Intronic T-DNA Insertion Renders Arabidopsis *opr3* a Conditional Jasmonic Acid-Producing Mutant[1[C][W][OA]]

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Jasmonic acid and its derived metabolites (JAs) orchestrate plant defense against insects and fungi. 12-Oxo-phytodienoic acid (OPDA), a JA precursor, has also been implicated in plant defense. We sought to define JAs and OPDA functions through comparative defense susceptibility characteristics of three Arabidopsis (*Arabidopsis thaliana*) genotypes: *aos*, lacking JAs and OPDA; *opda reductase3* (*opr3*), deficient in JA production but can accumulate OPDA; and transgenics that overexpress *OPR3*. *opr3*, like *aos*, is susceptible to cabbage loopers (*Trichoplusia ni*) but, relative to *aos*, *opr3* has enhanced resistance to a necrotrophic fungus. Gas chromatography-mass spectrometry reveals that *opr3* produces OPDA but no detectable JAs following wounding and looper infestation; unexpectedly, substantial levels of JAs accumulate in *opr3* upon fungal infection. Full-length *OPR3* transcripts accumulate in fungal-infected *opr3*, potentially through splicing of the T-DNA containing intron. Fungal resistance correlates with levels of JAs not OPDA; therefore, *opr3* resistance to some pests is likely due to JA accumulation, and signaling activities ascribed to OPDA should be reassessed because *opr3* can produce JAs. Together these data (1) reinforce the primary role JAs play in plant defense against insects and necrotrophic fungi, (2) argue for a reassessment of signaling activities ascribed to OPDA, and (3) provide evidence that mutants with intron insertions can retain gene function.

Plants are sessile organisms constantly challenged by diverse pests ranging from macroscopic insects to microbes. In response, plants have evolved an effective and diverse arsenal of toxic secondary metabolites to fight off and survive these challenges. For example, Arabidopsis (*Arabidopsis thaliana*) plants infested with cabbage loopers (*Trichoplusia ni*) or infected with necrotrophic pathogens, such as *Botrytis cinerea*, produce high levels of toxic glucosinolates and camalexin to resist its invaders (Jander et al., 2001; Lambrix et al., 2001; Kliebenstein et al., 2002; Sellam et al., 2007; Rowe et al., 2010). Production of such toxins involves signaling through the phytohormone jasmonic acid (JA) and several of its derivatives, including its biologically active form JA-Ile (Staswick and Tiryaki, 2004; Chen et al., 2005; Chehab et al., 2008; Suza and Staswick, 2008; Rowe et al., 2010). Here we refer to JA, JA-Ile, and other derivatives collectively as JAs, for simplicity.

JAs are important signaling molecules involved not only in plant defense (Albrecht et al., 1993; Howe et al., 1996; McConn and Browse, 1996; Creelman and Mullet, 1997; Staswick et al., 1998; Vijayan et al., 1998) but also in responses to abiotic stress (Parthier, 1990), mechanotransduction (Falkenstein et al., 1991), and reproduction (Creelman and Mullet, 1995; McConn and Browse, 1996; Hause et al., 2000; Ishiguro et al., 2001). JA synthesis is initiated by the oxidation of α-linolenic acid (18:3), released from chloroplast membranes, into 13-hydroperoxylinolenic acid. The latter is further dehydrated by ALLENE OXIDE SYNTHASE (AOS) and cyclized into (9*S*, 13*S*)-12-oxo-phytodienoic acid (OPDA) by allene oxide cyclase. OPDA REDUCTASE3 (OPR3) reduces OPDA to 3-oxo-2(2′[Z]-pentenyl) cyclopentane-1-octanoic acid that undergoes three rounds of β-oxidation in the peroxisomes to yield JA. Subsequently, JA is converted into JA-Ile (Staswick and Tiryaki, 2004; Suza and Staswick, 2008). The F-box protein, CORONATINE INSENSITIVE1 (COI1), mediates JA action, as binding of JA-Ile to COI1 results in the ubiquitination and degradation of JAZ proteins acting as transcriptional repressors of downstream gene targets (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Katsir et al., 2008; Melotto et al., 2008).

Mutants defective in JA biosynthesis and response have revealed roles for JAs in defense. These mutants include the triple *fad3 fad7 fad8* mutant, which lacks JA and its precursor metabolites (McConn and Browse, 1996), *opr3*, which contains a 17-kb T-DNA insertion in its second intron and is reported to block the JA biosynthesis pathway downstream of OPDA (Stintzi and Browse, 2000), and *coi1*, which produces normal levels of OPDA, JA, and its derived metabolites but is JA insensitive. All three mutants are male sterile; however, there are key differences in their resistance

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to various pathogens. In contrast to *fad3 fad7 fad8* and *coi1*, which are highly susceptible to the necrotrophic fungus, *Alternaria brassicicola*, as well as to the soil gnat, *Bradysia impatiens*, *opr3* is more resistant to these invaders (Stintzi et al., 2001; Zhang and Turner, 2008). *fad3 fad7 fad8* and *opr3* differ in that *opr3* can synthesize OPDA. Therefore, the observed resistance of *opr3* to *A. brassicicola* and *B. impatiens* has been attributed to the presence of OPDA. In addition, the susceptibility of *coi1* to the same plant invaders implicated OPDA in playing a defense role through COI1, similarly to JA (Stintzi et al., 2001). However, to date there is no experimental evidence that OPDA promotes COI1 and JAZ interaction (Thines et al., 2007), although such interaction could still take place through unknown mechanism(s). Reports also suggest that some of the OPDA actions may be *COI1* independent (Stintzi et al., 2001; Taki et al., 2005). Thus how OPDA might act to mediate defense in *opr3* remains an open question. Alternatively, there might be an unidentified explanation for the resistance of *opr3* to invaders.

