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RESEARCH ARTICLE

Molecular Dissection of Early Defense Signaling Underlying Volatile-mediated Defense Regulation and Herbivore Resistance in Rice

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Short title: Early signaling in volatile priming

One-sentence summary: The herbivore-induced plant volatile indole increases jasmonate-dependent plant resistance to herbivores by priming early defense signaling components, including a mitogen-activated protein kinase.

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ABSTRACT

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 are 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 receptor-like kinase *OsLRR-RLK1*. Pre-exposure to indole followed by simulated herbivory increased (i.e. primed) the transcription, accumulation, and activation of the mitogen-activated protein kinase OsMPK3 and the expression of the downstream WRKY transcription

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factor gene *OsWRKY70* and 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., 2018a). 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,

26 (*Z*)-3-hexenol increases the expression of the transcription factor gene *ZmWRKY12*
27 and the mitogen-activated protein kinase gene *ZmMAPK6,* which are likely involved
28 in transcriptional defense regulation. The same volatile also activates putative JA
29 biosynthesis genes such as *ZmAOS* and *ZmLOX5* (Engelberth et al., 2013). In
30 *Arabidopsis thaliana*, (*E*)-2-hexenal induces the expression of *AtWRKY40* and
31 *AtWRKY6* (Mirabella et al., 2015). *AtWRKY40* and *AtWRKY6* regulate γ-amino
32 butyric acid (GABA) metabolism, which mediates GLV-induced root growth
33 suppression in a JA-independent manner (Mirabella et al., 2008). Despite these
34 promising results, how HIPVs are integrated into early defense signaling to regulate
35 JA-dependent defenses remains unclear.

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

49 In the current study, to understand if and how indole is integrated into early
50 defense signaling in plants, we studied its role in rice (*Oryza sativa*). Rice is a useful
51 model, as several key players in early defense signaling have been identified in rice,
52 including receptor-like kinases (Ye, 2016; Hu et al., 2018b), MPKs (Wang et al., 2013;
53 Li et al., 2015; Liu et al., 2018), WRKY transcription factors (Wang et al., 2007; Hu
54 et al., 2015; Li et al., 2015; Hu et al., 2016; Huangfu et al., 2016) and jasmonate
55 biosynthesis genes (Zhou et al., 2009; Guo et al., 2014; Hu et al., 2015). By taking

56 advantage of the available knowledge and molecular resources in rice, we investigated
57 how indole is integrated into early defense signaling, and to what extent this
58 integration translates into enhanced herbivore resistance.
59

60 RESULTS

61 Caterpillar-induced indole increases herbivore resistance

62 To determine whether caterpillar attack induces the release of indole in rice, we
63 infested rice plants with fall armyworm (*Spodoptera frugiperda*) caterpillars and
64 measured indole release rates 12 - 20 h after the beginning of the attack. Indole
65 emissions increased with the severity of *S. frugiperda* attack and ranged from 9 to 25
66 ng h^{-1} per plant (Figure 1A-C). Based on these results, we calibrated capillary
67 dispensers to release indole at a physiologically relevant rate of 21 ng h^{-1} (Figure 1C)
68 and exposed rice plants to individual dispensers for 12 h. We then removed the
69 dispensers, added *S. frugiperda* larvae to control and indole pre-exposed plants and
70 measured larval weight gain and plant damage. Indole pre-exposure significantly
71 reduced larval damage and weight gain (Figure 1D, E). Thus, physiologically relevant
72 concentrations of indole are sufficient to increase rice resistance against a chewing
73 herbivore.
74

75 Indole pre-exposure increases the expression of early defense signaling genes

76 To explore the capacity of indole to regulate early defense signaling, we profiled the
77 expression of known early defense signaling genes (Figure 2), including two
78 receptor-like kinase (Ye, 2016; Hu et al., 2018b), two MPK (Wang et al., 2013; Li et
79 al., 2015), seven WRKY transcription factor (Qiu et al., 2008; Koo et al., 2009; Li,
80 2012; Han et al., 2013; Hu et al., 2015; Li et al., 2015; Huangfu et al., 2016) and five
81 jasmonate biosynthesis genes (Zhou et al., 2009; Fukumoto et al., 2013; Guo et al.,
82 2014; Hu et al., 2015). Control plants and the plants that were pre-exposed to indole
83 for 12 h were measured 0 min, 90 min and 360 min after simulated herbivore attack to
84 capture the impact of indole pre-exposure alone as well as the impact of indole
85 pre-exposure in combination with simulated herbivory. Higher defense gene

86 expression in indole pre-exposed plants that was not present at 0 min, but became
87 visible upon simulated herbivore attack, was interpreted as evidence for defense
88 priming. Herbivory was simulated by wounding the leaves and adding *S. frugiperda*
89 oral secretions (OS) as described (Erb et al., 2009; Fukumoto et al., 2013; Chuang et
90 al., 2014). The expression of *OsLRR-RLK1*, an early responsive receptor-like kinase
91 that localizes to the plasma membrane and regulates herbivore resistance (Hu et al.,
92 2018b), was directly induced by indole exposure and expressed at higher levels 90
93 min after simulated herbivore attack (Figure 2B). The transcription of *OsMPK3*,
94 encoding an MPK that acts downstream of *OsLRR-RLK1* to regulate
95 herbivore-induced defense and resistance (Wang et al., 2013; Hu et al., 2018b) was
96 not directly induced by indole but was primed for higher expression 90 min after
97 simulated herbivore attack (Figure 2C). *OsWRKY70*, encoding a positive regulator of
98 herbivore-induced defense that acts downstream of *OsMPK3* (Li et al., 2015), was
99 primed in a similar manner (Figure 2D). Three jasmonate biosynthesis genes,
100 *OsAOS1*, *OsAOC* and *OsOPR3* were equally primed by indole 90 min after elicitation
101 (Figure 2E). By contrast, *OsHI-RLK2*, *OsMPK6*, *OsWRKY13*, *OsWRKY24*,
102 *OsWRKY30*, *OsWRKY33*, *OsWRKY45*, *OsWRKY53* and the JA biosynthesis genes
103 *OsHI-LOX* did not respond to indole pretreatment. The induction of the JA-Ile
104 biosynthesis gene *OsJAR1* decreased upon indole pre-exposure (Figure 2B-E). Thus,
105 indole increases the expression of a specific subset of early defense signaling genes
106 that function upstream of JA biosynthesis.
107
108 **Indole pre-exposure increases OsMPK3 accumulation and activation upon**
109 **simulated herbivory**
110 To determine whether the transcriptional response of MPKs to indole pre-exposure
111 and elicitation by simulated herbivory is also reflected in protein abundance, we
112 performed protein gel blot analysis using OsMPK3 and OsMPK6-specific antibodies.
113 Pre-exposure to indole resulted in higher OsMPK3 abundance 90 min after elicitation
114 (Figure 3A). OsMPK6 accumulation was not altered by indole pre-exposure (Figure
115 3B). To further investigate whether indole pre-exposure increases OsMPK3 activation,

