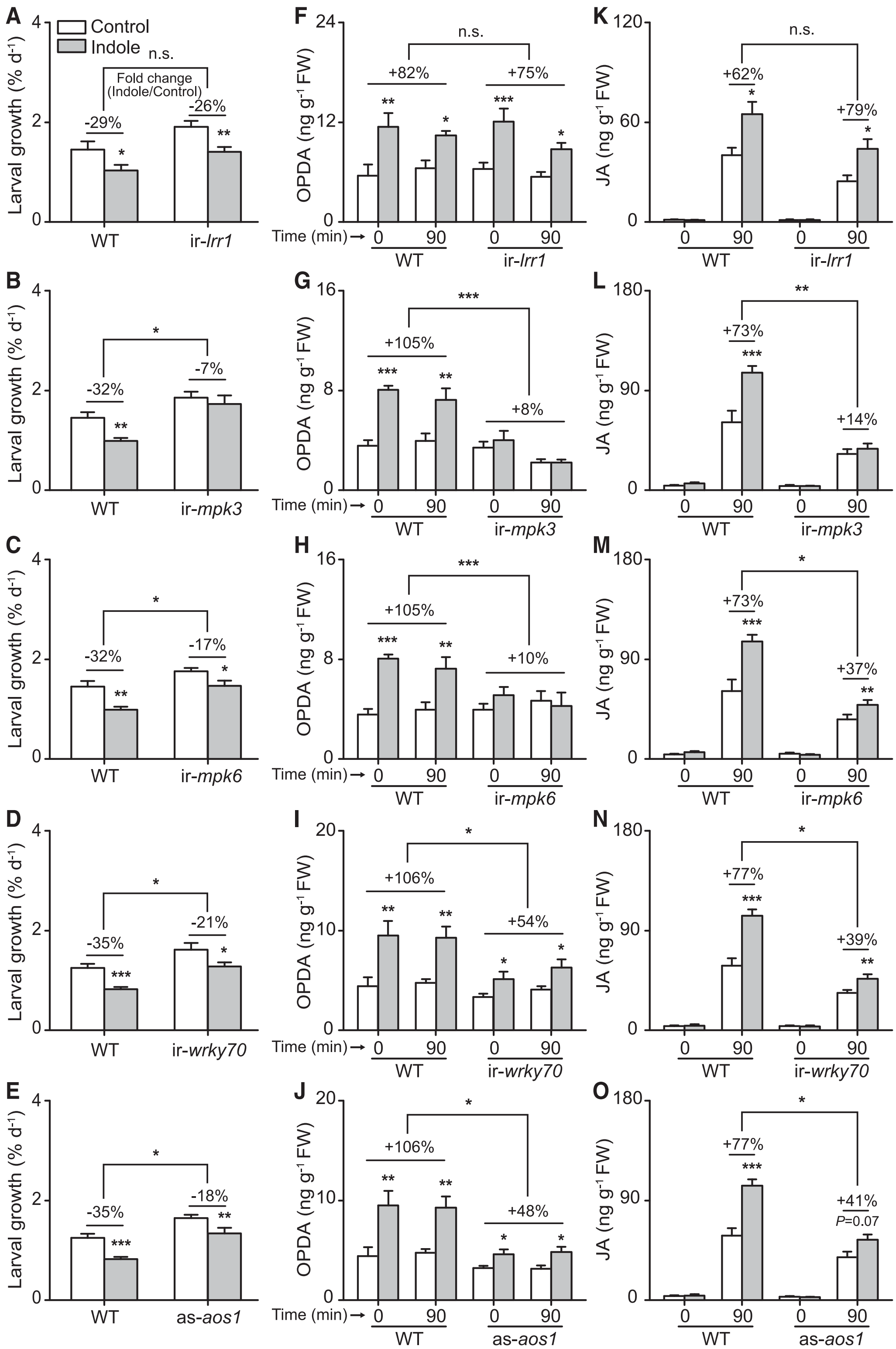

Figure 5. OsMPK3 Is Required for Indole-Dependent Jasmonate Accumulation and Herbivore Resistance.