With the aim toward gaining a better understanding of the potential role of OPDA in plant defense responses, we employed a set of Arabidopsis mutant genotypes differentially affected in the JA pathway and challenged them with *B. cinerea* or cabbage loopers. We demonstrate that upon *B. cinerea* infection, *opr3* is capable of producing full-length *OPR3* transcripts potentially through successful removal of the T-DNA-harboring intron and consequently accumulating JAs at substantial levels. Furthermore, resistance to *B. cinerea* and cabbage loopers in all examined genotypes correlates with accumulation levels of JAs. Therefore, previous interpretations about the sufficiency of OPDA in pathogen defense based on *opr*3 mutant resistance need to be reconsidered because *opr3* can accumulate JAs. We conclude that JAs are most likely responsible for Arabidopsis resistance to *B. cinerea* and cabbage loopers.

RESULTS

Introgression of *opr3* Mutation into Columbia-0 Background

We sought to elucidate and differentiate the functions and mechanisms of action of JAs and their precursor, OPDA, both of which have been implicated in insect and fungal defense responses. Toward this goal, we employed two mutants: *aos*, which lacks both OPDA and JA by loss of conversion of 13-hydroperoxylinolenic acid to 12,13-epoxyoctadecatrienoic acid, and *opr3*, reported to produce OPDA but be deficient in JA production due to a failure to convert OPDA to 3-oxo-2(2′[*Z*]-pentenyl)cyclopentane-1-octanoic acid (Stintzi and Browse, 2000; Stintzi et al., 2001). These two mutant lines have two different genetic backgrounds. Therefore, for comparative analyses, we reiteratively backcrossed *opr3*, originally in the Wassilewskija (Ws) background, eight times with the genetic background of the *aos* mutant, *gl-1* (Columbia-0 [Col-0]), and employed *gl-1* as the control background in all subsequent experiments. It should be noted that there are no significant detectable differences in AOS-derived metabolites between *gl-1* and Col-0 wild type (Chehab et al., 2008). The *aos* mutant as well as the resultant *opr3* are conditionally male sterile, rescued by exogenous methyl jasmonate (MeJA) application (Fig. 1A). In addition, like *aos*, *opr3* failed to accumulate JA and MeJA (referred to here as JAs for simplicity), even in response to wounding, which leads to increased accumulation of JAs in the control (*gl-1*; Fig. 1B). In contrast, *opr3* and *gl-1* lines accumulated statistically similar levels of OPDA before and after wounding, whereas *aos* had no detectable OPDA (Fig. 1C). These data obtained for *opr3* in the *gl-1* (Col-0) background are similar to that reported for Ws-background plants harboring the same *opr3* mutation (Stintzi and Browse, 2000). Stintzi et al. (2001) reported that wounded *opr3* accumulates JAs to less than 4% the levels observed in wild type. Growth conditions may account for the lower levels we observe for JAs and OPDA in wounded *gl-1* plants compared to some previously published reports.

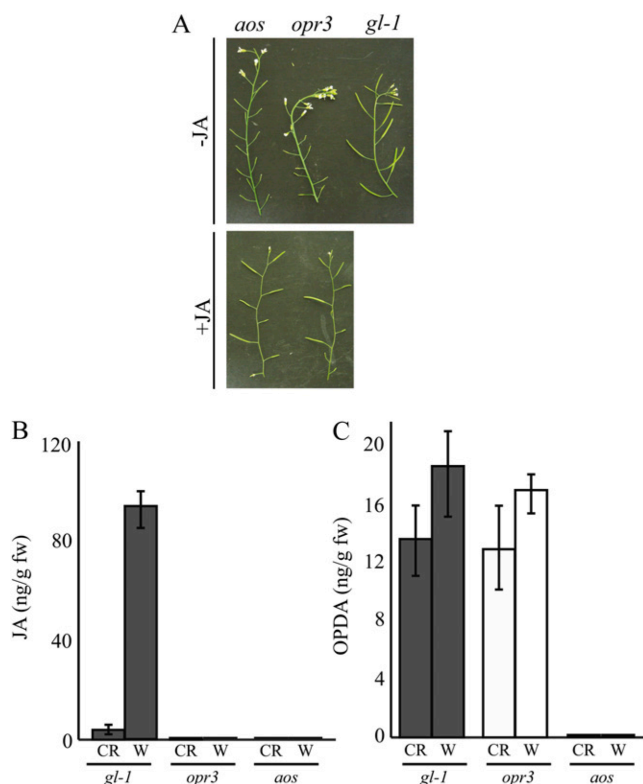

Figure 1. JA rescue of male fertility, and accumulation of JAs and OPDA in mechanically wounded Arabidopsis. A, Male fertility is restored in *aos* and *opr3* by the exogenous application of JA. B, Levels of JAs in untreated leaves (CR) and wounded leaves 2 h after mechanical damage (W). C, Levels of OPDA in untreated leaves (CR) and wounded leaves 2 h after mechanical damage (W). Means ± SD are shown. *n* = 3. [See online article for color version of this figure.]

opr3 Mutants Have Increased Susceptibility to Cabbage Loopers

The apparent ability of *opr3* to accumulate only OPDA but not JA provides a potentially powerful tool to uncouple the roles of these two metabolites in plant defense responses. JAs play an important role in plant defense against the cabbage looper, a chewing generalist lepidopteran (Jander et al., 2001; Lambrix et al., 2001; Kliebenstein et al., 2002; Chehab et al., 2008). We therefore first examined whether *OPR3* is necessary for enhanced resistance to cabbage loopers. No-choice bioassays were employed to assess the susceptibility of *opr3* to the cabbage looper. Loopers reared on *gl-1* had approximately 45% lower final weight than larvae feeding on *aos* or *opr3* (Fig. 2A). No statistical differences in looper weights were found between those reared on *aos* or *opr3* (Fig. 2A). Increased susceptibility of *opr3* and *aos* to looper attack was also evident by the remaining shoot mass of the plants following infestation; while *gl-1* retains relatively abundant shoot mass, both mutants are nearly devoid of rosette leaves 12 d after looper release (Fig. 2B). These results indicate that *OPR3* is essential for plant resistance to loopers and suggest that OPDA produced in *opr3* is not sufficient to confer looper resistance.