116 we measured OsMPK3 phosphorylation by immunoblot analysis using an
117 anti-phosphoERK1/2 (anti-pTEpY) antibody that interacts with doubly
118 phosphorylated (activated) MPK3 and MPK6 (Segui-Simarro et al., 2005; Anderson
119 et al., 2011; Schwessinger et al., 2015). Indole pre-exposure increased OsMPK3
120 activation 90 min after elicitation (Figure 3C). OsMPK6 may also exhibit a slightly
121 higher activation upon indole pre-treatment, but the gel blot analysis remains difficult
122 to interpret in this regard (Figure 3C). Thus, indole pre-exposure increases the elicited
123 accumulation and activation of MPKs involved in defense regulation such as
124 OsMPK3. As this effect only occurs upon elicitation by simulated herbivory, indole
125 pre-exposure primes rather than directly induces OsMPK3 accumulation and
126 activation.
127
128 **Indole pre-exposure induces OPDA and increases JA accumulation in plants**
129 **elicited by simulated herbivory**
130 To investigate whether the activation of early defense signaling components is
131 associated with higher accumulation of stress-related phytohormones, we quantified
132 12-oxophytodienoic acid (OPDA), JA and JA-isoleucine (JA-Ile), abscisic acid (ABA)
133 and salicylic acid (SA) in indole-exposed and control plants (Figure 4).
134 Indole pre-exposure increased the accumulation of OPDA before and after
135 elicitation (Figure 4A). JA concentrations increased in indole-exposed plants 90 and
136 360 min after elicitation (Figure 4B). The levels of JA-Ile, ABA, and SA were not
137 affected by indole pre-exposure (Figure 4C-E). To determine the total dose of indole
138 that is required for the increase in phytohormone concentrations, we exposed rice
139 plants to indole dispensers for 1-12 h and measured hormone accumulation 90
140 minutes after elicitation by simulated herbivory (Figure 4F-I). Exposure to indole
141 dispensers for 1 h (resulting in a total release of 21 ng from the dispensers) was
142 sufficient to increase OPDA and JA levels. Longer exposure did not significantly
143 increase OPDA and JA accumulation (Figure 4F-I). Thus, exposure of rice plants to
144 21 ng of indole over 1 h is sufficient to increase the production of oxylipin defense
145 regulators upon elicitation.

146

147 **OsMPK3 is required for indole-dependent jasmonate accumulation and**
148 **herbivore resistance**

149 To understand whether the early signaling components that are responsive to indole
150 are required for downstream responses, we measured JA accumulation upon
151 elicitation by simulated herbivory as well as herbivore resistance in control- and
152 indole pre-exposed wild type and transgenic plants, including the
153 *OsLRR-RLK1*-silenced line ir-*lrr1* (Hu et al., 2018b), the *OsMPK3*- and
154 *OsMPK6*-silenced lines ir-*mpk3* and ir-*mpk6* (Wang et al., 2013; Li et al., 2015) and
155 the *OsWRKY70*-silenced line ir-*wrky70* (Li et al., 2015). *OsLRR-RLK1* silencing did
156 not affect indole-dependent herbivore growth suppression, OPDA induction, or JA
157 accumulation (Figure 5A, F, K). By contrast, silencing *OsMPK3* completely
158 suppressed herbivore growth reduction, indole-dependent OPDA induction, and JA
159 accumulation (Figure 5B, G, L). The induction of JA by herbivore elicitation was still
160 clearly visible in *ir-mpk3* plants, demonstrating that the absence of indole-dependent
161 resistance is not due to a complete suppression of JA signaling. Silencing *OsMPK6*
162 reduced herbivore growth suppression and indole-dependent JA accumulation by
163 approximately 50% and led to an almost complete disappearance of OPDA induction
164 (Figure 5C, H, M). Silencing *OsWRKY70* also reduced herbivore growth suppression,
165 indole-dependent OPDA induction, and JA accumulation by approximately 50%
166 (Figure 5D, I, N). To exclude the potential allelic effects, we profiled the responses of
167 two independent *OsMPK3*- and *OsMPK6*-silenced lines in a separate experiment.
168 Consistent with our earlier results, OPDA induction was completely suppressed in
169 *OsMPK3*- and *OsMPK6*-silenced lines. Furthermore, JA accumulation following
170 simulated herbivory was compromised in both *OsMPK6*-silenced lines and
171 completely disappeared in the two *OsMPK3*-silenced lines (Supplemental Figure 1).
172 Thus, *OsMPK3* is required for, and *OsMPK6* and *OsWRKY70* contribute to, defense
173 regulation by the volatile indole.

