Jasmonic Acid and Its Precursor 12-Oxophytodienoic Acid Control Different Aspects of Constitutive and Induced Herbivore Defenses in Tomato[1][W][OPEN]

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The jasmonate family of growth regulators includes the isoleucine (Ile) conjugate of jasmonic acid (JA-Ile) and its biosynthetic precursor 12-oxophytodienoic acid (OPDA) as signaling molecules. To assess the relative contribution of JA/JA-Ile and OPDA to insect resistance in tomato (*Solanum lycopersicum*), we silenced the expression of OPDA reductase3 (OPR3) by RNA interference (RNAi). Consistent with a block in the biosynthetic pathway downstream of OPDA, *OPR3-RNAi* plants contained wild-type levels of OPDA but failed to accumulate JA or JA-Ile after wounding. JA/JA-Ile deficiency in *OPR3-RNAi* plants resulted in reduced trichome formation and impaired monoterpene and sesquiterpene production. The loss of these JA/JA-Ile -dependent defense traits rendered them more attractive to the specialist herbivore *Manduca sexta* with respect to feeding and oviposition. Oviposition preference resulted from reduced levels of repellant monoterpenes and sesquiterpenes. Feeding preference, on the other hand, was caused by increased production of cis-3-hexenal acting as a feeding stimulant for *M. sexta* larvae in *OPR3-RNAi* plants. Despite impaired constitutive defenses and increased palatability of *OPR3-RNAi* leaves, larval development was indistinguishable on *OPR3-RNAi* and wild-type plants, and was much delayed compared with development on the *jasmonic acid-insensitive1* (*jai1*) mutant. Apparently, signaling through JAI1, the tomato ortholog of the ubiquitin ligase CORONATINE INSENSITIVE1 in Arabidopsis (*Arabidopsis thaliana*), is required for defense, whereas the conversion of OPDA to JA/JA-Ile is not. Comparing the signaling activities of OPDA and JA/JA-Ile, we found that OPDA can substitute for JA/JA-Ile in the local induction of defense gene expression, but the production of JA/JA-Ile is required for a systemic response.

Oxylipins comprise a vast array of bioactive metabolites that are generated from membrane lipids as a result of lipid peroxidation (Mosblech et al., 2009; Farmer and Mueller, 2013). Among them are jasmonates, a family of signaling molecules that act as phytohormones in the regulation of developmental processes and stress responses in plants, including tuber and trichome formation, leaf senescence, reproductive development, secondary metabolism, mechanotransduction, symbiotic interactions, plant responses to wounding, and defenses against insects and pathogens (Koo and Howe, 2009; De Geyter et al., 2012; Wasternack and Hause, 2013). Jasmonates are synthesized via the octadecanoid pathway (reviewed by Schaller and Stintzi, 2008, 2009; Wasternack and Kombrink, 2010) from polyunsaturated fatty acids that are liberated by lipases from galactolipids in plastid membranes. Catalyzed by 13-lipoxygenases, molecular oxygen is introduced to yield their 13-hydroperoxy derivatives. These fatty acid hydroperoxides are converted to 12-oxophytodienoic acids (OPDAs; including linolenic acid [18:3]-derived OPDA and dinor [dn]-OPDA derived from 16:3) by allene oxide synthase (AOS) and allene oxide cyclase, which concludes the plastid-localized part of the pathway. The cyclopentenone ring of OPDA and dn-OPDA is subsequently reduced to the corresponding cyclopentanone in peroxisomes. The reaction is catalyzed by OPDA reductase3 (OPR3), which is the only peroxisomal member of a small family of related enone reductases and is the only one accepting the biologically relevant stereoisomer of dn-OPDA or OPDA as a substrate (Schaller et al., 2000; Stintzi and Browse, 2000; Strassner et al., 2002; Breithaupt et al., 2006, 2009). After reduction by OPR3, the alkanoic acid side chain of the resulting cyclopentanones is shortened by β-oxidation to yield (+)-7-iso-jasmonic acid (JA), which epimerizes to the more stable trans isomer (−)-JA (Wasternack and Hause, 2013).

Until recently, JA has been viewed as the end product of the pathway and as the bioactive hormone. It becomes increasingly clear, however, that biological activity extends to and may even differ between the various JA metabolites and conjugates, and the question as to which of the jasmonate family members are responsible for any one of the different physiological responses remains an urgent question in the field. With

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respect to biological activity, the isoleucine (Ile) conjugate of JA (JA-Ile) is the best characterized family member, and it is also the only jasmonate for which the molecular basis of its gene-regulatory activity has been elucidated. The conjugation of JA to Ile is catalyzed by the amino acid conjugate synthetase JASMONATE RESISTANT1 (JAR1) via an activated acyl-adenylate intermediate in an ATP-dependent reaction (Staswick et al., 2002; Staswick and Tiryaki, 2004; Westfall et al., 2012). JA-Ile, more precisely, the (+)-7-iso-JA-L-Ile isomer retaining cis configuration at C3 and C7 of the cyclopentanone ring as established by allene oxide cyclase and OPR3, is perceived by CORONATINE INSENSITIVE1 (COI1) and JASMONATE ZIM DOMAIN (JAZ) in a coreceptor complex (Chini et al., 2007; Thines et al., 2007; Fonseca et al., 2009; Yan et al., 2009; Sheard et al., 2010). After binding of JA-Ile, COI1 mediates the ubiquitin-dependent degradation of JAZ repressors resulting in the activation of JA-dependent gene expression (Browse, 2009; Pauwels and Goossens, 2011; Kazan and Manners, 2012).