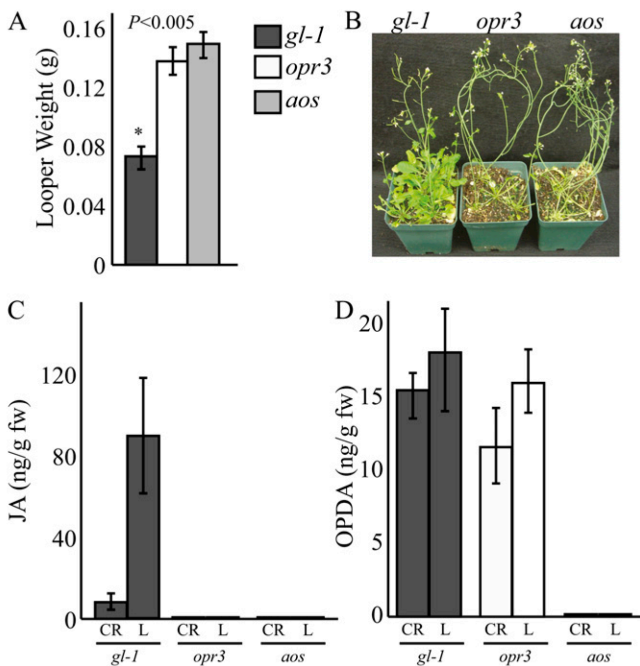

Figure 2. *opr3* has increased susceptibility to cabbage loopers. A, Looper weights 12 d after release of a newly hatched larva in an arena containing five plants of indicated genotype. Means ± SEM of two independent experiments, each with approximately 15 arenas per genotype, are shown. B, Representative photos of *gl-1*, *opr3*, and *aos* showing tissue damage. C, Levels of JAs in control (CR) and looper-infested (L) leaves 72 h post infestation. Means ± SD are shown. $n = 3$. D, Levels of OPDA in control (CR) and looper-infested (L) leaves 72 h post infestation. Means ± SD are shown. $n = 3$. [See online article for color version of this figure.]

To verify whether OPDA, and not JAs, accumulate in *opr3* upon looper infestation, we measured levels of JAs and OPDA in tissues from looper-challenged plants (Fig. 2, C and D). *gl-1* plants produced inducible levels of JAs upon looper infestation, whereas *opr3* and *aos* had no detectable JAs (Fig. 2C). *gl-1* and *opr3* accumulated OPDA, but *aos* had none (Fig. 2D). These results confirm that OPDA is not sufficient for cabbage looper resistance and that the JA-related metabolites produced upon looper infestation (Fig. 2) are comparable to those produced after mechanical wounding (Fig. 1). Together these data indicate that *OPR3* function and the ability to accumulate JAs are necessary for Arabidopsis resistance to loopers.

opr3 Has Partial Resistance to *B. cinerea* and Produces Camalexin

The JA signaling pathway is also implicated in necrotrophic pathogen responses, therefore we next addressed whether OPDA accumulation in *opr3* is sufficient for fungal resistance. *B. cinerea* conidia were applied to *gl-1*, *opr3*, and *aos* leaves. The mean diameters of the necrotic area are similar for *gl-1* and *opr3* at 48 h post inoculation (hpi) but are 30% larger for *aos* (Fig. 3A). At 72 hpi, *opr3* lesions were 30% larger than those on *gl-1*. In contrast, *aos* lesions were nearly 400% larger than *gl-1* (Fig. 3A). Therefore, *opr3* is more resistant than *aos* to the fungus, although it is less resistant than *gl-1*. Stintzi et al. (2001) previously reported enhanced resistance of *opr3* against a different necrotrophic pathogen, *A. brassicicola.* Camalexin, a primary Arabidopsis phytoalexin important for pathogen growth inhibition, accumulates in *opr3* to nearly 75% the levels of that found in *gl-1* (Fig. 3B). In contrast, *aos* lacks detectable camalexin (Fig. 3B; Chehab et al., 2008). Depending on the invading pathogen, JA may be required for camalexin biosynthesis (Rowe et al., 2010). Together, these data appear to be consistent with the possible interpretation that *OPR3* function is not necessary for enhanced resistance to necrotrophic fungi. However, this interpretation is likely false as the following results indicate.

JA Accumulates in *opr3* Infected with *B. cinerea*

To examine whether the partial resistance of *opr3* to *B. cinerea* is evidence for JA-independent defense, we measured levels of JA and OPDA in *B. cinerea*-infected leaves at 48 and 72 hpi. As expected, *gl-1* accumulated high levels of both OPDA and JA in infected leaves, whereas neither metabolite is detectable in *aos* (Fig. 3. C and D). *opr3* also had higher levels of OPDA than *gl-1* (Fig. 3D). However, an unexpected and critically important finding is that *opr3* leaves produced substantial levels of JAs, approximately 30% of the total JAs produced by *gl-1* at both recorded time points (Fig. 3C). Thus, *opr3* can accumulate substantial amounts of JAs at least under specific conditions. This finding indicates that *opr3* phenotypes, such as