174

175 **The jasmonate signaling pathway contributes to indole-induced herbivore**
176 **resistance**

177 To study the connection between the regulation of jasmonates and the decrease in
178 herbivore performance in indole-exposed plants, we tested *as-aos1* plants, which
179 accumulate lower levels of jasmonates upon herbivore elicitation compared to wild
180 type (Hu et al., 2015). OPDA, induction, JA accumulation and herbivore growth
181 suppression were reduced by approximately 50% in *as-aos1* plants vs. the wild type
182 (Figure 5E, J, O). Across the different genotypes, herbivore growth suppression was
183 strongly correlated with OPDA and JA over-accumulation: Genotypes that responded
184 to indole with stronger OPDA induction and JA accumulation upon elicitation also
185 reduced larval growth more strongly after pre-exposure (Figure 6A, B). Again, JA-Ile
186 did not respond significantly to indole pre-treatment (Supplemental Figure 2), and
187 there was no correlation between the effects of indole on JA-Ile and herbivore growth
188 suppression (Figure 6C). Together, these findings implicate the jasmonate-signaling
189 pathway in indole-induced herbivore resistance.
190

191 **DISCUSSION**

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

198 HIPVs such as GLVs have been shown to regulate early defense genes that likely
199 act upstream of stress hormone signaling (Ton et al., 2007; Frost et al., 2008; Erb et
200 al., 2015; Hu et al., 2018a). Here, we demonstrate that indole pre-exposure at
201 physiological doses also results in marked changes in the expression of early defense
202 signaling genes. The receptor-like kinase gene *OsLRR-RLK1* was directly induced by
203 exposure to indole, while the MPK OsMPK3 and the WRKY transcription factor gene
204 *OsWRKY70* were primed for stronger activation and expression. Experiments with

205 transgenic plants revealed that *OsMPK3* gene expression is required, and *OsWRKY70*
206 contributes, to indole-induced downstream responses. As *OsWRKY70* is regulated by
207 and acts downstream of *OsMPK3* (Li et al., 2015), we infer that indole acts upstream
208 of *OsMPK3*. The finding that the indole-induced priming was not altered in an
209 *OsLRR-RLK1*-silenced line further suggests that the expression of this receptor-like
210 kinase, which can regulate *OsMPK3*, is not directly required for indole-induced
211 priming. Experiments with additional, independently silenced lines, and, ideally, an
212 *OsLRR-RLK1* null mutant, would be required to completely rule out the involvement
213 of this gene in indole-dependent responses. In maize, GLV exposure has been shown
214 to directly increase the expression of *ZmMAPK6* and *ZmWRKY12* (Engelberth et al.,
215 2013). By contrast, we found that *OsMPK3* and *OsWRKY70* were not directly induced
216 by indole, but responded more strongly to elicitation by simulated herbivory. This
217 finding is in line with recent comparative work in maize showing that GLVs directly
218 induce defense genes, while indole primes their expression (Hu et al., 2018a). Thus,
219 while GLVs and indole both strengthen the jasmonate signaling pathway, their mode
220 of action and integration into early defense signaling are likely different.

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

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 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* following 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 following 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 minutes 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), while it did not change ABA

265 accumulation in rice in the current experiments. ABA is regulated by a wide variety
266 of biotic and abiotic parameters (Nambara and Marion-Poll, 2005). Whether the
267 absence of ABA overaccumulation reflects differences in experimental conditions or
268 whether it is the result of differences in the signaling networks that connect indole
269 responses to hormonal signaling remains to be elucidated.

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

290 In summary, we propose the following model. Plant leaves that are attacked by
291 herbivores release the volatile indole. Through as yet unknown perception
292 mechanisms, indole primes OsMPK3 in non-attacked tissues. When these tissues
293 come under attack, OsMPK3 is activated more strongly, which boosts downstream
294 responses, including the transcription of *OsWRKY70* and jasmonate biosynthesis

295 genes, which again results in an overaccumulation of bioactive oxylipins such as
296 OPDA and JA. Enhanced jasmonate signaling then boosts plant defense responses and
297 thereby reduces herbivore growth and damage. This study provides a mechanistic
298 basis for the regulatory potential and mode of action of HIPVs in plant defense
299 priming.
300

301 **METHODS**

302 **Plant and insect resources**

303 The rice (*Oryza sativa*) cultivar Xiushui 110 was used in this study. In addition, the
304 transgenic line ir-*lrr1* and its corresponding wild type line Xiushui 110 as well as the
305 transgenic lines ir-*mpk3*, ir-*mpk6*, ir-*wrky70*, as-*aos1* and their corresponding wild
306 type Xiushui 11 were used. These genotypes have been described and characterized
307 previously, including multiple independently transformed lines to exclude allelic
308 effects (Wang et al., 2013; Hu et al., 2015; Li et al., 2015; Hu et al., 2018b). Rice
309 seeds were pre-germinated and sown in plastic pots (11 cm height, 4 cm diameter)
310 using commercial potting soil (Aussaaterde, Ricoter Erdaufbere-itung AG,
311 Switzerland). Plants were grown in a greenhouse (26°C ± 2°C, 55% relative humidity,
312 14:10 h light/dark, with 250 μmol*m^{-2}*s^{-1} additional light supplied by Philips Master
313 GreenPower 600W 400V E40 High Pressure Sodium bulbs [Philips Lighting
314 Switzerland AG, Switzerland]). Plants were watered three times per week, and used
315 for experiments 30 days after sowing. Fall armyworm (*Spodoptera frugiperda*) larvae
316 were provided by University of Neuchâtel and reared on an artificial diet as
317 previously described (Maag et al., 2014). Oral secretions (OS) were collected from
318 third instar *S. frugiperda* larvae that had been feeding on rice leaves for 48 h, and
319 diluted 1:1 with sterilized Milli-Q water before use.
320

321 **Quantification of herbivore-induced indole emissions**

322 To determine the natural emission rates of indole, we infested rice plants with 3, 5 or
323 8 third-instar *S. frugiperda* larvae for 12 h, resulting in the consumption of approx.
324 10%, 30% and 50% of total leaf area, respectively. Following infestation, volatiles

325 were collected using a dynamic headspace sampling system and Super-Q traps (n=8,
326 [individual plants]). Briefly, the rice plants were enclosed in cooking bags (PET, 35 ×
327 40 cm, max. 200 °C, Migros supermarket, Switzerland). Purified air from a multiple
328 air-delivery system entered the bags via Teflon tubing at a rate of 0.8 L min^{-1} and was
329 pulled out through the Super-Q trap (Volatile Collection Trap LLC., UK) at a rate of
330 0.6 L min^{-1}. Before collection, the Super-Q traps were rinsed with 3 mL of methylene
331 chloride (>99.8%, GC, Sigma, USA). Volatiles were collected for 8 h. After
332 collection, the traps were extracted with 200 μL of methylene chloride containing two
333 internal standards (n-octane and nonyl-acetate, each 1 μg in 200 μL methylene
334 chloride). Then, a 1 μL aliquot of each sample was injected into GC/MS (Agilent
335 7820A GC interfaced with an Agilent 5977E MSD, USA) in pulsed split mode onto
336 an apolar column (HP-5MS, 30 m, 0.25 mm ID, 0.25 μm film thickness, Alltech
337 Associates, Inc., USA) for analysis. Helium at constant flow (1 mL min^{-1}) was used as
338 the carrier gas. After injection, the column temperature was maintained at 40 °C for 1
339 min, increased to 250 °C at 6 °C min^{-1} followed by a post-run of 3 min at 250 °C. The
340 quadrupole MS was operated in the electron ionization mode at 70 eV, a source
341 temperature of 230 °C, quadrupole temperature of 150 °C, with a continuous scan
342 from m/z 50 to 300. The detector signal was processed with HP GC Chemstation
343 software. Absolute emission rates of indole were determined based on peak areas and
344 calculated using a standard curve of synthetic indole (> 98%, GC, Sigma, USA).
345

