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Intronic T-DNA Insertion Renders *Arabidopsis opr3* a Conditional JA Producing Mutant

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ABSTRACT

Jasmonic acid and its derived metabolites (JAs) orchestrate plant defense against insects and fungi. OPDA (12-oxo-phytodienoic acid), a JA precursor, has also been implicated in plant defense. We sought to define JAs and OPDA functions through comparative defense susceptibilities characteristics of three Arabidopsis genotypes: aos, lacking JAs and OPDA; opr3, deficient in JA production but can accumulate OPDA; and transgenics that overexpress OPR3. opr3, like aos, is susceptible to cabbage loopers but, relative to aos, opr3 has enhanced resistance to a necrotrophic fungus. GC-MS 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 (i) reinforce the primary role JAs play in plant defense against insects and necrotrophic fungi, (ii) argue for a reassessment of signaling activities ascribed to OPDA, and (iii) provide evidence that mutants with intron insertions can retain gene function.
INTRODUCTION

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 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-Isoleucine (JA-Ile) (Chen et al., 2005; Chehab et al., 2008; Rowe et al., 2010; Staswick and Tiryaki, 2004; Suza and Staswick, 2008). 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), mechano-transduction (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 (13-HPOT). The latter is further dehydrated by allene oxide synthase (AOS) and cyclized into (9S, 13S)-12-oxo-phytodienoic acid (OPDA) by allene oxide cyclase. OPDA reductase 3 (OPR3) reduces OPDA to 3-oxo-2(2’[Z]-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0) which 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 insensitive 1 (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 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, *Bradyvia 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 a yet unidentified explanation for the resistance of *opr3* to invaders.

With the aim towards 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 *Botrytis 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 *opr3* 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 Col-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. Towards this goal, we
employed two mutants: *aos*, which lacks both OPDA and JA by loss of conversion of 13-HPOT to 12,13-EOT (12,13-epoxyoctadecatrienoic acid), and *opr3*, reported to produce OPDA but is deficient in JA production due to a failure to convert OPDA to OPC-8:0 (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 Ws-background, 8 times with the genetic background of the *aos* mutant, *gl-1* (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 (Figure 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*) (Figure 1B). In contrast, *opr3* and *gl-1* lines accumulated statistically similar levels of OPDA before and after wounding, whereas *aos* had no detectable OPDA (Figure 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* accumulate JAs to less than 4% the levels observed in wild type. Differences between our observation and that reported by Stintzi et al. (2001) might be due to differences in growth conditions. Growth conditions may also account for the lower levels we observe for JAs and OPDA in wounded *gl-1* plants compared to some previously published reports.

**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* (Figure 2A). No statistical differences in looper weights were found between those reared on *aos* or *opr3* (Figure 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 days after looper release (Figure 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.