The phenotype of the *jar1* mutant indicates that JAR1 activity and conjugation of JA to Ile is required for some but not all jasmonate-regulated processes. Processes that depend on JAR1 include JA-induced root growth inhibition (Staswick et al., 1992), resistance against certain pathogens (Staswick et al., 1998; van Loon et al., 1998; Ryu et al., 2004), and protection against ozone damage (Overmyer et al., 2000; Rao et al., 2000), whereas JA-induced cell cycle arrest (Zhang and Turner, 2008; Noir et al., 2013), anthocyanin accumulation (Loreti et al., 2008; Chen et al., 2007), and some wound responses in Arabidopsis (*Arabidopsis thaliana*; Suza and Staswick, 2008; Zhang and Turner, 2008) are JAR1 independent. Bioactive jasmonates other than JA-Ile must therefore exist. Similarly in coyote tobacco (*Nicotiana attenuata*), silencing of JAR4 and JAR6, the two functionally redundant homologs of JAR1 in Arabidopsis, identified JA-Ile as one but not the only jasmonate signal for induced resistance against herbivores (Kang et al., 2006; Wang et al., 2008).

Signaling activity has in fact been demonstrated for other members of the jasmonate family. 12-hydroxyjasmonate (12-OH-JA, tuberonic acid) and its *O*-glucoside have long been implicated in potato (*Solanum tuberosum*) tuber formation (Yoshihara et al., 1989), and the latter is also responsible for nyctinastic leaf movements in *Albizzia* (Nakamura et al., 2011). cis-Jasmone acts as a signal for the induction of indirect defense and COI1-independent gene expression (Matthes et al., 2010). In addition, OPDA has also been identified as a signaling molecule that differs in activity from JA/JA-Ile (Stintzi et al., 2001; Dave and Graham, 2012; Park et al., 2013). OPDA is much more active than JA/JA-Ile in mechanotransduction (Stelmach et al., 1998; Blechert et al., 1999; Escalante-Pérez et al., 2011) and it sustains resistance of Arabidopsis plants against herbivores and pathogens in absence of JA/JA-Ile (Stintzi et al., 2001; Zhang and Turner, 2008; Stotz et al., 2011). OPDA also elicits the synthesis of diterpenoid-derived volatiles in lima bean (*Phaseolus lunatus*) and the accumulation of phytoalexins in soybean (*Glycine max*) more efficiently than JA (Koch et al., 1999; Fliegmann et al., 2003). Seed dormancy is regulated by OPDA in Arabidopsis (Dave et al., 2011), and in tomato (*Solanum lycopersicum*) OPDA rather than JA/JA-Ile is also required for embryo development (Goetz et al., 2012). A distinct set of genes is controlled by OPDA in Arabidopsis, which shows only partial overlap with those regulated by JA/JA-Ile and includes COI1-dependent as well as COI1-independent genes (Stintzi et al., 2001; Taki et al., 2005; Mueller et al., 2008; Ribot et al., 2008; Stotz et al., 2013).

OPDA is not perceived by the COI1-JAZ coreceptor complex (Thines et al., 2007; Sheard et al., 2010), and the mechanisms underlying OPDA signaling through COI1 are not understood. COI1-independent gene activation, on the other hand, can be attributed in part to the strong electrophilicity and high reactivity of its α,β-unsaturated carbonyl group, a characteristic feature of reactive electrophiles that are known for their cytotoxic and gene-regulatory activities (Alméras et al., 2003; Taki et al., 2005; Mueller et al., 2008; Farmer and Mueller, 2013). Furthermore, the specific interaction of OPDA with the plastidic Cyclophilin20-3 (CYP20-3) was recently shown to mediate the readjustment of cellular redox homeostasis after wounding (Park et al., 2013). The contribution of CYP20-3 to OPDA signaling is further supported by the JA/OPDA/coronatine-insensitive phenotypes of the *cyp20-3* loss-of-function mutant with respect to increased resistance to *Pseudomonas syringae*, OPDA-induced root growth inhibition, and attenuated expression of OPDA-responsive genes after OPDA application (Park et al., 2013).

Although the collective data clearly support a role for OPDA as a signaling molecule in its own right, the validity of this conclusion was recently questioned (Chehab et al., 2011). In many of the studies cited above, the Arabidopsis *opr3* mutant was used as a tool to distinguish between JA/JA-Ile- and OPDA-specific signaling. *opr3* harbors a Transfer-DNA insertion within the second intron of the *OPR3* gene (Sanders et al., 2000; Stintzi and Browse, 2000). Upon *Botrytis cinerea* infection, the intron appears to be spliced, giving rise to functional *OPR3* transcripts (Chehab et al., 2011). Therefore, *opr3* does not seem to behave as a true null mutant under certain conditions, particularly pathogen infection. The authors thus argue for JA/JA-Ile as the primary defense signal, and for a reassessment of defense signaling activities ascribed to OPDA (Chehab et al., 2011).

We addressed this question by performing a loss-of-function analysis in tomato. The tomato *OPR3* gene was silenced by RNA interference (RNAi) and the resulting transgenic plants were analyzed with respect to oxylipin content, herbivore resistance, and defense signaling. We found *OPR3-RNAi* plants to be impaired in trichome formation and secondary metabolite production. The loss of these JA/JA-Ile-dependent defense traits rendered them more attractive to the specialist herbivore *Manduca sexta* with respect to feeding and oviposition. Despite impaired constitutive defenses,

larval development was indistinguishable on *OPR3-RNAi* and wild-type plants, and was much delayed compared with development on the *jasmonic acid-insensitive1* (*jai1*) mutant. Signaling through JAI1 is thus required for insect resistance, whereas JA/JA-Ile production is not. Consistent with this notion, we found OPDA to be active as a signal for defense gene induction after wounding. Systemic signaling, however, relied on JA/JA-Ile formation.