346 **Indole exposure**

347 To expose rice to synthetic indole, we covered plants of different genotypes
348 individually with passively ventilated plastic cylinders (40 cm height, 4 cm diameter)
349 made of transparent plastic sheets (Rosco Laboratories Inc., USA). The plants were
350 placed into the greenhouse (26 °C ± 2 °C, 55% relative humidity, 14:10 h light/dark,
351 50,000 lm m^{-2}), and indole or control dispensers were added into the cylinders. After
352 12 h of exposure, the cylinders were carefully removed and the plants were subjected
353 to OS elicitation (see "Plant elicitation" below). Indole and control dispensers were
354 made as described previously (Erb et al., 2015). Briefly, the dispensers consisted of 2

mL amber glass vials (11.6 × 32 mm^{-2}; Sigma) containing 20 mg of synthetic indole (> 98%, GC, Sigma, USA). The vials were closed with open screw caps that contained a PTFE/rubber septum, which was pierced with a 1 µL micropette (Drummond, Millan SA, Switzerland). 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 approx. 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 jasmonic acid (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, pre-weighed 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

385 of the cages was moved every day to provide sufficient food for the larvae. Larval
386 mass was determined 7 days after the start of the experiment. To quantify damage, the
387 remaining leaf pieces were scanned, and the removed leaf area was quantified using
388 Digimizer 4.6.1 (Digimizer) (*n*=15, [individual larvae]).
389

390 **Phytohormone quantification**

391 Rice leaves were harvested at 0, 90 and 360 min after the start of OS elicitation and
392 ground in liquid nitrogen (*n*>5, [individual plants]). The phytohormones OPDA, JA,
393 JA-Ile, SA, and ABA were extracted with ethyl acetate spiked with isotopically
394 labeled standards (1 ng for d_5-JA, d_6-ABA, d_6-SA, and $^{13}C_6$-JA-Ile) and analyzed by
395 UHPLC-MS/MS as described (Glauser et al., 2014).
396

397 **Gene expression analysis**

398 qRT-PCR was used to measure the expression levels of different genes. Rice leaves
399 were harvested at 0, 90 and 360 min after the start of OS elicitation and ground in
400 liquid nitrogen (*n*>4, [individual plants]). Total RNA was isolated from the leaves
401 using a GeneJET Plant RNA Purification Kit (Thermo Scientific, USA). One µg of
402 each total RNA sample was reverse transcribed with SuperScript® II Reverse
403 Transcriptase (Invitrogen, USA) to synthesize cDNA. The qRT-PCR assay was
404 performed on the LightCycler® 96 Instrument (Roche, Switzerland) using the KAPA
405 SYBR FAST qPCR Master Mix (Kapa Biosystems, USA). A linear standard curve
406 was constructed using a serial dilution of cDNA that was pooled from all plants, and
407 generated by plotting the threshold cycle (Ct) against the $\log_{10}$ of the dilution factors.
408 The relative transcript levels of the target genes in samples were determined
409 according to the standard curve. A rice actin gene *OsACTIN* was used as an internal
410 standard to normalize cDNA concentrations. The primers used for qRT-PCR for all
411 tested genes are listed in Supplemental Table 1.
412

413 **MPK protein and activation detection**

414 Rice leaves were harvested at 0 and 90 min after the start of OS elicitation and ground

415 in liquid nitrogen. Total proteins were extracted from pooled leaves of six replicates at
416 each time point as described (Wu et al., 2007). Forty μg of total proteins were
417 separated by SDS-PAGE and transferred onto Bio Trace pure nitrocellulose blotting
418 membrane (Bio-Rad, USA). Immunoblotting was performed as described previously
419 (Hu et al., 2015). The primary antibody anti-MPK3 (Beijing Protein Innovation,
420 China; cat. no. AbP80147-A-SE, 1:1000 dilution) or anti-MPK6 (Beijing Protein
421 Innovation, China; cat. no. AbP80140-A-SE, 1:1000 dilution) was used to detect total
422 OsMPK3 or OsMPK6 protein, respectively. The rabbit monoclonal
423 anti-phospho-ERK1/2 (anti-pTEpY) antibody (Cell Signaling Technologies, USA; cat.
424 no. 4370, 1:2000 dilution), which is specific for the activated (phosphorylated) form
425 of the p44/42 MPKs (Thr202/Tyr204) (Segui-Simarro et al., 2005; Anderson et al.,
426 2011), was used to detect the active OsMPK3 and OsMPK6. The Anti-Plant beta
427 Actin Mouse antibody (CMCTAG, USA; cat. no. AT0004, 1:5000 dilution) was used
428 for a loading control and was detected on a replicate blot. Antigen-antibody
429 complexes were detected with horseradish peroxidase-conjugated anti-rabbit (Thermo
430 Scientific, USA; cat. no. 31460, 1:10000 dilution) or anti-mouse (Sigma, USA; cat. no.
431 AP308P, 1:5000 dilution) secondary antibody (Thermo Scientific, USA) followed by
432 chemiluminescence detection with Pierce™ ECL Western Blotting Substrate (Thermo
433 Scientific, USA).
434
435 **Statistical analyses**
436 Differences in levels of gene expression and phytohormones were analyzed by
437 analysis of variance (ANOVA, Supplemental File 1) followed by pairwise
438 comparisons of Least Squares Means (LSMeans), which were corrected using the
439 False Discovery Rate (FDR) method (Benjamini and Hochberg, 1995). The data
440 normality was verified by inspecting residuals using the "plotresid" function of the R
441 package "RVAideMemoire" (Herve, 2015). The variance homogeneity was tested
442 through Shapiro-Wilk's tests using the "shapiro.test" function in R. Datasets that did
443 not fit assumptions were log- or asinh-transformed to meet the requirements of
444 normality and equal variance. Differences in larval growth and leaf damage were