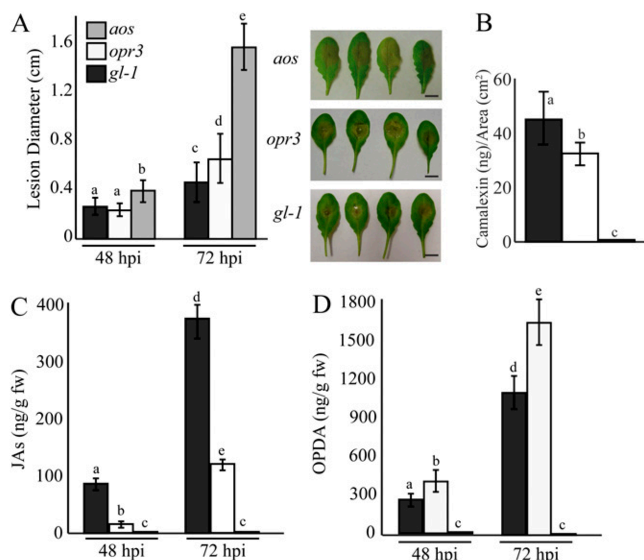

Figure 3. *opr3* has increased resistance to *B. cinerea* relative to *aos* and accumulates camalexin and JAs. A, *aos*, *opr3*, and *gl-1* leaf lesion diameters at 48 and 72 hpi with fungal conidia. Mean lesion diameters ± SD are shown. $n = 40$. Photographs of representative leaves of each genotype 72 hpi. Bar = 1 cm. B, Leaf camalexin levels at 72 hpi. Means ± SD. $n = 30$. C, Levels of leaf JAs at 48 and 72 hpi with fungal conidia. Means ± SD. $n = 8$. D, Levels of leaf OPDA at 48 and 72 hpi with fungal conidia. Means ± SD. $n = 8$. Within any given treatment, letters indicate significant differences ($P < 0.005$, Tukey's test).

enhanced resistance to *B. cinerea* and camalexin accumulation, may be consequences of JA production rather than OPDA accumulation. The interpretation that JAs are the active metabolites conferring these phenotypes is consistent with the observation that *opr3* accumulates less JAs, has less camalexin, and has lower *B. cinerea* resistance compared to *gl-1* that accumulates higher levels of JAs and camalexin and has superior *B. cinerea* resistance (Fig. 3, A–C).

opr3 Is Not a Loss-of-Function Mutant

The *opr3* mutation is a 17-kb T-DNA insertion within the second *OPR3* intron. Previous findings identified *opr3* as a likely null mutant, given the large DNA insertion, the lack of detectable *OPR3* transcripts, the JA-dependent male fertility phenotype, and an apparent deficiency in JA accumulation (Stintzi and Browse, 2000; Stintzi et al., 2001). The discovery of easily detectable levels of JA in *opr3* in response to *B. cinerea* (Fig. 3C) led us to investigate the molecular basis of the finding by examining the *OPR3* transcript levels in plants subjected to mechanical wounding, cabbage loopers, or *B. cinerea* infection. In *gl-1*, full-length mature *OPR3* transcripts were detectable by semi-quantitative reverse transcription (RT)-PCR, using primers targeted to sequences including the start and stop translational codons, in untreated plants and increased approximately 2-fold 2 h after wounding and 72 h post infestation with cabbage loopers, and 8-fold 72 hpi with *B. cinerea* (Fig. 4A, left section). In contrast, *opr3* lacked detectable full-length *OPR3* transcripts when subjected to wounding or cabbage loopers (Fig. 4A, right section). However, full-length *OPR3* transcripts were detected in *opr3* subjected to fungal infection (Fig. 4A, right section). These full-length RT-PCR products from *opr3* were confirmed to be derived from the *OPR3* locus by restriction digests and DNA sequencing that indicated that the second intron was precisely excised. These results were corroborated by quantitative RT-PCR using primers that flank the T-DNA-harboring second intron (Fig. 4, B and C). Note that *aos* lacks detectable *OPR3* transcripts under all treatment conditions (Fig. 4, B

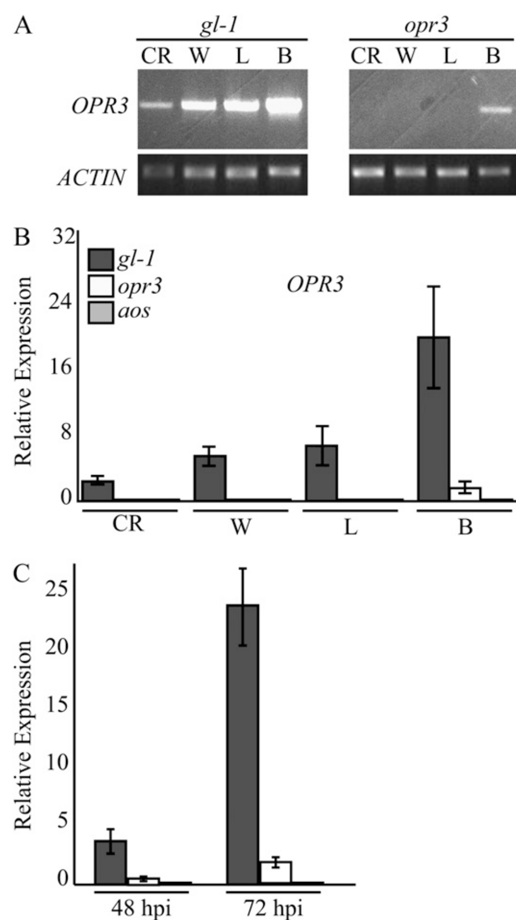

Figure 4. *OPR3* transcript levels. A, Semiquantitative RT-PCR of full-length *OPR3* and *ACTIN* transcripts in *gl-1* and *opr3* that were not treated (CR), wounded (W), looper infested for 72 h (L), or *B. cinerea* infected 72 hpi (B). Primers used to generate the full-length *OPR3* transcript amplified the full open reading frame from the start codon to the stop codon. B, Quantitative RT-PCR of *OPR3* transcripts relative to *TUB4* transcripts in *gl-1* and *opr3* that were not treated (CR), wounded (W), looper infested for 72 h (L), or *B. cinerea* infected 72 hpi (B). Means ± SD. $n = 6$. C, Quantitative RT-PCR analysis of *OPR3* transcripts relative to *TUB4* transcripts in *gl-1*, *opr3*, and *aos* at 48 and 72 hpi with *B. cinerea*. Means ± SD. $n = 6$.

and C), confirming the dependence on JAs for *OPR3* expression. *OPR3* transcript accumulation was detected in *opr3* at 48 hpi, and the transcript level increased 4-fold in abundance by 72 hpi with *B. cinerea* (Fig. 4C). Furthermore, *OPR3* transcript abundance in infected *opr3* (Fig. 4C) correlated with JA accumulation and *B. cinerea* resistance: JAs and evidence of *B. cinerea* resistance were also detected in *opr3* at 48 hpi and increased at 72 hpi (Fig. 3, A and C). Finally to rule out the possibility of *opr3* seed stock contamination, similar analyses were performed on individual *opr3* plants. Three individual *opr3* plants each showed (1) the expected male-sterile phenotype, (2) an absence of detectable wild-type *OPR3* gene, (3) the presence of the mutant allele, and (4) detectable accumulation of full-length *OPR3* transcripts by RT-PCR (Supplemental Fig. S1). Furthermore, JAs were detected in extracts from the pooled infected leaves from these three plants (Supplemental Fig. S1). Overall these results demonstrate that *opr3* is not a null mutant but instead is able to produce full-length mature *OPR3* transcripts under certain conditions, likely through splicing out the intron harboring the 17-kb T-DNA insertion, albeit with low efficiency.