RESULTS

Silencing of *SlOPR3* Expression

To assess the relative importance of JA/JA-Ile and the jasmonate precursor OPDA for herbivore defenses in tomato, we silenced the expression of *OPR3* by RNAi. As previously shown for Arabidopsis and tomato plants that are deficient in JA/JA-Ile biosynthesis or signaling, transgenic plants expressing the OPR3 hairpin construct turned out to be sterile. However, similar to JA- and JA-Ile-deficient Arabidopsis mutants, in which fertility can be restored by application of jasmonates (McConn and Browse, 1996; Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002), we were able to obtain seeds from these plants by methyl jasmonate treatment of flower buds and squeezing of the anther cone to facilitate pollen release. T1 plants were grown from these seeds and OPR3 protein levels were analyzed on western blots. Ten independent RNAi lines in which OPR3 was undetectable were chosen for further analysis (Supplemental Fig. S1). Confirming the specificity of silencing, expression of the closest OPR3 homolog was found to be unaffected in three randomly chosen *OPR3-RNAi* lines (P3, J30, and J55; Supplemental Fig. S2).

To assess the efficiency of OPR3 silencing and the effect of OPR3 deficiency on jasmonate content, we compared the levels of jasmonates downstream of the OPR3 reaction in RNAi lines and wild-type plants. The results are shown in Figure 1A for JA, its bioactive derivative JA-Ile, and its inactivation product 12-OH-JA. In unwounded plants, 12-OH-JA levels were similar in *OPR3-RNAi* and wild-type plants, whereas JA and JA-Ile levels were significantly reduced (230 versus 370 pmol/g fresh weight and 95 versus 140 pmol/g fresh weight, respectively; Student's *t* test at $P < 0.05$ for JA and $P < 0.01$ for JA-Ile). Forty min after wounding, a strong increase was observed for JA (19-fold), JA-Ile (35-fold), and 12-OH-JA (6-fold) in wild-type plants, but not in *OPR3-RNAi* plants (Fig. 1A). Two h after wounding, there was a further increase of 12-OH-JA in wild-type plants with a concomitant decrease in JA (Fig. 1B) as a result of hormone inactivation by ω-hydroxylation (Miersch et al., 2008; Heitz et al., 2012; Koo and Howe, 2012). No such increase was observed in *OPR3-RNAi* plants (Fig. 1B). By contrast, the OPR3 substrate OPDA accumulated to similar levels 2 h after wounding in both wild-type and *OPR3-RNAi* plants (Fig. 1B). We conclude that OPR3 activity was efficiently reduced in silenced plants, resulting in a depletion of downstream products. Any residual OPR3 activity that may still be present in RNAi lines is insufficient to sustain the burst of JA/JA-Ile and the accumulation of hydroxylated JA catabolites in wounded leaves. Furthermore, the sterile phenotype of *OPR3-RNAi* plants suggests that there may also be a strong reduction of OPR3 activity and basal JA/JA-Ile levels in developing flowers.

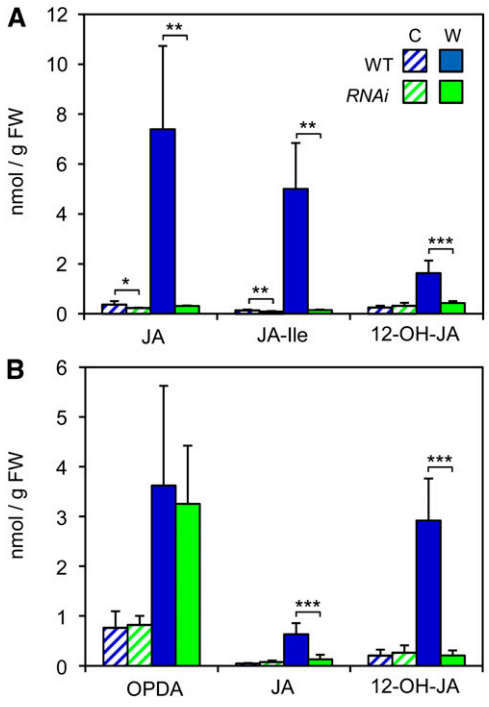

Figure 1. Jasmonate levels in wild-type and *OPR3-RNAi* plants. A, Jasmonates in wild-type (WT, dark blue) and *OPR3-RNAi* leaf tissue (*RNAi*, green) 40 min after wounding (W, filled bars) compared with unwounded controls (C, hatched bars). JA, JA-Ile, and 12-OH-JA were quantified by liquid chromatography-tandem mass spectrometry after solid-phase extraction of methanolic extracts. Jasmonate levels are given in nanomoles per gram of fresh weight (FW) as the mean ± SD of six biological replicates for wild-type plants. For *OPR3-RNAi* plants, three biological replicates were performed on each of three independent transgenic lines. B, OPDA, JA, and 12-OH-JA were quantified by GC-MS in wild-type and *OPR3-RNAi* plants 2 h after wounding (color scheme as in A). The experiment involved eight and four biological replicates on independent *RNAi* lines for wounded plants and unwounded controls, respectively. Asterisks indicate significant differences between *OPR3-RNAi* and wild-type plants (*$P <$ 0.05, **$P < 0.01$, and ***$P < 0.001$).