445 determined by two-sided Student's *t*-tests. The relative priming intensity was
446 calculated based on the fold changes of larval growth, OPDA or JA levels in the
447 indole-exposed plants relative to control-exposed plants. The differences in fold
448 changes were compared using Student's *t*-tests. The correlations (fold changes of
449 OPDA, JA or JA-Ile vs. fold changes of larval growth) were tested through Pearson's
450 product-moment correlation using the "cor. test" function in R (Puth et al., 2014). All
451 the analyses were conducted using R 3.2.2 (R Foundation for Statistical Computing,
452 Vienna, Austria). The numbers of replicates for each experiment are given in the
453 figures and denote independent biological replicates (i.e. individual plants, individual
454 larvae).
455
456 **Accession Numbers**
457 Sequence data from this article can be found in the Rice Annotation Project under
458 accession numbers *OsLRR-RLK1* (Os06g47650), *OsHI-RLK2* (Genbank accession
459 number XM_015757324), *OsMPK3* (Os03g17700), *OsMPK6* (Os06g06090),
460 *OsWRKY70* (Os05g39720), *OsWRKY53* (Os05g27730), *OsWRKY45* (Os05g25770),
461 *OsWRKY33* (Os03g33012), *OsWRKY30* (Os08g38990), *OsWRKY24* (Os01g61080),
462 *OsWRKY13* (Os01g54600), *OsHI-LOX* (Os08g39840), *OsAOS1* (Os03g55800),
463 *OsAOC* (Os03g32314), *OsOPR3* (Os08g35740), *OsJAR1* (Os05g50890), and
464 *OsACTIN* (Os03g50885).
465
466 **Supplemental Data**
467 **Supplemental Figure 1.** Effect of indole pre-exposure on jasmonate accumulation in
468 two independent *OsMPK3*- and *OsMPK6*-silenced lines.
469 **Supplemental Figure 2**. Herbivore-induced jasmonic acid-isoleucine (JA-Ile) levels
470 in MPK, WRKY and JA-impaired plants after indole exposure.
471 **Supplemental Table 1**. Primers used for qRT-PCR of target genes.
472 **Supplemental Files 1**. ANOVA tables.
473

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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 manuscript.

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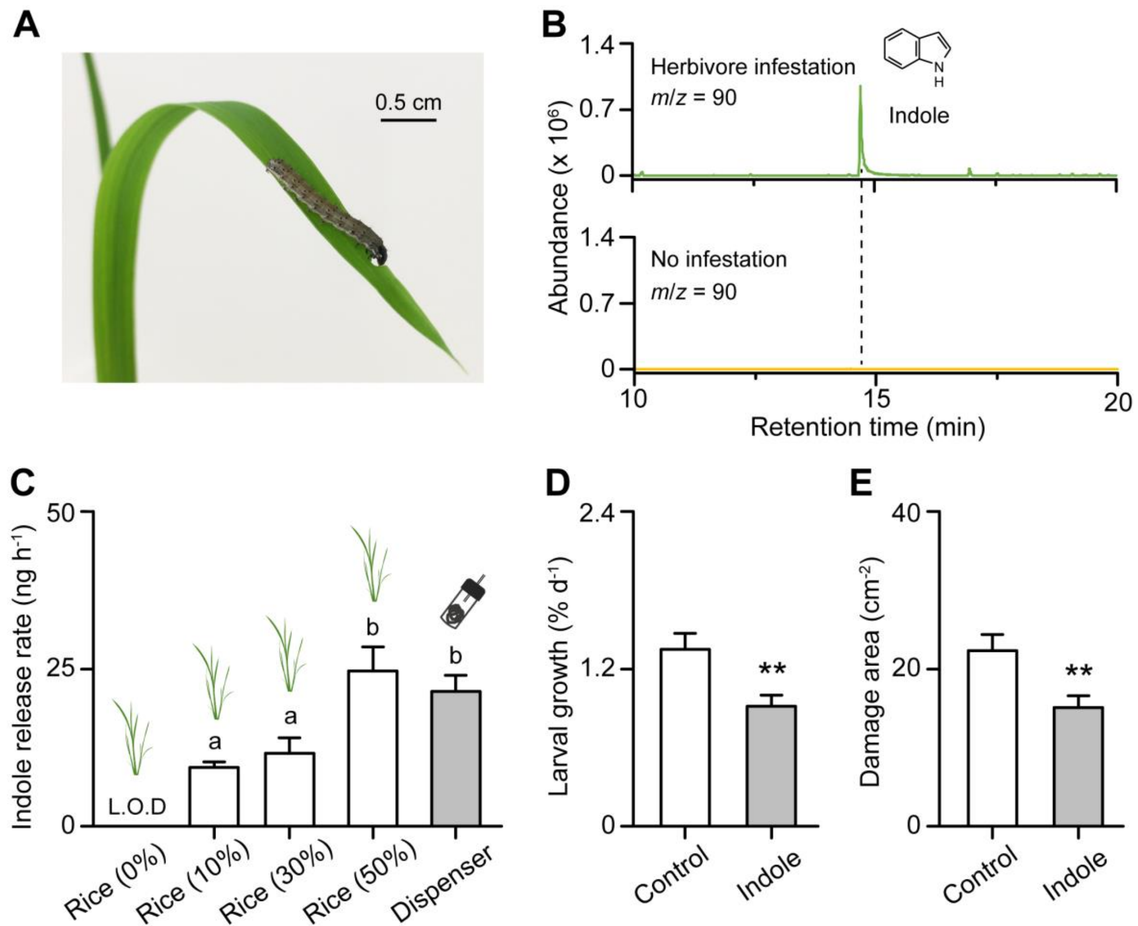

Figure 1. Indole is an herbivore-induced plant volatile that increases rice resistance to *Spodoptera frugiperda* larvae at physiological doses. **(A)** An *S. frugiperda* caterpillar feeding on a rice leaf. **(B)** Extracted ion chromatograms of GC/MS headspace analyses of control and *S. frugiperda* 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*=6-8 [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 approx. 21 ng h^{-1} or control dispensers for 12 h prior to 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, **, $P < 0.01$).