The Arabidopsis genome contains five other OPR-encoding genes. Although biochemical studies established that OPR3 is the reductase responsible for converting OPDA to OPC:8, the involvement of the other *OPRs* in JA production has not been ruled out (Schaller et al., 2000). We examined transcript levels of the other five *OPRs* upon fungal infection and found that only *OPR2* showed an increase in transcript level in response to infection (Supplemental Fig. S2). Although it is unlikely that reductases other than OPR3 convert OPDA to OPC:8 (Breithaupt et al., 2001, 2006; Malone et al., 2005; Hall et al., 2007, 2008; Beynon, et al., 2009; Schaller and Stintzi, 2009), whether *OPR2* or other *OPR*s are involved in the production of JAs and thus play a role in fungal resistance remains to be determined.

The data presented strengthen the hypothesis that JAs may be necessary for defense against necrotrophic fungi resistance and that OPDA is not sufficient to confer resistance. To further investigate the potential role for JAs in resistance against *B. cinerea*, we supplied *aos* with exogenous JA and monitored fungal growth. Because *aos* lacks both OPDA and JAs (Fig. 2, C and D; Chehab et al., 2008), the effects of exogenous JA, without potential interference by endogenous OPDA, could be assessed. Fungal spores were spotted on *gl-1* and *aos* leaves, and the leaves were subsequently sprayed with water or JA. Lesion diameters were measured at 72 hpi. *B. cinerea*-infected *gl-1* leaves did not show statistically significant differences in the lesion diameters whether sprayed with water or increasing concentrations of JA (Fig. 5, A and B). This indicates that exogenous JA application was neither toxic to the fungus nor inhibitory to fungal infectivity. Furthermore, these data suggest that the endogenously produced JAs in *gl-1* are likely accumulating to a maximally beneficial level. In contrast, the mean lesion diameters on *aos* leaves decreased relative to the increased JA concentrations applied (Fig. 5, A and B). *aos* leaves sprayed with 0.5 mM JA had a mean lesion diameter not significantly different than that measured on *gl-1* (Fig. 5, A and B). These results indicate that JA treatment is sufficient to confer *B. cinerea* resistance to *aos*.

To verify whether JAs, and not OPDA, were responsible for *aos* fungal resistance, we measured OPDA levels in fungal-infected *aos* and *gl-1* leaves treated with 0.5 mM JA. OPDA was absent in *aos* but present in *gl-1* (Fig. 5C). Thus, JAs, in the absence of OPDA, can induce at least some defense mechanisms against *B. cinerea*. These results suggest that OPDA may have no

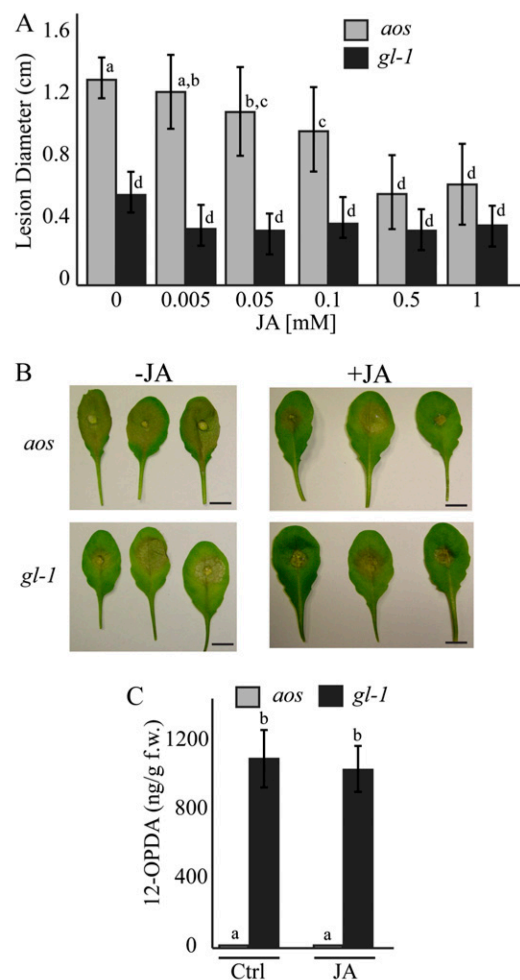

Figure 5. JAs are required for defense against *B. cinerea*. A, Leaf lesion diameters of Col-0 and *aos* sprayed with indicated JA concentrations at 72 hpi with fungal conidia. Mean lesion diameters ± SD are shown. n = 30. B, Representative leaves of each genotype 72 hpi. Bar = 1 cm. C, OPDA levels in *aos* and Col-0 leaves with no JA treatment (Ctrl) and with 0.5 mM JA (JA) 72 hpi with fungal conidia. Letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences at $P < 0.05$, Tukey's test.

direct role in fungal defense but acts only as a JA precursor.