Herbivore Defense in *OPR3*-Silenced Tomato Plants

To investigate the relative contribution of OPDA and JA/JA-Ile to insect resistance, we performed bioassays comparing the wild-type tomato with either *OPR3*-silenced plants that are impaired in the production of JA/JA-Ile or with the *jai1-1* mutant that is unable to respond to JA-Ile (Li et al., 2004). In dual-choice tests, *M. sexta* larvae strongly preferred leaf discs of *OPR3-RNAi* and *jai1-1* mutants over the corresponding wild-type background (cvs UC82B and Castlemart, respectively; Fig. 2A). The data suggest that the mutants lack herbivore

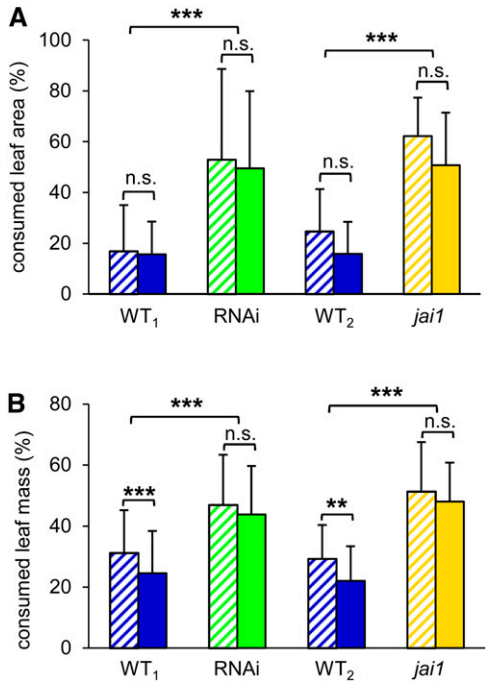

Figure 2. Bioassays assessing the relative contribution of OPDA and JA/JA-Ile to insect resistance. A, Dual-choice tests revealing a preference of *M. sexta* larvae for *OPR3-RNAi* and *jai1-1* mutants over wild-type plants. Leaf discs from either *OPR3-RNAi* (three independent transgenic lines) or *jai1-1* mutant plants and the corresponding wild-type controls (WT_1, cv UC82B; and WT_2, cv Castlemart) were offered to third-instar *M. sexta* larvae. The leaf area consumed within 4 h of feeding is indicated in percentages ± SD ($n = 33$, $P < 0.001$). B, No-choice tests assessing palatability of *OPR3-RNAi* and *jai1-1* mutants compared with the wild type. A single forth-instar *M. sexta* larva was offered 500 mg of leaf discs from either *OPR3-RNAi* plants, *jai1-1* mutants, or the respective controls. The leaf mass that was consumed within 30 min is indicated in percentages ± SD ($n = 100$ for *OPR3-RNAi* [four independent transgenic lines] and cv UC82B; $n = 55$ for *jai1-1* and cv Castlemart). In A and B, the wild type is shown in dark blue, *OPR3-RNAi* in green, and *jai1-1* in yellow. Both experiments were performed with leaf material from healthy plants (hatched bars) and from plants that were wounded 24 h before the experiment (filled bars). Asterisks indicate significant differences (Wilcoxon rank sum test; $^{**}P < 0.01$ and $^{***}P < 0.001$). n.s., Not significant.

defense traits that are only present in wild-type plants and thus depend on JA/JA-Ile biosynthesis and signaling. Alternatively, the mutants may produce compounds that stimulate feeding of *M. sexta* larvae.

Consistent with either possibility, the palatability of *OPR3-RNAi* and *jai1-1* plants was found to be higher compared with the respective wild types. In no-choice experiments, the larvae consumed about twice as much leaf disc material of the mutants deficient in JA/JA-Ile production and signaling (Fig. 2B). Interestingly, palatability of the wild type was further reduced when plants were wounded 24 h before the feeding trial (Fig. 2B). This wound-induced effect was not seen in *OPR3-RNAi* and *jai1-1* plants, suggesting that OPDA cannot substitute for JA/JA-Ile as a signal in either the wound-induced reduction of leaf palatability (Fig. 2B) or in jasmonate-dependent formation of constitutive defense traits affecting feeding preference of the specialist *M. sexta* (Fig. 2A). In addition to feeding preference, JA/JA-Ile deficiency was also found to affect host plant choice for oviposition. In dual-choice tests presenting wild-type tomato and *OPR3-RNAi* plants to ovipositing *M. sexta* females in a two-channel olfactometer, a clear preference was observed for *OPR3*-silenced plants over the wild-type control (Fig. 3).

Comparison of Constitutive Herbivore Defense Traits in *OPR3-RNAi* and Wild-Type Plants

To identify the factors that might be responsible for the observed differences in attractiveness for feeding and oviposition, we compared nutritional quality, trichome density, and trichome metabolites of *OPR3*-silenced and wild-type plants. Total nitrogen content was unaffected by JA/JA-Ile deficiency in RNAi plants (Fig. 4A). Likewise, the levels of starch, total sugars, and reducing sugars were all indistinguishable between RNAi plants and controls (Fig. 4A). Only the amount of bound carbon was slightly reduced in *OPR3*-silenced plants compared with control plants (Fig. 4A). The pronounced differences in palatability and attractiveness as a host for feeding and ovipositing *M. sexta* can thus not be explained by differences in nutritional quality.