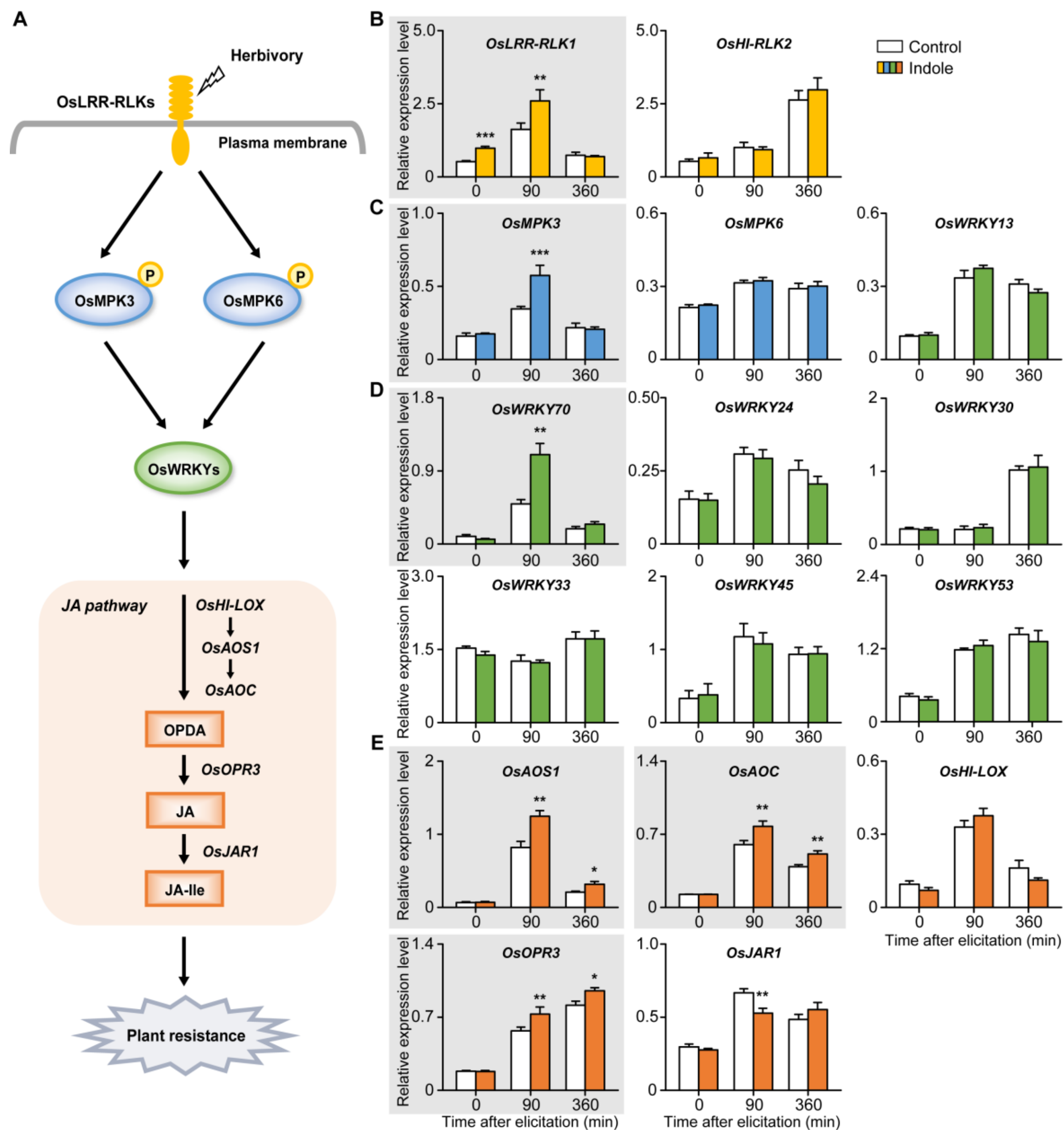

Figure 2. Indole pre-exposure increases the expression of early defense signaling genes. **(A)** Current model of herbivore-induced defense signaling in rice, including leucine-rich repeat receptor-like kinases (LRR-RLKs), mitogen-activated protein kinases (MPKs), WRKY transcription factors, jasmonate biosynthesis genes and oxylipins. P, phosphorylation **(B – 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 *Spodoptera frugiperda* oral secretions (+SE, *n*=4-6 [individual plants]). OPDA, 12-oxophytodienoic acid; JA, jasmonic acid; JA-Ile, JA-isoleucine. 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.

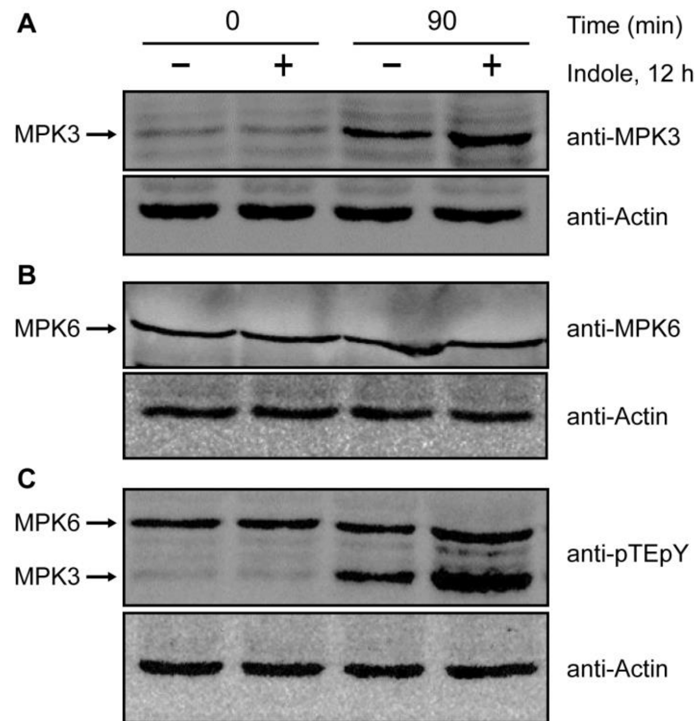

Figure 3. Indole pre-exposure increases OsMPK3 accumulation and activation upon simulated herbivory. **(A – C)** Protein accumulation and activation of OsMPK3 and OsMPK6 with (+) or without (-) indole pre-exposure before (0 min) and after elicitation by simulated herbivory (90 min). Leaves from 6 replicate 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, (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.