Overexpression of *OPR3* Reduces OPDA Levels

The previously described experiment demonstrates that the exogenous application of JA is sufficient to elicit defense against *B. cinerea*. However, exogenous application of metabolites can fail to induce responses comparable to those produced in vivo (Chehab et al., 2008). Furthermore, to examine whether OPDA itself may also have a role in fungal resistance, we generated plants that overexpress *OPR3* in an attempt to reduce levels of OPDA and increase endogenous levels of JAs relative to wild type. All lines examined had altered levels of OPDA and JAs (Supplemental Fig. S3, A and B). The representative line, which we denote here as *OPR3-OE*, had approximately 35% higher basal and wound-induced JAs and approximately 40% lower basal and wound-induced OPDA as compared to wild type.

OPR3-OE Is Resistant to *B. cinerea*, and OPDA Levels Do Not Correlate with Resistance

As expected, fungal-infected *OPR3-OE* accumulated 45% less OPDA and 30% more JAs as compared to Col-0 (Fig. 6, B and C). At 72 hpi, *OPR3-OE* lesions were 25% smaller in diameter than those of Col-0 (Fig. 6A). Independent *OPR3* overexpressing lines behaved similarly to *OPR3-OE* (Supplemental Fig. S4). These experimental results support a lack of correlation between OPDA accumulation and fungal resistance. Instead, there is a strong inverse correlation between mean fungal lesion diameter and accumulation of JAs, thus further supporting the central functional role for JAs in fungal resistance.

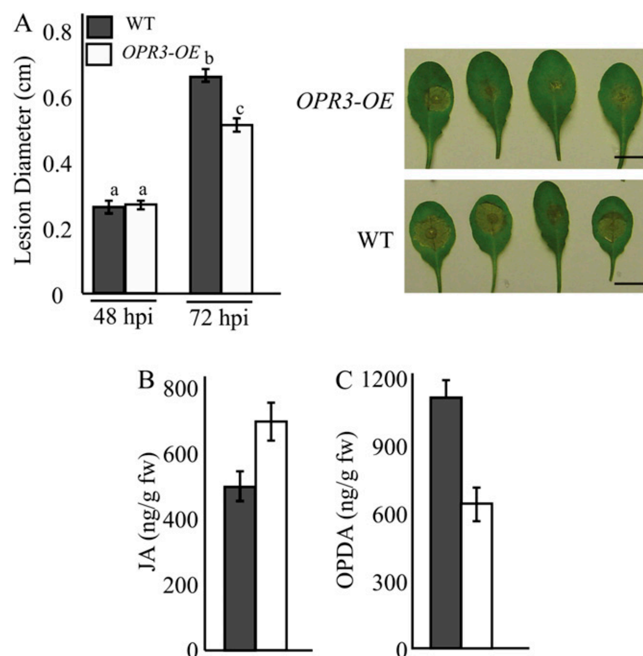

Figure 6. *OPR3-OE* plants are more resistant to *B. cinerea*. A, Leaf lesion diameters of *OPR3-OE* and Col-0 48 hpi and 72 hpi with fungal conidia. Representative leaves of each genotype at 72 hpi. Bar = 1 cm. B, Levels of JAs in Col-0 (WT) and *OPR3-OE* leaves 72 hpi with fungal conidia. C, OPDA levels in Col-0 (WT) and *OPR3-OE* leaves 72 hpi with fungal conidia. Mean lesion diameters ± SD. $n = 40$. Bars with different letters indicate significant differences ($P < 0.005$, Tukey's test) within a treatment.

DISCUSSION

Plant defense against necrotic fungi and chewing insects depends upon the JA response pathway. Elucidation of downstream responses requires the identification of the active compounds. JA-Ile action has been defined; JA-Ile binds COI1 and mediates destruction of the JAZ repressor proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007; Katsir et al., 2008; Melotto et al., 2008). In contrast, although OPDA had been implicated as an active oxylipin, its mechanism of action remained unclear. OPDA, unlike JA-Ile, does not bind COI1; yet COI1 is thought to be required for some, but not all putative OPDA actions (Ribot et al., 2008). Much of the evidence that OPDA has defense activity comes from previous characterization of the mutant *opr3*. *opr3*, with a 17-kb T-DNA insertion in an *OPR3* intron, manifests resistance to *A. brassicicola* and *B. impatiens* comparable to wild type (Stintzi et al., 2001). The interpretation that such an observed resistance results from a direct role for OPDA needs to be reevaluated in the light of the data presented here indicating that *opr3* can accumulate JAs upon *B. cinerea* infection (Fig. 3C). Our discovery that *opr3* is a not a null mutant, capable of generating mature full-length *OPR3* transcripts (Fig. 4) and accumulating JA (Fig. 3C) indicates that JA and its derived metabolites are likely necessary for necrotrophic fungal defense.

Furthermore, because *opr3* accumulates OPDA but is sensitive to cabbage loopers, we conclude that OPDA is not sufficient for defense against this insect and that JAs are also likely the primary metabolites involved in looper resistance (Fig. 2). Similarly, tomato (*Solanum lycopersicum*) plants able to produce OPDA but not JAs due to a mutation in *ACX1A*, essential for β-oxidation steps of JA biosynthesis, are susceptible to *Manduca sexta* (Li et al., 2005). Together, these data, in addition to recently published reports (Kang et al., 2006), support the conclusion that JAs are required for plant defense against chewing lepidopterans.

Our data also present a strong correlation between *B. cinerea* resistance and levels of JAs. Wild type is most resistant to *B. cinerea* infection with the highest accumulation of JAs, whereas *opr3* is moderately resistant and accumulates moderate levels of JAs. *aos* is highly susceptible to the fungus and generates no JAs (Fig. 3, A–D). Furthermore, transgenic plants that overexpress *OPR3* accumulate more JAs than wild type and exhibit

greater fungal resistance (Fig. 6). OPDA levels, in contrast, are higher in *opr3* mutants and lower in *OPR3-OE* as compared to wild type, inversely correlated with relative fungal resistance (Figs. 3 and 6). In contrast to our observation, a previous report showed a decrease in the levels of OPDA in wounded *opr3* leaves (Stintzi et al., 2001); such variation might be attributed to differences in growing conditions. Overall, our data strongly support the conclusion that OPDA is not directly sufficient for plant fungal defense. There are other reports that suggest a role for OPDA in the expression regulation of stress-responsive genes (Taki et al., 2005; Ribot et al., 2008) and inhibition of Arabidopsis seed germination (Dave et al., 2011). Convincing evidence to support a role of OPDA as a direct signal in plant defense will require the identification and characterization of a true null *OPR3* mutant incapable of converting OPDA to JA.