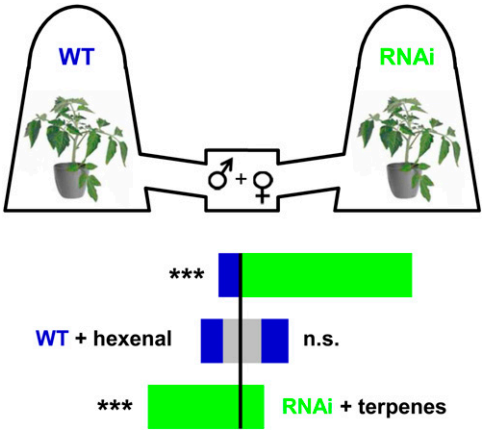

Figure 3. *M. sexta* oviposition preference for *OPR3-RNAi* plants as a result of impaired terpene production. To assess oviposition preference, tomato wild-type and *OPR3-RNAi* plants were offered to a pair of mating *M. sexta* in a two-channel olfactometer. A choice for the wild type (WT) is indicated by blue bars, a choice for *OPR3-RNAi* in green, and no choice in gray. When no further odorants were added, the preference for RNAi plants was statistically significant ($n = 35$, $\chi^2 = 48.8$, $^{***}P = 0.0001$). In a wild-type/wild-type comparison, the addition of cis-3-hexenal on one side of the olfactometer had no influence on oviposition preference ($n = 16$, $\chi^2 = 0.87$, $P = 0.64$). By contrast, the addition of a mixture of terpenes reflecting the terpene content of wild-type trichomes in the *OPR3-RNAi/OPR3-RNAi* comparison had a significant deterrent effect ($n = 21$, $\chi^2 = 22.6$, $^{***}P < 0.0001$). n.s., Not significant.

Next we looked at trichome development, which is known to be regulated by jasmonates (Li et al., 2004; Boughton et al., 2005; Yoshida et al., 2009) and is a well-established resistance factor (Kennedy, 2003; Dalin et al., 2008; Kang et al., 2010). There are glandular and non-glandular trichomes in tomato, with glandular trichomes types IV and VI being most relevant for insect resistance (Kennedy, 2003). The latter were found to be affected in *OPR3-RNAi* plants. In three independent RNAi lines, the density of type VI trichomes was reduced by about two-thirds (Fig. 4B), and their metabolite content differed from that of wild-type trichomes. Gas chromatography-mass spectrometry (GC-MS) analyses of trichome extracts detected significant differences for 10 compounds that were identified as cis-3-hexenal, five monoterpenes (α-pinene, 2-carene, limonene, and α- and β-phellandrene), three sesquiterpenes (α-humulene, δ-elemene, and β-caryophyllene), and one unknown compound (Fig. 4C; Supplemental Figs. S3 and S4; Supplemental Tables S1–S3). The differences were particularly strong for monoterpenes that were reduced by an average factor of 24 in *OPR3-RNAi* compared with wild-type trichomes, whereas sesquiterpenes showed on average a 9-fold reduction. On the other hand, cis-3-hexenal was increased 2.5-fold in *OPR3*-silenced plants over control levels (Fig. 4C).

cis-3-Hexenal and Terpenes Affect Feeding Preference and Oviposition Behavior

To address the question of whether differences in trichome density or content are responsible for the feeding preference of *M. sexta* larvae for JA/JA-Ile-deficient