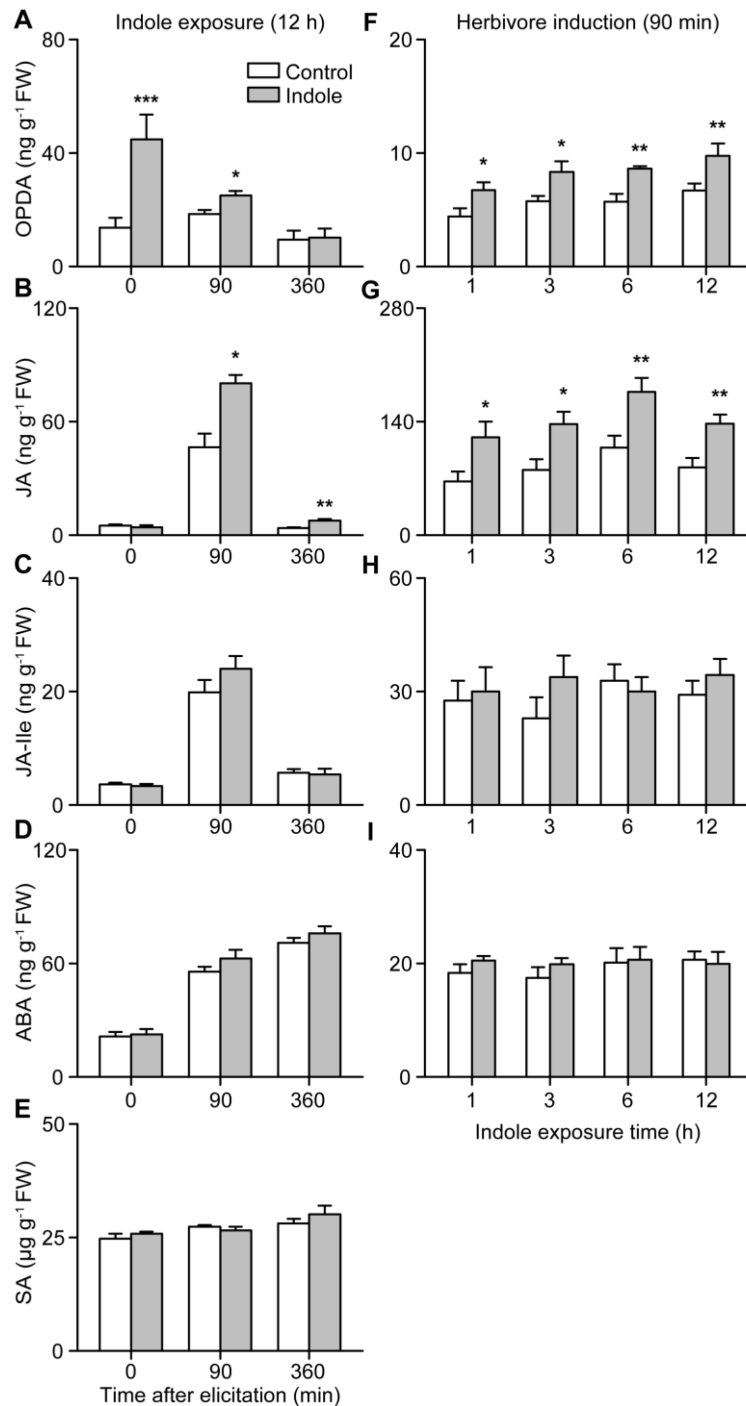

Figure 4. Indole pre-exposure induces OPDA and increases JA accumulation in plants elicited by simulated herbivory. **(A – E)** Average concentrations of **(A)** OPDA, **(B)** JA, **(C)** JA-isoleucine (JA-Ile), **(D)** abscisic acid (ABA) and **(E)** salicylic acid (SA) in indole pre-exposed and control plants at different time points after elicitation (+SE, *n*=5-6 [individual plants]). Rice plants were pre-exposed to indole for 12 h before elicitation. (**F – 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*=5-6 [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; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

