Intronic T-DNA Insertion Renders Arabidopsis opr3 a Conditional Jasmonic Acid-Producing Mutant

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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; opr3 reductase3 (opr3), deficient in JA production but can accumulate OPDA; and transgenics that overexpress OPRA. 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; McCann 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; McCann 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 (9S, 13S)-12-oxo-phytodienoic acid (OPDA) by allene oxide cyclase. OPDA REDUCTASE3 (OPR3) reduces OPDA to 3-oxo-2(2’-[2]-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 (McCann 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 coil, 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, *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-harbouring 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 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(\(\beta\)-[2\(\beta\)]-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](https://plantphysiol.org)

**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 ± so are shown. \(n = 3\). [See online article for color version of this figure.]
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; Kliewenstein 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 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.

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. 2C 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.

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.]

op3 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 plant defense against 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
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 3](https://plantphysiol.org)  
**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).

![Figure 4](https://plantphysiol.org)  
**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 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. OP3R 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, OP3R 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 sterile phenotype, (2) an absence of detectable wild-type OP3R gene, (3) the presence of the mutant allele, and (4) detectable accumulation of full-length OP3R 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 OP3R 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 OP3R 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 OP2 show an increase in transcript level in response to infection (Supplemental Fig. S2). Although it is unlikely that reductases other than OP3R 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 OP2 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 (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
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, indicating that the exogenous application of JA is sufficient to elicit defense against this fungus and generates no JAs (Fig. 3, C).

**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, 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 actx1/5 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 actx1/5 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 AIAOS, 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′-CACCAGGCCCCGGA-CACAAGG-3′ and reverse: 5′-TCAGAGGCGGGAAAAAGGA-3′. PCR amplification was conducted in 25 μL of 10× 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 (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% glutosinate 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. 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′-ACCGACCACTCCGGCCGCAGTTTC-3′ and reverse primer 5′-CTGTGAATCCTGTCACCAATCTT-3′) according to manufacturer’s instructions. The interactive PCR round was performed exactly as in the primary PCR reaction by 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′-AGCTGATGCAGCAGCAGACC-3′ and reverse primer 5′-GCGGGCTGATACACCATC-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 the 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 transcription 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. PCR was cycled 40 times with primers designed for OPRI (AT1G76680), OPRI2 (AT1G76690), OPRI3 (AT2G06050), OPRI4 (AT1G7990) AT1G18020, and OPRI6 (AT1G09400). Primers used for OPRI3 and OPRI4 were therefore primer 5'-ACGGACACTCTCCCGCCTTTC-3', reverse primer 5'-CTGTAACCTGTCCCCAAAATCT-3'. Primers used for OPRI, OPRI2, OPRI4, and OPRI6 are the same ones reported in Beynon et al. (2009). TUBULIN4 primers (forward primer 5'-CTGTCCCCGTACACCGAGACG-3', reverse primer 5'-AAGGAAACCGAAGACAGCAAG-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 RT-PCR

Two microliters of cDNA produced as described above were used for semi-quantitative RT-PCR performed in 25 μL of buffer containing 1.5 mM MgCl2, 100 μM of each dNTP, 500 μM 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 translation start codon (forward: 5'-CACATGACCGGCGGACAAGG-3') and the 3'-OPR3 primer was specific for sequences containing the stop codon (reverse: 5'-TCAGACGGCCGAAAAGCCA-3'). ACTIN-specific primers (forward primer: 5'-GATCTAACCCAGGGTGTTAC-3', reverse primer 5'-GATCTAGCTGCTACATCTG-3') were used as a control. 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 Cape Town, 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 15% 20 mm plastic petri dishes with 1% agar. Each dish contained a single genotype. Each experiment was used at least four dishes per genotype, containing 10 leaves per dish. Leaves were inoculated with 4 μL droplets of 2.5% 106 conidia/mL in half-strength-filtered grape juice and incubated at room temperature. Lesion area (cm²) was digitally measured from images (118 pixels/cm²) 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. 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 larva were individually determined. Student’s t-tests were performed to compare larva 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 x 0.25 mm x 0.1 μm) 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 ratios ions as follows: JA-ME (trans 11.98 min, cis 12.28 min, 224) and 12-OPDA-ME (trans 17.82 min, 306).

Sequence data from this article can be found in the GenBank/EMBL database libraries under accession numbers OPRI, NM_202428; OPRI2, NM_106319; OPRI3 (Ws), AF295663; OPRI4 (Col-0), NM_201702; OPRI4, AF344314; OPRI5, NM_179352; and OPRI6, NM_108810.

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. OPRI transcript levels.

Supplemental Figure S3. Comparisons of accumulation of JAIs and OPDA in mechanically wounded Col-0 (wild type) and OPRI overexpression lines (OPRI3-oe, Line X, Line Y).

Supplemental Figure S4. OPRI overexpression lines are resistant to B. cinerea.

ACKNOWLEDGMENTS

We thank Seichi Matsuda for gas chromatography-mass spectrometry use, Neil Bruce for providing the OPRI overexpression line Y, and John Browse for providing the opr3 seeds. We are grateful to members of the Bram Lab for scientific discussions and critical review of the manuscript.

Received February 14, 2011; accepted April 8, 2011; published April 12, 2011.

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