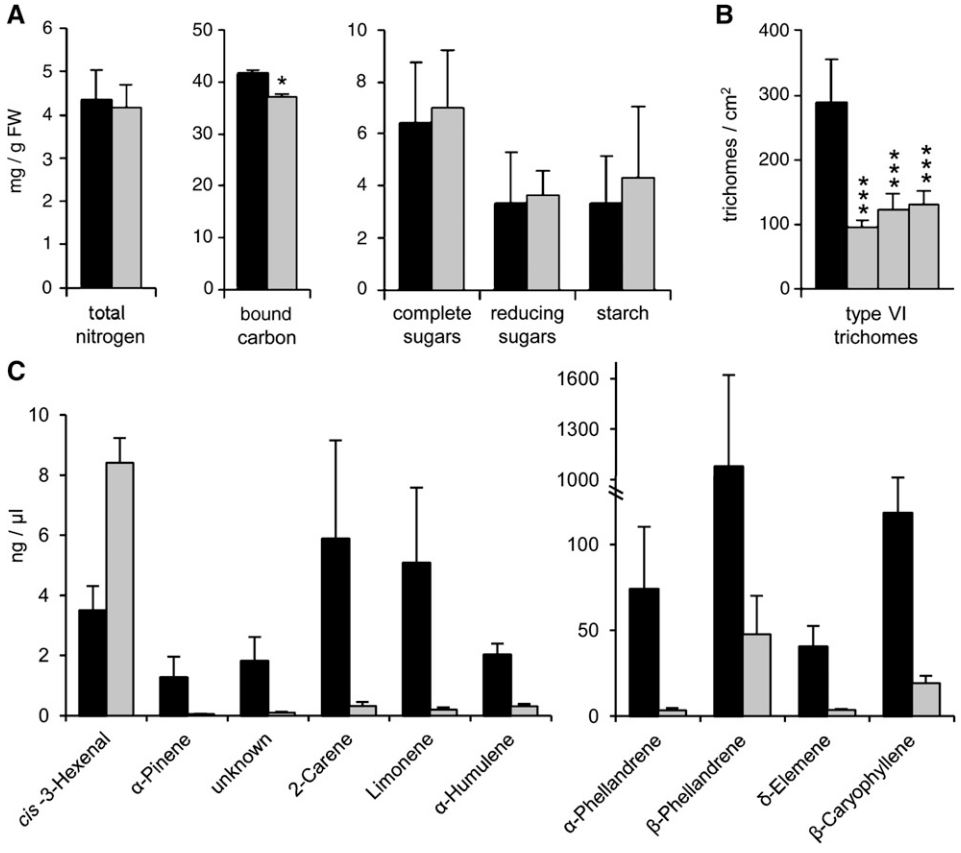

Figure 4. Effect of *OPR3* silencing on nutritional quality, trichome density, and volatile production. A, Nutritional quality. Total leaf nitrogen, bound carbon, and carbohydrate content (complete [total] sugars, reducing sugars, and starch) were analyzed in the wild type (black bars; $n = 4$ for total carbon and nitrogen, $n = 15$ for carbohydrates), and in *OPR3-RNAi* plants (gray bars; $n = 12$ for total carbon and nitrogen, $n = 15$ for carbohydrates) in milligrams per gram of fresh weight (FW). B, Trichome density. Trichome density is given as the number of type VI trichomes per square centimeter of leaf area for the wild type (black bar; $n = 26$) and three independent *OPR3-RNAi* lines (gray bars; $n = 15$, 15, and 5 for lines J55 [left], P3 [center], and J18 [right bar], respectively). C, Trichome volatiles. GC-MS analyses identified significant differences for 10 compounds identified as cis-3-hexenal, five monoterpenes (α-pinene, 2-carene, limonene, and α- and β-phellandrene), three sesquiterpenes (α-humulene, δ-elemene, and β-caryophyllene), and one unknown (mass spectrum in Supplemental Fig. S4) that were quantified in nanograms per microliter of trichome extract (1 μL corresponding to 2 mg of leaf material) in two technical replicates performed on each of two independent RNAi lines. Data in A to C show the mean ± SD for wild type in black and *OPR3-RNAi* in gray. The identification of δ-elemene may be an artifact because it may have formed from germacrene C in the injector during gas chromatography (Quintana et al., 2003). Asterisks in A and B indicate significant differences ($^*P < 0.05$ and $^{***}P < 0.001$). FW, fresh weight.

plants, dual-choice tests were performed using an artificial diet to which trichome extracts from either wild-type or *OPR3*-silenced plants were added. Similar to the preference for leaves of *OPR3-RNAi* plants over wild-type leaves (Fig. 2A), the larvae also consumed much more of the artificial diet supplemented with extracts from *OPR3-RNAi* trichomes compared with the diet with wild-type extract (Fig. 5A). This observation suggests that changes in trichome density and/or composition may be responsible for the observed feeding behavior. To account for the reduced density of trichomes in *OPR3-RNAi* plants (Fig. 4B) and a correspondingly lower concentration of trichome constituents in trichome extracts, the experiment was repeated with the amount of wild-type extract reduced to one-third, but feeding preference for *OPR3-RNAi* diet remained the same (Fig. 5A). Therefore, the observed feeding behavior may be caused either by a deterrent effect of terpenes that are more abundant in the wild type or alternatively, by an attractive effect of cis-3-hexenal that is increased in *OPR3-RNAi* trichomes. The latter seems to be the case, because any feeding preference was lost when the extracts from wild-type trichomes were complemented with cis-3-hexenal to match the concentration in *OPR3-RNAi* trichomes (Fig. 5A). To confirm this conclusion, we performed dual-choice tests comparing an artificial diet to which commercially available terpenes were added in a blend reflecting the terpene content of either wild-type or *OPR3-RNAi* trichomes, as well as cis-3-hexenal, again comparing wild-type and *OPR3-RNAi* concentrations. Although differences in terpene concentration had no effect on feeding behavior, a strong stimulatory effect was observed for cis-3-hexenal (Fig. 5B), explaining the observed feeding preference of *M. sexta* larvae for *OPR3-RNAi* plants (Fig. 2A).

To address the question of whether differences in cis-3-hexenal content can also account for the differences in host plant selection for oviposition, we offered *M. sexta* females a choice between two wild-type tomato plants, with cis-3-hexenal added on one side of the olfactometer and the solvent control on the other. In this experiment, the insects did not distinguish between the two plants and many made no choice at all (Fig. 3). By contrast, when two *OPR3-RNAi* plants were offered the wild-type blend of terpenes added on one side, a repellant activity of terpenes was observed (Fig. 3). We conclude that oviposition and feeding behavior are differentially affected by secondary metabolites, with terpenes having a repellant effect on ovipositing *M. sexta* females and cis-3-hexenal stimulating the feeding of the larvae.

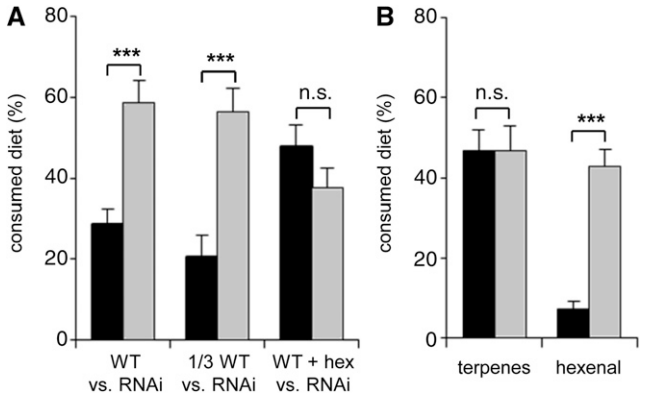

Figure 5. cis-3-Hexenal acts as a feeding stimulant for *M. sexta* larvae. A, Dual-choice tests were performed with an artificial diet to which extracts from wild-type (WT; black bars) or *OPR3-RNAi* trichomes (gray bars) were added (WT versus RNAi, left). To account for the reduced trichome density of *OPR3-RNAi* plants, the experiment was repeated with the amount of wild-type extract reduced to one-third (1/3 WT versus RNAi, center). In the last comparison, wild-type extract was complemented by the addition of cis-3-hexenal to match the concentration observed in *OPR3-RNAi* trichomes (WT+hex versus RNAi, right). B, Dual-choice tests were performed with an artificial diet to which synthetic compounds (a terpene blend or cis-3-hexenal) were added in concentrations reflecting the composition of the wild type (black bars) or *OPR3-RNAi* trichomes (gray bars). The consumed diet in A and B is given in percentages as the mean of 33 experiments for each genotype and treatment ± SE. Asterisks indicate statistically significant differences (Wilcoxon rank sum test; $***P < 0.001$). n.s., Not significant.