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 (Figures 2C and 2D). gl-1 plants produced inducible levels of JAs upon looper infestation, whereas opr3 and aos had no detectable JAs (Figure 2C). gl-1 and opr3 accumulated OPDA, but aos had none (Figure 2D). These results confirm that OPDA is not sufficient for cabbage looper resistance and that the JA-related metabolites produced upon looper infestation (Figure 2) are comparable to those produced after mechanical wounding (Figure 1). Together these data indicate that OPR3 function and the ability to accumulate JAs are necessary for plant 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 hours post inoculation (hpi) but are 30% larger for aos (Figure 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 (Figure 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 (Figure 3B). In contrast, aos lacks detectable camalexin (Figure 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* (Figures 3C and 3D). *opr3* also had higher levels of OPDA than *gl-1* (Figure 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 (Figure 3C). Thus, *opr3* can accumulate substantial amounts of JAs at least under specific conditions. This finding indicates that *opr3* phenotypes, such as 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* which accumulates higher levels of JAs and camalexin and superior *B. cinerea* resistance (Figures 3A, B and 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* (Figure 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 RT-PCR, using primers targeted to sequences including the start and stop translational codons, in untreated plants and increased approximately two-fold 2 hrs after wounding and 72 hrs post infestation with cabbage loopers, and 8-fold 72 hpi with *B. cinerea* (Figures 4A, left panel). In contrast, *opr3* lacked detectable full-length *OPR3* transcripts when subjected to wounding or cabbage loopers (Figures 4A, right panel). However, full-length *OPR3* transcripts were detected in *opr3* subjected to fungal infection (Figures 4A, right panel). 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 (Figure 4B and 4C). Note that *aos* lacks detectable *OPR3* transcripts under all treatment
conditions (Figure 4B and 4C), 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 (Figure 4C). Furthermore, OPR3 transcript abundance in infected opr3 (Figure 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 (Figure 3A and 3C). 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 (i) the expected male-sterile phenotype, (ii) an absence of detectable wild-type OPR3 gene, (iii) the presence of the mutant allele, and (iv) detectable accumulation of full-length OPR3 transcripts by RT-PCR. (Figure S1). Furthermore, JAs were detected in extracts from the pooled infected leaves from these three plants (Figure 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 5 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 (Figure S2). Although it is unlikely that reductases other than OPR3 convert OPDA to OPC:8 (Breithaupt et al., 2001; Malone et al., 2005; Breithaupt et al., 2006; Hall et al., 2007; Hall et al., 2008; Beynon, et al., 2009; Schaller and Stintzi, 2009), whether OPR2 or other OPRs 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 (Figure 2C and 2D; 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 (Figures 5A and 5B). 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 (Figures 5A and 5B). *aos* leaves sprayed with 0.5 mM JA had a mean lesion diameter not significantly different than that measured on *gl-1* (Figures 5A and 5B). 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* (Figure 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 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 (Supplementary Figures S2A and S2B). The representative line, which we denote here as *OPR3-OE*, had ~35% higher basal and wound-induced JAs and ~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 (Figures 6B and 6C). At 72 hpi, *OPR3-OE* lesions were 25% smaller in diameter than those of Col-0 (Figure 6A). Independent *OPR3* overexpressing lines behaved similarly to *OPR3-OE* (Supplementary Figure 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.

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 re-evaluated in the light of the data presented here indicating that opr3 can accumulate JAs upon B. cinerea infection (Figure 3C). Our discovery that opr3 is a not a null mutant, capable of generating mature full-length OPR3 transcripts (Figure 4) and accumulating JA (Figure 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 (Figure 2). Similarly, tomato plants able to produce OPDA but not JAs due to a mutation in ACXIA, essential for beta-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 (Figures 3A, C and D). Furthermore, transgenic plants that overexpress OPR3 accumulate more JAs than wild type and exhibit greater fungal resistance (Figure 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 (Figures 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 (Figures 5A and 5B). 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 beta-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 (Figure 3A) and JAs are undetectable in B. cinerea-infected aos (Figure 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 (Figure 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 (Figure 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 non-autonomous 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.

**METHODS**

**Plant Growth Conditions, and Generation and Molecular Characterization of Transgenic and Mutant Lines.** Transgenic and mutant 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 (ABRC, 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 ABRC, was amplified by PCR with oligonucleotides designed for Gateway cloning. Primers used for amplification were: Forward: 5’-CACATGACGCGGCACAAGG-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 MgCl₂, 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 by a LR reaction (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-days 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 8 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’-ACGGACCACACTCCCGCCGTTTTC-3’ and reverse primer 5’-CGTGAACTGCTTCCACAACTT-3’) according to manufacturer’s instructions. The interative 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 Reverse-Transcription PCR.** Leaves were ground in liquid nitrogen, and RNA was extracted using the Tri reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. Extracted RNA was quantified using NanoDrop 100 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), 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, Carlsbad, CA) after DNase treatment (Roche Diagnostics, Basel, Switzerland). Quantitative real-time PCR was performed with Thermocycler ABI SYBR Green PCR master mix (Applied Biosystems, Carlsbad, CA) 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’-ACGGACCACCTCCGCGCGTTTTC-3’, reverse primer 5’-CGTGAACTGCTTCCACAACCTTT-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’-CTGTTTCCGTACCCCTCAAGC-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).