(A) to **(E)** Average growth rate of *S. frugiperda* caterpillars feeding on **(A)** ir-*lrr1*, **(B)** ir-*mpk3*, **(C)** ir-*mpk6*, **(D)** ir-*wrky70*, and **(E)** as-*aos1* lines and wild-type (WT) plants that were pre-exposed to indole or control (±SE, n = 15 [individual larvae]). WT, wild type; n.s., not significant.

The Jasmonate Signaling Pathway Contributes to Indole-Induced Herbivore Resistance

To study the connection between the regulation of jasmonates and the decrease in herbivore performance in indole-exposed plants, we tested *as-aos1* plants, which accumulate lower levels of jasmonates upon herbivore elicitation compared with wild type (Hu et al., 2015). OPDA induction, JA accumulation, and herbivore growth suppression were reduced by ~50% in as-*aos1* plants versus the wild type (Figures 5E, 5J, and 5O). Across the different genotypes, herbivore growth suppression was strongly correlated with OPDA and JA over-accumulation: Genotypes that responded to indole with stronger OPDA induction and JA accumulation upon elicitation also reduced larval growth more strongly after pre-exposure (Figures 6A and 6B). Again, JA-Ile did not respond significantly to indole pretreatment (Supplemental Figure 2), and there was no correlation between the effects of indole on JA-Ile and herbivore growth suppression (Figure 6C). Together, these findings implicate the jasmonate-signaling pathway in indole-induced herbivore resistance.

DISCUSSION

HIPVs regulate plant defense responses and increase herbivore resistance in many different plant species. However, how volatiles influence early defense signaling, and whether the resulting increase in defense responsiveness increases herbivore resistance, are not well understood. This study helps fill these gaps in our knowledge by identifying early defense regulators that are involved in volatile defense regulation and plant resistance to herbivory.

HIPVs such as GLVs have been shown to regulate early defense genes that likely act upstream of stress hormone signaling (Ton et al., 2007; Frost et al., 2008; Erb et al., 2015; Hu et al., 2019). Here, we demonstrate that indole pre-exposure at physiological doses also results in marked changes in the expression of early defense signaling genes. The receptor-like kinase gene *OsLRR-RLK1* was directly induced by exposure to indole, while the MPK OsMPK3 and the WRKY transcription factor gene *OsWRKY70* were primed for stronger activation and expression. Experiments with transgenic plants revealed that *OsMPK3* gene expression is required, and *OsWRKY70* contributes, to indole-induced downstream responses. As *OsWRKY70* is regulated by and acts downstream of *OsMPK3* (Li et al., 2015), we infer that indole acts upstream of *OsMPK3*. The finding that the indole-induced priming was not altered in an *OsLRR-RLK1*-silenced line further suggests that the expression of this receptor-like kinase, which can regulate *OsMPK3*, is not directly required for indole-induced priming. Experiments with additional, independently silenced lines, and, ideally, an *OsLRR-RLK1* null mutant, would be required to completely rule out the involvement of this gene in indole-dependent responses. In maize, GLV exposure has been shown to directly increase the expression of *ZmMAPK6* and *ZmWRKY12* (Engelberth et al., 2013). By contrast, we found that *OsMPK3* and *OsWRKY70* were not directly induced by indole, but responded more strongly to elicitation by simulated herbivory. This finding is in line with recent comparative work in maize showing that GLVs directly induce defense genes, while indole primes their expression (Hu et al., 2019). Thus, while GLVs and indole both strengthen the jasmonate signaling pathway, their mode of action and integration into early defense signaling are likely different.

Priming mechanisms have been elucidated for various nonvolatile chemicals. In Arabidopsis, β-aminobutyric acid (BABA) acts via the aspartyl-tRNA synthetase IBI1 and induces the expression of a lectin receptor kinase gene *LecRK-VI.2*, which is in turn required for BABA-induced priming (Singh et al., 2012; Luna et al., 2014). Furthermore, thiadiazole-7-carbothioic acid *S*-methyl ester treatment increases mRNA levels and inactive protein levels of MPK3 and MPK6, which are then activated more strongly upon stress and thereby enhance defense responses (Beckers et al., 2009). Our work shows that naturally occurring volatiles such as indole act by modulating similar components of early defense signaling, but in a different manner. For instance, indole exposure primes MPK activity but does not directly induce MPK accumulation (Figure 3). It also induces the transcription of a receptor-like kinase gene, but this does not seem to be required to activate downstream responses. We conclude that indole reprograms early signaling through mechanisms that differ from those used by nonvolatile chemical elicitors such as BABA and thiadiazole-7-carbothioic acid *S*-methyl ester.