Performance of *M. sexta* Larvae Is Impaired on *jai1-1* But Unaffected on *OPR3-RNAi* Host Plants

Considering that *M. sexta* larvae showed a strong preference for leaves of *OPR3-RNAi* and *jai1-1* mutants over wild-type controls, and also consumed much more leaf material of the plants that were deficient in JA/JA-Ile biosynthesis or signaling, we suspected that both genotypes might be similarly impaired in resistance against *M. sexta* and sustain faster development of the larvae than the wild type. This was clearly not the case. There was no difference in growth between *M. sexta* larvae reared on *OPR3-RNAi* or wild-type plants. In an experiment with eighty 3-d-old, 10-mg larvae placed on each of the two genotypes, their development was indistinguishable until they entered the wandering stage at day 17, with an average mass of 4.36 and 4.14 g on wild-type and *OPR3-RNAi* plants, respectively (Fig. 6A). Therefore, resistance against *M. sexta* larvae appears to be at wild-type levels in *OPR3-RNAi* plants, despite the fact that these plants fail to produce JA/JA-Ile as a defense signal in response to wounding (Fig. 1) or *M. sexta* feeding (Supplemental Fig. S5). Apparently, silencing of *OPR3* expression and loss of JA/JA-Ile production do not compromise resistance against *M. sexta*, whereas impaired JA/JA-Ile signaling does. On the *jai1-1* mutant, larvae developed much faster than on wild-type or *OPR3-RNAi* plants. They entered the wandering stage 3 d earlier at an average weight of 4.67 g on *jai1-1* when larvae reared on the wild type had attained only 1.25 g (Fig. 6B).

In contrast with the short-term feeding assays testing for host plant attractiveness and palatability in which freshly prepared leaf discs were used that did not have sufficient time to mount an induced wound response, intact plants were used for the assessment of larval performance over numerous days, allowing the plants to

respond to insect feeding. Induced defenses are thus likely to become more relevant in these experiments. The observation that *OPR3-RNAi* plants behaved like the wild type, whereas *jai1-1* mutants sustained faster development of the herbivore, suggests that induced compared with constitutive defense traits are more relevant with respect to the control of larval performance. It also implies that JAI1/COI1 signaling is required for induced defense, whereas OPR3 and hence JA/JA-Ile production are not. The OPR3 substrate OPDA may thus substitute for JA/JA-Ile as a signal molecule for the activation of induced defenses.

OPDA Is Sufficient for Local Induction of Herbivore Defense Genes But Not for Systemic Signaling

To test whether herbivore defense can be activated in the absence of JA/JA-Ile, we analyzed transcript levels for proteinase inhibitor II (PI-II), a systemic wound-response protein (Schaller and Ryan, 1996) and a well-established marker for induced defense against insects in tomato (Farmer and Ryan, 1992; Li et al., 2003). Wounding caused the accumulation of *PI-II* transcripts in leaves of *OPR3-RNAi* plants (Fig. 7, local leaves), indicating that JA/JA-Ile production is not required for defense gene activation at the site of wounding. The incomplete octadecanoid pathway terminating with OPDA production may thus be sufficient for the induction of herbivore defense gene expression. Indeed, treatment with the JA/JA-Ile precursors linolenic acid or OPDA induced the accumulation of *PI-II* transcripts in both *OPR3-RNAi* plants and wild-type controls (Fig. 8). The data indicate that conversion of the precursors to JA/JA-Ile is not necessary and confirm OPDA as a signal for herbivore defense gene induction.

Grafting experiments between *OPR3-RNAi* and wild-type plants were then performed to address the question of whether the formation of OPDA is also sufficient for systemic wound signaling (Fig. 7). In each of these experiments, the root stock was wounded and the expression of the *PI-II* wound-response marker was analyzed 8 h later in both the wounded leaves and the unwounded scion. In a homologous control graft using only wild-type plants, *PI-II* transcripts accumulated in response to wounding in both the root stock and the scion, indicating that the systemic wound signal is able to pass the graft junction (Fig. 7). Similarly, when *OPR3-RNAi* was grafted onto the wild type (RNAi/WT), *PI-II* transcripts accumulated both locally and systemically, indicating that the *OPR3-RNAi* scion responded to the systemic wound signal that is generated in the wild-type root stock (Fig. 7). However, in the reciprocal graft between *OPR3-RNAi* root stock and the wild-type scion (WT/RNAi), there was no systemic induction of the wound-response marker. The wounded *OPR3-RNAi* root stock responded to wounding as indicated by the local accumulation of *PI-II* transcripts, but it failed to produce a signal for the induction of this defense gene in systemic tissues (Fig. 7).