**Semi-Quantitative Reverse Transcription PCR.** Two µL of cDNA produced as described above was used for semi-quantitative RT-PCR performed in 25 µL of buffer containing 1.5 mM MgCl₂, 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’-CACATGACGGCGCGACAAGG-3’) and the 3’ OPR3 primer was specific for sequences containing the stop codon (Reverse: 5’-TCAGAGGCGGGAAAAGGA-3’). ACTIN1-specific primers (Forward primer: 5’-GATCCTAAACCGAGCGTGGTAC-3’ and Reverse primer 5’-GACCTGACTGTCATCTACTGC-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 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 x 20 mm plastic Petri dishes with 1% agar. Each dish contained a single genotype. Each experiment used at least 4 dishes per genotype, containing 10 leaves per dish. Leaves were inoculated with 4 µL droplets of 2.5x10⁴...
conidia/mL in half-strength filtered grape juice and incubated at room temperature. Lesion area (cm²) 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 (*T. ni*) were purchased from Benzon Research, Inc. (Easton, PA). 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 days, the fresh weights of looper larvae were individually determined. Students t-tests were performed to compare larval 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, Deerfield, IL) 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 GC-MS using a Hewlett and Packard 6890 series gas chromatograph coupled to an Agilent Technologies 5973 network mass selective detector operated in electronic ionization (EI) mode. One µL of sample was injected in splitless mode at 250°C and separated using an Restek Rtx-35ms column (30 m x 0.25 mm x 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⁺ m/z 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).

**Supplemental Data**

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

Supplementary Figure S2. OPR transcript levels
Supplementary Figure S3. Comparisons of accumulation of JAs and OPDA in mechanically wounded Col-0 (WT) and OPR3-overexpression lines (OPR3-OE, Line X, Line Y).

Supplementary Figure S4. OPR3 overexpression lines are resistant to B. cinerea.

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REFERENCES


**FIGURE LEGENDS**

**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 hours after mechanical
damage (W). C, Levels of OPDA in untreated leaves (CR) and wounded leaves 2 hours after mechanical damage (W). Means ± standard deviations (SD) are shown. n =3.

**Figure 2.** *opr3* has increased susceptibility to cabbage loopers (*Trichoplusia ni*). A, Looper weights 12 days after release of a newly hatched larva in an arena containing 5 plants of indicated genotype. Means ± standard error of the mean (SEM) of two independent experiments, each with ~15 arenas per genotype, are shown. B, Represented photos of *gl-1, opr3* and *aos* showing tissue damage. C, Levels of JAs in control (CR) and looper-infested (L) leaves 72 hour post infestation. Means ± standard deviations (SD) are shown. n =3. D, Levels of OPDA in control (CR) and looper-infested (L) leaves 72 hour post infestation. Means ± standard deviations (SD) are shown. n =3.

**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 hrs post inoculation (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).

**Figure 4.** *OPR3* transcript levels. A, Semi-quantitative 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 hours (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 hours (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.

**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).

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.

Supplementary Figure S1. Phenotypic and molecular analysis of individual opr3 mutant plants. A, Inflorescences from three opr3 and gl-1 plants whose rosette leaves were used for B. cinerea infection, oxylipin measurements, genotyping and semi-quantitative RT-PCR. Note that each opr3 inflorescence fails to fill siliques. Number corresponding to each plant is shown above each inflorescence. B, Genotyping individual plants showing primary and iterative PCR rounds (first and second lanes, respectively) for each opr3 plant and a single primary PCR reaction (single lane) for each gl-1 control. All three opr3 plant DNAs fail to amplify a genomic OPR3 region (g. OPR3) spanning the T-DNA insert site but show T-DNA insertion site specific bands. C, Semi-quantitative RT-PCR of full-length OPR3 and ACTIN transcripts from each individual opr3 and gl-1 plant 72 hpi with B. cinerea. D, Levels of leaf JAs from the three opr3 and gl-1 plant samples at 72 hpi with B. cinerea. Mean = ±SD n=3. E, Levels of leaf OPDA from the three opr3 and gl-1 samples at 72 hpi with B. cinerea. Mean = ±SD n=3.