Most HIPVs that enhance defenses have also been shown to prime jasmonate biosynthesis. Indole does the same in maize (Erb et al., 2015) and, as shown here, rice. Our experiments with transgenic plants show that the priming of JA requires *OsMPK3* and is enhanced by *OsWRKY70*, both of which are primed by indole exposure (Figure 5). We thus infer that JA priming results from the modulation of *OsMPK3*-dependent early defense signaling by volatile indole. As indole-exposure primes JA biosynthesis genes, the capacity of plants to synthesize JA upon herbivore elicitation is likely increased through the higher

Figure 5. (continued).

(F) to **(J)** Average concentrations of herbivore-induced OPDA in the different transgenic lines and wild-type plants that were pre-exposed to indole or control dispensers (±SE, $n = 6$ [individual plants]). FW, fresh weight; WT, wild type; n.s., not significant.

(K) to **(O)** Average concentrations of herbivore-induced JA in the different transgenic lines and wild-type plants that were pre-exposed to indole or control dispensers (±SE, $n = 6$ [individual plants]). Note that wild-type, ir-*mpk3*, and ir-*mpk6* plants as well as wild-type, ir-*wrky70*, and as-*aos1* plants were measured together within the same experiments. The wild-type data are, therefore, identical in the respective figures (e.g. same wild-type data for ir-*mpk3* and ir-*mpk6* figures; same wild-type data for ir-*wrky70* and as-*aos1* figures). Percentages refer to fold changes of indole-exposed plants relative to control-exposed plants. Asterisks above bars indicate significant differences between volatile exposure treatments within the same plant genotype (two-way ANOVA followed by pairwise comparisons through FDR-corrected LSMeans; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Asterisks above lines represent significant differences between indole-dependent fold changes of wild-type and transgenic lines (Student's *t*-tests, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). FW, fresh weight; WT, wild type; n.s., not significant.

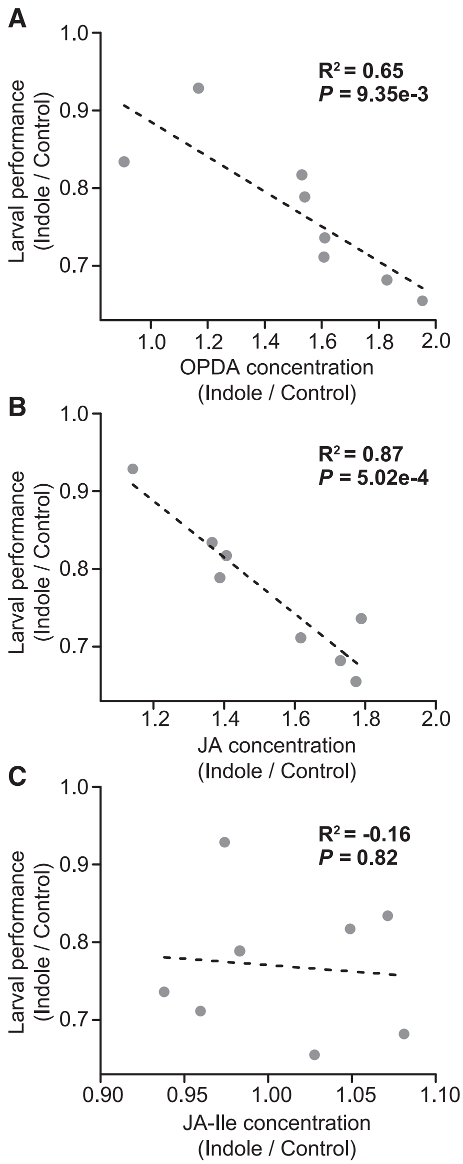

Figure 6. Correlations among Indole-Induced Regulation of OPDA, JA, and Herbivore Resistance.