The strong correlation between levels of JAs and resistance suggests that JAs may be the physiologically functional signals for resistance. Indeed, *B. cinerea*-induced lesions on *aos* leaves were reduced in diameter by treatment with exogenous JA (Fig. 5, A and B). This increased JA-induced resistance was independent of OPDA, confirming the central role for JAs in mediating fungal defense.

Recently, Schilmiller et al. (2007) reported that JA-deficient Arabidopsis *acx1/5* mutants, defective in genes involved in β-oxidation steps of JA biosynthesis, accumulate JA upon fungal infection and are resistant to *A. brassicicola.* These mutants fail to produce JA in response to looper infestation or mechanical wounding. One possible conclusion was that the observed JA accumulation in fungal-treated *acx1/5* leaves might be from JA or JA precursor production by the fungus. Although this might also be a possible explanation for the results presented here, we believe it is unlikely because *aos* remains highly susceptible to *B. cinerea* infection (Fig. 3A) and JAs are undetectable in *B. cinerea*-infected *aos* (Fig. 3C). The demonstration that *OPR3* transcripts are generated in *opr3* is strong evidence that JAs are most likely produced by the plant; we therefore conclude that the observed increased resistance of *opr3* is most likely due to its ability to produce JAs.

OPR3 expression is progressively increased in wild-type plants that are wounded, looper infested, or fungal infected, respectively. However, only fungal-infected *opr3* plants accumulated detectable levels of intact full-length *OPR3* transcripts (Fig. 4A). We propose that proper splicing of the *OPR3* RNA in *opr3* is inefficient due to the presence of the 17-kb T-DNA insert, with only less than 4% of primary transcript being successfully spliced upon *B. cinerea* infection as compared to wild type (Fig. 4). Thus, we hypothesize that only under certain conditions, such as *B. cinerea* infection, when *OPR3* primary transcripts accumulate to very high levels does such inefficient splicing produce detectable properly processed mRNA. An alternative possibility is that fungal infection somehow promotes productive *OPR3* transcript splicing. The second *OPR3* intron also contains a nonautonomous transposable element, Tnat1, which could also potentially play a role in intron removal. Verification of the mechanism by which full-length mature *OPR3* transcripts are generated upon specific stimuli is a focus of our current studies. The unexpected finding that intronic insertions may be removed, albeit at low efficiency, to generate functional transcripts should promote caution among researchers working with insertion mutants, especially those mutants with insertions within introns. Perhaps even very large insertions can be tolerated, and the mutated gene can thereby retain at least partial function.

MATERIALS AND METHODS

Plant Growth Conditions, and Generation and Molecular Characterization of Transgenic and Mutant Lines

Transgenic and mutant Arabidopsis (*Arabidopsis thaliana*) plants were grown as previously described (Chehab et al., 2006). Experiments were performed with 5-week-old plants. *aos* seeds (CS6149) were purchased from the Arabidopsis Biological Resource Center (Columbus, OH). PCR analyses confirmed the presence of the T-DNA insertion within *AtAOS*, as previously described (Park et al., 2002). To generate *OPR3-OE* and *Line X*, the *OPR3* cDNA clone, purchased from the Arabidopsis Biological Resource Center, was amplified by PCR with oligonucleotides designed for Gateway cloning. Primers used for amplification were: forward: 5′-CACATGACGGCGGCA-CAAGG-3′ and reverse: 5′-TCAGAGGCGGGAAAAAGGA-3′. PCR amplification was conducted in 25 μL of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μM of each dNTP, 500 nM each of forward and reverse primer, 0.625 units of *Taq* DNA polymerase, and 50 ng of DNA template. Amplification was conducted at 94°C for 1 min, 94°C for 30 s, 54°C for 1 min, 72°C for 90 s, with a 10-min extension step at 72°C. The amplified product was cloned into the pENTR/D-TOPO vector and subcloned into the Gateway destination vector pB2GW7.0 (Karimi et al., 2002). The plasmid was sequenced and transformed into the EHA101 *Agrobacterium* strain. Col-0 transformation was performed by the floral-dip method (Clough and Bent, 1998). T1 transgenics were selected with 1:1,000 finale (equivalent to 5.78% glufosinate ammonium) twice a week starting at 10 to 12 d of age. Single-insert transgenic lines were propagated to generate the homozygous seed stocks used in this report. The *Line Y OPR3* overexpression line was a generous gift from Dr. Neil Bruce (Beynon et al., 2009). To introgress the *opr3* mutation into the *gl-1* (Col-0) background of the *aos* mutant, *opr3* (Ws) stamens were used to pollinate *gl-1*. Pollen from F1 seedlings, verified by kanamycin resistance, was used to fertilize *gl-1* eight times reiteratively after which the resultant F1 plants were allowed to self fertilize to obtain the conditionally male sterile *opr3*. Verification of the presence of the T-DNA insertion in the *OPR3* locus was performed by PCR and sequencing.

Genomic DNA Isolation and Genomic DNA PCR

Leaves from individual Arabidopsis plants were ground in liquid nitrogen, and the genomic DNA was isolated using the CTAB method for amplification by PCR. The genomic DNA (*g. OPR3*) flanking the T-DNA insert was amplified by PCR using TaKaRa Ex *Taq* DNA polymerase and the following primers (forward primer 5′-ACGGACCACTCCCGGCGGTTTTC-3′ and reverse primer 5′-CGTGAACTGCTTCCACAACTT-3′) according to manufacturer's instructions. The interactive PCR round was performed exactly as in the primary PCR reaction but using 2 μL from the 20 μL primary PCR reaction as template. The presence of the T-DNA insertion was confirmed using the following primers (forward primer 5′-AGTGACTGGCGATGCTGTC-3′ and reverse primer 5′-GGCGGCTGATACACCATC-3′). The annealing temperature for the primer pairs was 56°C. The PCR profile was 30 cycles, each consisting of 94°C for 2 min, annealing temperature for 1 min, and 72°C for 1 min; for the final step, the temperature was held at 72°C for 10 min.