Supplementary Figure S2. OPR transcript levels. Quantitative RT-PCR of OPR1, OPR2, OPR4/5 and OPR6 transcripts in gl-1 leaves 72 hpi with B. cinerea conidia. Transcript levels were normalized to TUB4 transcripts. Means ± SEM. n = 3.

Supplementary Figure S3. Comparisons of accumulation of JAs and OPDA in mechanically wounded Col-0 (WT) and OPR3-overexpression lines (OPR3-OE, Line X, Line Y). A, Levels of JAs in untreated (CR) WT, OPR3-OE, Line X and Line Y leaves 2 hours after mechanical
damage. B, OPDA levels in untreated (CR) WT, OPR3-OE, Line X and Line Y leaves 2 hours after mechanical damage. Means ± SD. n = 3.

**Supplementary Figure S4.** OPR3 overexpression lines are resistant to *B. cinerea*. A, Leaf lesion diameters of Line X in comparison to Col-0 (WT) 48 hpi and 72 hpi with fungal conidia. B, Leaf lesion diameters of Line Y in comparison to Col-0 (WT) 48 hpi and 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.
Figure 1. JA rescue of male fertility and accumulation of JA and OPDA in mechanically wounded Arabidopsis. (A) Male fertility is restored in aos and opr3 by the exogenous application of JA. (B) Levels of JA (MeJA & JA) and (C) OPDA in untreated leaves (CR) and wounded leaves 2 hours after mechanical damage (W). The means ± standard deviations (SD) of three independent biological replicates are shown.
Figure 2. opr3 has increased susceptibility to cabbage looper (Trichoplusia ni) attack. (A) Mean weight of loopers 12 days after release of a newly hatched larva in an arena containing five plants of indicated genotype. Means ± SE of two independent experiments, with ~15 arenas per genotype, are shown. (B) Extent of damage observed on representative gl-1, opr3 and aos plants. (C) JA and (D) OPDA levels determined in control (CR) and looper-infested (L) leaves.
Figure 3. opr3 mutant plants have increased resistance to B. cinerea as compared to aos and accumulate camalexin and JA. (A) Leaf lesion diameter was measured for aos, opr3, and gl-1 48 and 72 hours post inoculation (hpi) with fungal conidia. Mean lesion diameter ± SD of 40 inoculated leaves is shown. Representative photographs of each genotype 72 hpi are shown. Bar = 1 cm. (B) Leaf camalexin at 72 hpi. Means ± SD of 30 replicates for each genotype are shown. Within any given treatment, bars with different letters indicate significant differences (P<0.005, Tukey’s test). (C) Levels of JA and (D) OPDA in leaves 72 hpi with fungal conidia.
Figure 4. OPR3 transcript levels. A, Semi-quantitative 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 hours (L), or B. cinerea infected 72 hpi (B). 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 hours (L), or B. cinerea infected 72 hpi (B). Means ± SD. n = 6. C, Quantitative RT-PCR analysis of OPR3 transcript levels relative to TUB4 transcripts in gl-1, opr3 and aos at 48 and 72 hpi with B. cinerea. Means ± SD. n = 6.
Figure 5. JAs are required for defense against B. cinerea. (A) Leaf lesion diameter of Col-0 and aos sprayed with indicated JA concentrations at 72 hpi with fungal conidia. Mean lesion diameters ± SD of 30 inoculated leaves are shown. (B) Representative plants 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 72 (JA) hpi with the fungal conidia. Letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences at (p<0.05, Tukey’s test).
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