(A) to **(C)** Correlations among the fold changes of herbivore-induced **(A)**, JA **(B)**, and JA-Ile **(C)** concentrations in indole-exposed plants relative to control-exposed plants and fold changes of *S. frugiperda* larval performance on indole-exposed plants relative to control-exposed plants. Circles denote individual genotypes. R^2 and P values of Pearson product-moment correlations are shown.

abundance of rate-limiting enzymes (Haga et al., 2008; Yara et al., 2008; Riemann et al., 2013). *OsAOC*, for instance, which catalyzes allene oxide to OPDA, is encoded by only a single copy gene, and *OsAOC*-defective rice plants are jasmonate-deficient (Riemann et al., 2013; Lu et al., 2015). Indole exposure also directly induces the accumulation of the JA precursor OPDA. In theory, this bigger pool may increase the formation of JA upon elicitation through the induction of *OsOPR3* after herbivore attack. However, our experiments show that OPDA depletion upon elicitation is not strictly required for JA priming. Thus, there is currently no evidence that direct OPDA induction is the key mechanism behind the priming of JA biosynthesis in indole pre-exposed plants.

Apart from the similarities of hormonal responses of maize and rice to indole, there seem to be a few differences as well. For instance, while both JA and JA-Ile accumulation was higher in indole pre-exposed maize plants after simulated herbivore attack, JA, but not JA-Ile, responded to indole in rice. The absence of JA-Ile overaccumulation was associated with a slight suppression of *OsJAR1* expression in indole pre-exposed plants 90 min after simulated herbivore attack. OsJAR1 conjugates JA to different amino acids, including Ile (Xiao et al., 2014), and the reduced inducibility of the corresponding gene may thus be responsible for the absence of JA-Ile overaccumulation. Our experiments add to the growing body of evidence that jasmonates other than JA-Ile have the capacity to act as regulators of plant physiological processes (Wang et al., 2008; Machado et al., 2017; Monte et al., 2018). Another difference between maize and rice was that indole pre-exposure increased ABA levels in maize (Erb et al., 2015), whereas it did not change ABA accumulation in rice in this experiments. ABA is regulated by a wide variety of biotic and abiotic parameters (Nambara and Marion-Poll, 2005). Whether the absence of ABA overaccumulation reflects differences in experimental conditions or is the result of differences in the signaling networks that connect indole responses to hormonal signaling remains to be elucidated.

OsMPK3, OsWRKY70, and JA are part of the same signaling cascade and are positive regulators of rice resistance to chewing herbivores (Zhou et al., 2009; Wang et al., 2013; Li et al., 2015). Indole primes these defense-signaling components, and silencing their expression reduces indole-induced resistance against *S. frugiperda*, which illustrates that indole increases plant resistance by enhancing early defense signaling and JA biosynthesis. Previous work has shown that indole can also directly protect plants by repelling and increasing the mortality of *S. littoralis* caterpillars (Veyrat et al., 2016; Ye et al., 2018). In this experiment, we minimized direct effects of indole on *S. frugiperda* by pre-exposing the plants to synthetic indole and removing the dispensers before putting the caterpillars on the plant. Furthermore, indole exposure enhances rather than suppresses *S. littoralis* growth, despite the increased mortality (Veyrat et al., 2016), and is thus unlikely to be directly responsible for the lower weight gain of *S. frugiperda*. Thus, the suppression of caterpillar growth in indole pre-exposed plants results from the capacity of indole to enhance early defense signaling and JA biosynthesis rather than its direct effects on caterpillar physiology. A recent study documented that pathogen-induced monoterpenes pinenes can trigger systemic acquired resistance, an effect that is dependent on SA biosynthesis (Riedlmeier et al., 2017). Thus, plant volatiles can trigger resistance against both pathogens and herbivores by enhancing plant defenses through phytohormonal signaling pathways.

In summary, we propose the following model. Plant leaves that are attacked by herbivores release the volatile indole. Through as yet unknown perception mechanisms, indole primes OsMPK3 in nonattacked tissues. When these tissues come under attack, OsMPK3 is activated more strongly, which boosts downstream responses, including the transcription of *OsWRKY70* and jasmonate biosynthesis genes, which again results in an over-accumulation of bioactive oxylipins such as OPDA and JA. Enhanced jasmonate signaling then boosts plant defense responses and thereby reduces herbivore growth and damage. This study provides a mechanistic basis for the regulatory potential and mode of action of HIPVs in plant defense priming.