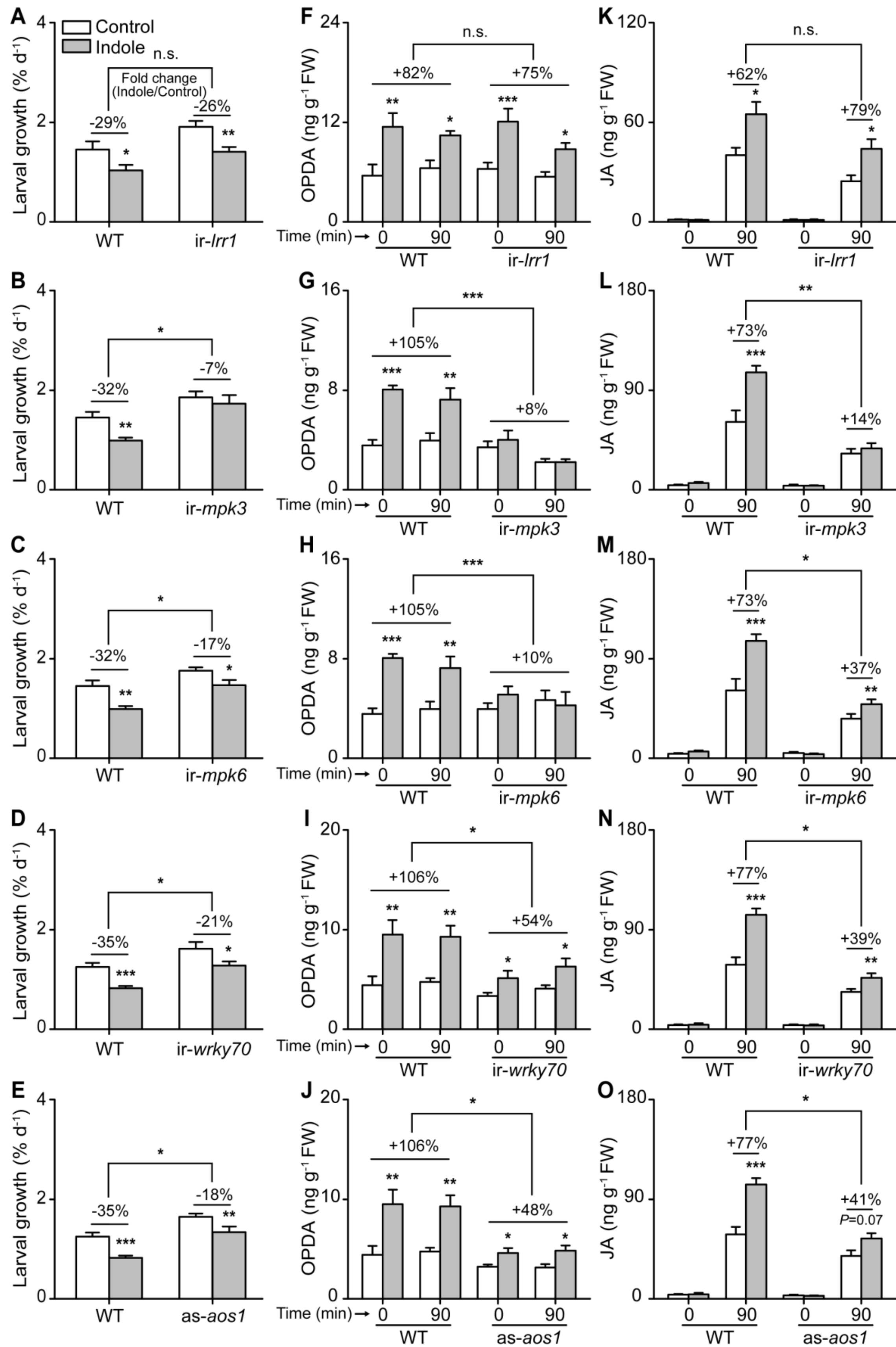

Figure 5. OsMPK3 is required for indole-dependent jasmonate accumulation and herbivore resistance. **(A – E)** Average growth rate of *Spodoptera frugiperda* caterpillars feeding on **(A)** ir-

lrr1, **(B)** ir-*mpk3*, **(C)** ir-*mpk6*, **(D)** ir-*wrky70*, **(E)** as-*aos1* lines and wild-type (WT) plants that were pre-exposed to indole or control (+SE, *n*=15 [individual larvae]). **(F – J)** Average concentrations of herbivore-induced 12-oxophytodienoic acid (OPDA) in the different transgenic lines and WT plants that were pre-exposed to indole or control dispensers (+SE, *n*=6 [individual plants]). **(K – O)** Average concentrations of herbivore-induced jasmonic acid (JA) in the different transgenic lines and WT plants that were pre-exposed to indole or control dispensers (+SE, *n*=6 [individual plants]). Note that WT, ir-*mpk3* and ir-*mpk6* plants as well as WT, ir-*wrky70* and *as-aos1* plants were measured together within the same experiments. The WT data are, therefore, identical in the respective figures (e.g. same WT data for ir-*mpk3* and ir-*mpk6* figures; same WT data for *ir-wrky70* and *as-aos1* figures). FW, fresh weight. n.s. not significant. 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 WT and transgenic lines (Student's *t*-tests, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

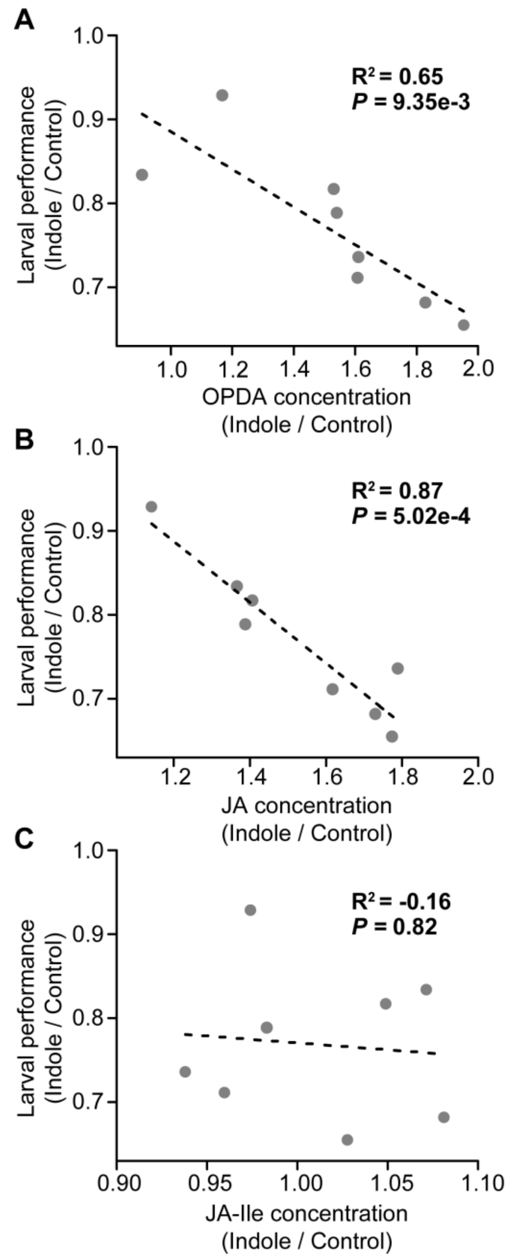

Figure 6. Correlations between indole-induced regulation of OPDA, JA and herbivore resistance. **(A – C)** Correlations between the fold changes of herbivore-induced 12-oxophytodienoic acid (OPDA, A), jasmonic acid (JA, B), and JA-isoleucine (JA-Ile, C) concentrations in indole-exposed plants relative 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.

Molecular dissection of early defense signaling underlying volatile-mediated defense regulation and herbivore resistance in rice
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