Quantitative RT-PCR

Leaves were ground in liquid nitrogen, and RNA was extracted using the Tri reagent (Molecular Research Center) according to the manufacturer's instructions. Extracted RNA was quantified using NanoDrop 100 spectrophotometer (Thermo Fisher Scientific), and 1 μg was reverse transcribed into cDNA using a poly (dT) reverse primer and Superscript III reverse transcriptase as instructed by the manufacturer (Invitrogen) after DNase treatment (Roche Diagnostics). Quantitative real-time PCR was performed with Thermocycler ABI SYBR green PCR master mix (Applied Biosystems) in an ABI PRISM 7000 (Applied Biosystems) system cycled 40 times with primers designed for *OPR1* (AT1G76680), *OPR2* (AT1G76690), *OPR3* (AT2G06050), *OPR4/5* (AT1G17990/AT1G18020), and *OPR6*(AT1G09400). Primers used for *OPR3* are: forward primer 5′-ACGGACCACTCCCGGCGGTTTTC-3′, reverse primer 5′-CGTGAACTGCTTCCACAACTT-3′. Primers used for *OPR1*, *OPR2*, *OPR4/5*, and *OPR6* are the same ones reported in Beynon et al. (2009). *TUBULIN4* primers (forward primer 5′-CTGTTTCCGTACCCTCAAGC-3′, reverse primer 5′-AGGGAAACGAAGACAGCAAG-3′) were used as a control to normalize gene expression in each sample. Quantification was conducted as previously described (Tsai et al., 2007).

Semiquantitative RT-PCR

Two microliters of cDNA produced as described above was used for semiquantitative RT-PCR performed in 25 μL of buffer containing 1.5 mM $MgCl_2$, 100 μM of each dNTP, 500 nM of each forward and reverse primer, 0.625 units of *Taq* DNA polymerase. PCR conditions used were as described above. The 5′ *OPR3* primer was specific for sequences containing the translational start codon (forward: 5′-CACATGACGGCGGCACAAGG-3′) and the 3′ OPR3 primer was specific for sequences containing the stop codon (reverse: 5′-TCAGAGGCGGGAAAAAGGA-3′). *ACTIN1*-specific primers (forward primer: 5′-GATCCTAACCGAGCGTGGTTAC-3′ and reverse primer 5′-GACCTGACTCGTCATACTCTGC-3′) were used as a control. RT-PCR reactions were performed at least twice with independent RNA preparations.

B. cinerea Assays and Camalexin Measurements

B. cinerea isolate 'Grape' was obtained from the laboratory of Melanie Vivier (University of Capetown, South Africa; Denby et al., 2004). The grape (*Vitis vinifera*) isolate has previously been shown to be sensitive to JA-dependent defenses as well as camalexin (Rowe et al., 2010). Rosette leaves from 5-week-old Arabidopsis were excised and placed in 145 × 20 mm plastic petri dishes with 1% agar. Each dish contained a single genotype. Each experiment used at least four dishes per genotype, containing 10 leaves per dish. Leaves were inoculated with 4 μL droplets of 2.5×10^4 conidia/mL in half-strength-filtered grape juice and incubated at room temperature. Lesion area (cm^2) was digitally measured from images (118 pixels/cm) of infected leaves using Image J (Abramoff et al., 2004) with scale objects included in images. Camalexin was extracted from individual infected leaves and quantified as described (Kliebenstein et al., 2005).

Cabbage Looper Development Assay

Eggs of the cabbage looper (*Trichoplusia ni*) were purchased from Benzon Research, Inc. One newly hatched larva was transferred with a fine brush to a pot containing five soil-grown 5-week-old plants of the specified genotype. Each pot was confined in a screened cage. After 12 d, the fresh weights of looper larvae were individually determined. Student's *t* tests were performed to compare larvae weights.

Quantification of OPDA and Jasmonates

MeJA, JA, and 12-OPDA were extracted as described (Chehab et al., 2008). The produced methyl ester volatiles were captured on HaySep-Q (Grace Davison Discovery Sciences) columns by vapor-phase extraction as described (Engelberth et al., 2003). The trapped metabolites were then eluted with 150 μL of dichloromethane and analyzed by gas chromatography-mass spectrometry using a Hewlett and Packard 6890 series gas chromatograph coupled to an Agilent Technologies 5973 network mass selective detector operated in electronic ionization mode. One microliter of sample was injected in splitless mode at 250°C and separated using an Restek Rtx-35ms column (30 m × 0.25 mm × 0.1 mm) held at 40°C for 1 min after injection, and then at increasing temperatures programmed to ramp at 15°C/min to 250°C (10 min), with helium as the carrier gas (constant flow rate 0.7 mL/min). Measurements were carried out in selected ion monitoring (Sellam et al., 2007) mode with retention times and M^+ mass-to-charge ratio ions as follows: JA-ME (trans 11.98 min, cis 12.28 min, 224) and 12-OPDA-ME (trans 17.82 min, cis 18.31 min, 306).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers *OPR1*, NM_202428; *OPR2*, NM_106319; *OPR3* (Ws), AF293653; *OPR3* (Col-0), NM_201702; *OPR4*, AF344314; *OPR5*, NM_179352; and *OPR6*, NM_100810.

Supplemental Data

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

Supplemental Figure S1. Phenotypic and molecular analysis of individual *opr3* mutant plants.

Supplemental Figure S2. *OPR* transcript levels.

Supplemental Figure S3. Comparisons of accumulation of JAs and OPDA in mechanically wounded Col-0 (wild type) and *OPR3* overexpression lines (*OPR3-OE*, *Line X*, *Line Y*).

Supplemental Figure S4. *OPR3* overexpression lines are resistant to *B. cinerea*.

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