METHODS

Plant and Insect Resources

The rice (*Oryza sativa*) cv Xiushui 110 was used in this study. In addition, the transgenic line ir-*lrr1* and its corresponding wild-type line Xiushui 110 as well as the transgenic lines ir-*mpk3*, ir-*mpk6*, ir-*wrky70*, as-*aos1*, and their corresponding wild-type Xiushui 11 were used. These genotypes have been described and characterized previously, including multiple independently transformed lines to exclude allelic effects (Wang et al., 2013; Hu et al., 2015, 2018; Li et al., 2015). Rice seeds were pregerminated and sown in plastic pots (11 cm in height, 4 cm in diameter) using commercial potting soil (Aussaaterde; Ricoter Erdaufbere-itung). Plants were grown in a greenhouse (26°C ± 2°C, 55% relative humidity, 14-:10-h light/dark, with 250 μmol*m^{-2}*s^{-1} additional light supplied by Master GreenPower 600W 400V E40 High Pressure Sodium bulbs [Philips Lighting Switzerland]). Plants were watered three times per week, and used for experiments 30 d after sowing. Fall armyworm (*Spodoptera frugiperda*) larvae were provided by University of Neuchâtel and reared on an artificial diet as described in Maag et al. (2014). OS were collected from third-instar *S. frugiperda* larvae that had been feeding on rice leaves for 48 h, and diluted 1:1 with sterilized Milli-Q water (Millipore) before use.

Quantification of Herbivore-Induced Indole Emissions

To determine the natural emission rates of indole, we infested rice plants with 3, 5, or 8 third-instar *S. frugiperda* larvae for 12 h, resulting in the consumption of ~10%, 30%, and 50% of total leaf area, respectively. After infestation, volatiles were collected using a dynamic headspace sampling system and Super-Q traps (n = 8, [individual plants]). Briefly, the rice plants were enclosed in cooking bags (polyethylene terephthalate , 35 × 40 cm, maximum 200°C; Migros Supermarket). Purified air from a multiple air-delivery system entered the bags via Teflon tubing at a rate of 0.8 liters min^{-1} and was pulled out through the Super-Q trap (Volatile Collection Trap) at a rate of 0.6 liters min^{-1}. Before collection, the Super-Q traps were rinsed with 3 mL of methylene chloride (>99.8%, gas chromatography [GC]; Sigma-Aldrich). Volatiles were collected for 8 h. After collection, the traps were extracted with 200 μL of methylene chloride containing two internal standards (*n*-octane and nonyl-acetate, each 1 μg in 200 μL of methylene chloride). Then, a 1 μL aliquot of each sample was injected into a GC/MS (mass spectrometry; cat. no. 7820A GC interfaced with a cat. no. 5977E MSD; Agilent) in pulsed split mode onto an apolar column (HP-5MS, 30 m, 0.25 mm ID, 0.25 μm film thickness; Alltech Associates) for analysis. Helium at constant flow (1 mL min^{-1}) was used as the carrier gas. After injection, the column temperature was maintained at 40°C for 1 min, increased to 250°C at 6°C min^{-1} followed by a post-run of 3 min at 250°C. The quadrupole MS was operated in the electron ionization mode at 70 eV, a source temperature of 230°C, quadrupole temperature of 150°C, with a continuous scan from *m/z* 50 to 300. The detector signal was processed with HP GC Chemstation software. Absolute emission rates of indole were determined based on peak areas and calculated using a standard curve of synthetic indole (>98%, GC; Sigma-Aldrich).

Indole Exposure

To expose rice to synthetic indole, we covered plants of different genotypes individually with passively ventilated plastic cylinders (40 cm in height, 4 cm in diameter) made of transparent plastic sheets (Rosco Laboratories). The plants were placed into the greenhouse (26°C ± 2°C, 55% relative humidity, 14-h/10-h light/dark, 50,000 lumens m^{-2}), and indole or control dispensers were added into the cylinders. After 12 h of exposure, the cylinders were carefully removed and the plants were subjected to OS elicitation (see "Plant Elicitation"). Indole and control dispensers were made as described in Erb et al. (2015). Briefly, the dispensers consisted of 2-mL amber glass vials (11.6 × 32 mm^{-2}; Sigma-Aldrich) containing 20 mg of synthetic indole (>98%, GC; Sigma-Aldrich). The vials were closed with open screw caps that contained a polytetrafluoroethylene/rubber septum, which was pierced with a 1-μL micropipette (Drummond; Millan). The vials were sealed with Parafilm and wrapped in aluminum foil for heat-protection and to avoid photodegradation. GC/MS analyses using the approach described above showed that these dispensers release a~21 ng h^{-1} volatile indole, which corresponds to the amounts emitted by a single rice plant under attack by *S. frugiperda* (Figure 1). Control dispensers consisted of empty glass vials. Dispensers were prepared 24 h before the start of the experiments. As we used a passively ventilated cylinder system, indole may accumulate at levels that are higher than expected under natural conditions. To test whether plant defense responses are affected by the potential accumulation of indole over time, we exposed rice plants to dispensers for 1 h, 3 h, 6 h, and 12 h and measured priming of JA as a downstream defense marker (see sections "Plant Elicitation" and "Phytohormone Quantification"). We found that JA priming is independent of the duration of indole exposure (Figure 4). We therefore proceeded in using this system and an exposure time of 12 h for the remaining experiments.

Plant Elicitation

After indole exposure, the cylinders and dispensers were removed. Rice plants were elicited by wounding two leaves over an area (~0.5 cm^{-2}) on both sides of the central vein with a razor blade, followed by the application of 10 μL of *S. frugiperda* OS. This treatment results in plant responses similar to those under real herbivore attack (Erb et al., 2009; Fukumoto et al., 2013; Chuang et al., 2014). Leaves were then harvested at different time intervals and flash-frozen for further analysis.

Herbivore Performance

One starved, preweighed second instar larva was individually introduced into each cylindrical mesh cage (1-cm height and 5-cm diameter). The cages were clipped onto the leaves of rice plants that had been pre-exposed to indole or control. The position of the cages was moved every day to provide sufficient food for the larvae. Larval mass was determined 7 d after the start of the experiment. To quantify damage, the remaining leaf pieces were scanned, and the removed leaf area was quantified using a model no. 4.6.1 (Digimizer; n = 15 [individual larvae]).

Phytohormone Quantification

Rice leaves were harvested at 0, 90 and 360 min after the start of OS elicitation and ground in liquid N (n > 5 [individual plants]). The phytohormones OPDA, JA, JA-Ile, SA, and ABA were extracted with ethyl acetate spiked with isotopically labeled standards (1 ng for d_5-JA, d_6-ABA, d_6-SA, and $^{13}C_6$-JA-Ile) and analyzed by ultra-high performance liquid chromatography tandem mass spectrometry as described in Glauser et al. (2014).

Gene Expression Analysis

Quantitative real time-PCR (qRT-PCR) was used to measure the expression levels of different genes. Rice leaves were harvested at 0, 90, and 360 min after the start of OS elicitation and ground in liquid N (n > 4 [individual plants]). Total RNA was isolated from the leaves using a GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific). One μg of each total RNA sample was reverse transcribed with SuperScript II Reverse Transcriptase (Invitrogen) to synthesize complementary DNA (cDNA). The qRT-PCR assay was performed on the LightCycler 96 Instrument (Roche) using the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems). A linear standard curve was constructed using a serial dilution of cDNA that was pooled from all plants, and generated by plotting the threshold cycle (*Ct*) against the $\log_{10}$ of the dilution factors. The relative transcript levels of the target genes in samples were determined according to the standard curve. A rice actin gene *OsACTIN* was used as an internal standard to normalize cDNA concentrations. The primers used for qRT-PCR for all tested genes are listed in Supplemental Table.

MPK Protein and Activation Detection

Rice leaves were harvested at 0 and 90 min after the start of OS elicitation and ground in liquid N. Total proteins were extracted from pooled leaves of six replicates at each time point as described in Wu et al. (2007). Forty μg of total proteins were separated by SDS-PAGE and transferred onto BioTrace pure nitrocellulose blotting membrane (Bio-Rad). Immunoblotting was performed as described in Hu et al. (2015). The primary antibody anti-MPK3 (cat. No. AbP80147-A-SE, 1:1,000 dilution; Beijing Protein Innovation) or anti-MPK6 (cat. No. AbP80140-A-SE, 1:1000 dilution; Beijing Protein Innovation) was used to detect total OsMPK3 or OsMPK6 protein, respectively. The rabbit monoclonal anti-phospho-ERK1/2 (anti-pTEpY) antibody (cat. No. 4370, 1:2,000 dilution; Cell Signaling Technologies), which is specific for the activated (phosphorylated) form of the p44/42 MPKs (Thr-202/Tyr-204; Seguí-Simarro et al., 2005; Anderson et al., 2011), was used to detect the active OsMPK3 and OsMPK6. The Anti-Plant beta Actin Mouse antibody (cat. No. AT0004, 1:5,000 dilution; CMCTAG) was used for a loading control and was detected on a replicate blot. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated anti-rabbit (cat. No. 31460, 1:10,000 dilution; Thermo Fisher Scientific) or anti-mouse (cat. No. AP308P, 1:5,000 dilution; Sigma-Aldrich) secondary antibody (Thermo Fisher Scientific) followed by chemiluminescence detection with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

Statistical Analyses

Differences in levels of gene expression and phytohormones were analyzed by analysis of variance (ANOVA; Supplemental File) followed by pairwise comparisons of Least Squares Means (LSMeans), which were corrected using the False Discovery Rate (FDR) method (Benjamini and Hochberg, 1995). The data normality was verified by inspecting residuals using the “plotresid” function of the R package “RVAideMemoire” (Herve, 2019). The variance homogeneity was tested through Shapiro-Wilk’s tests using the “shapiro.test” function in R. Data sets that did not fit assumptions were log- or asinh-transformed to meet the requirements of normality and equal variance. Differences in larval growth and leaf damage were determined by two-sided Student’s *t*-tests. The relative priming intensity was calculated based on the fold changes of larval growth, OPDA, or JA levels in the indole-exposed plants relative to control-exposed plants. The differences in fold changes were compared using Student’s *t*-tests. The correlations (fold changes of OPDA, JA, or JA-Ile versus fold changes of larval growth) were tested through Pearson’s product-moment correlation using the “cor. test” function in R (Puth et al., 2014). All the analyses were conducted using R 3.2.2 (R Foundation for Statistical Computing). The numbers of replicates for each experiment are given in the figures and denote independent biological replicates (i.e. individual plants, individual larvae).

Accession Numbers

Sequence data from this article can be found in the Rice Annotation Project under accession numbers *OsLRR-RLK1* (Os06g47650), *OsHI-RLK2* (GenBank accession number XM_015757324), *OsMPK3* (Os03g17700), *OsMPK6* (Os06g06090), *OsWRKY70* (Os05g39720), *OsWRKY53* (Os05g27730), *OsWRKY45* (Os05g25770), *OsWRKY33* (Os03g33012), *OsWRKY30* (Os08g38990), *OsWRKY24* (Os01g61080), *OsWRKY13* (Os01g54600), *OsHI-LOX* (Os08g39840), *OsAOS1* (Os03g55800), *OsAOC* (Os03g32314), *OsOPR3* (Os08g35740), *OsJAR1* (Os05g50890), and *OsACTIN* (Os03g50885).

Supplemental Data

Supplemental Figure 1. Effect of indole pre-exposure on jasmonate accumulation in two independent *OsMPK3*- and *OsMPK6*-silenced lines.

Supplemental Figure 2. Herbivore-induced JA-Ile levels in MPK, WRKY, and JA-impaired plants after indole exposure.

Supplemental Table. Primers used for qRT-PCR of target genes.

Supplemental File. ANOVA data.

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AUTHOR CONTRIBUTIONS

M.E., Y.L., and L.H. conceived the project; M.E. and Y.L. acquired project funding; L.H., M.Y., Y.L., and M.E. designed research; L.H., M.Y., and G.G. performed experiments; L.H., M.Y., Y.L., and M.E. analyzed and interpreted data; L.H., M.Y., and M.E. prepared and wrote the first draft; all authors read and approved the article.

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