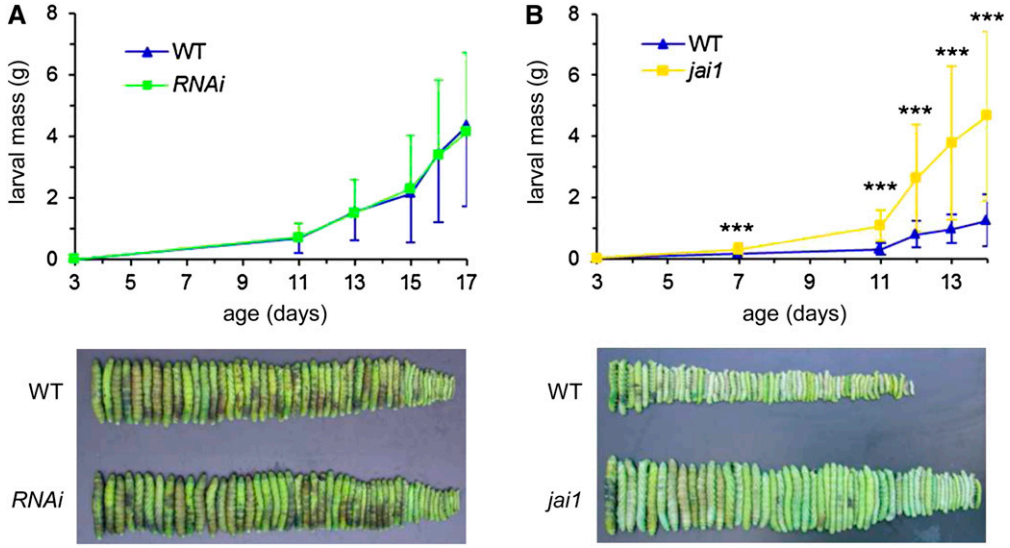

Figure 6. Although the development of *M. sexta* larvae is indistinguishable on *OPR3-RNAi* and wild-type (WT) plants, it is much faster on *jai1* mutants. A, Eighty 3-d-old larvae were placed on each of the two genotypes to be compared (wild-type cv UC82B, dark blue; and *OPR3-RNAi*, green). Larvae were weighed collectively at age 3 d and individually at age 11, 13, 15, 16, and 17 d. On the last day, the larvae were photographed (bottom) and the experiment was terminated because they were about to enter the wandering stage for pupation. B, In the same way, the development of 75 larvae was compared on the wild type (cv Castlemart, blue) and the *jai1-1* mutant (yellow). The weight of the larvae was determined at age 7, 11, 12, 13, and 14 d. This experiment had to be terminated 3 d earlier, because larvae feeding on *jai1-1* had already entered the wandering stage at age 14 d. Data in A and B show the mean weight of the larvae ± SD. Asterisks indicate significant differences (Student's *t* test; $***P < 0.001$).

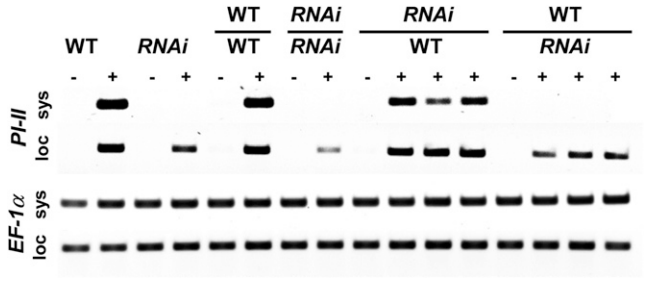

Figure 7. OPDA is sufficient for local defense gene induction but not for systemic wound signaling. The expression of the *PI-II* wound-response marker gene was analyzed by RT-PCR 8 h after wounding (+) in both the wounded leaf (loc) as well as systemic unwounded leaves (sys) of wild-type (WT) and *OPR3-RNAi* (*RNAi*) plants. The corresponding leaves of unwounded plants (−) were analyzed as controls. The same experiment was also performed with grafted plants. The different graft combinations are indicated as fractions, with the genotype of the scion above, and that of the root stock below the fraction line. One experiment (−,+) is shown for the control grafts (WT/WT and *RNAi/RNAi*), whereas one control and three wounded plants with independent RNAi lines as root stock or scion were analyzed in the case of the informative grafts (*RNAi*/WT and WT/*RNAi*; −,+++). RT-PCR amplification of *EF-1α* is shown as a control for RNA integrity and cDNA synthesis. PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide.

DISCUSSION

Since the seminal study of Green and Ryan (1972), who reported the systemic accumulation of antinutritive proteinase inhibitors in tomato and potato plants after local injury by insect herbivores, the wound response in Solanaceae has developed into a model system for induced insect resistance and long-distance signaling in plants (Howe and Schaller, 2008). The central role of jasmonates as signal molecules in plant defense was also discovered in this system (Farmer and Ryan, 1990, 1992) and has since been confirmed in many loss-of-function studies for jasmonate biosynthesis or signaling, mainly in tomato (Howe et al., 1996; Li et al., 2003, 2004, 2005) and coyote tobacco (Halitschke et al., 2004; Kessler et al., 2004; Kang et al., 2006; Paschold et al., 2007). However, to our knowledge, the question as to whether individual traits that contribute to insect resistance may be differentially controlled by different members of the jasmonate family has hardly been addressed (Wang et al., 2008; Vandoorn et al., 2011).

We show here that only a subset of herbivore defense traits is affected by JA/JA-Ile deficiency in *OPR3-RNAi* plants, implying that the ones that are still intact are controlled by other signals. A similar conclusion was reached by Wang et al. (2008), who compared herbivore resistance and defense responses in transgenic coyote tobacco plants that were either silenced for LOX3 (thus lacking all jasmonates) or for JAR4/JAR6 (thus lacking JA-Ile and other JA amino acid conjugates). In LOX3- compared with JAR4/JAR6-silenced plants, the levels of direct defenses were lower and herbivore resistance was not fully restored by JA-Ile. It was concluded that JA-Ile cannot be the only defense signal, and that JA, its precursors, or its metabolites are also active in plant herbivore interactions (Wang et al., 2008). Comparing insect resistance and induced herbivore defense responses in *OPR3-RNAi* plants, the *jai1* mutant, and the wild type, we could locate the additional jasmonate signal upstream of OPR3 in the octadecanoid pathway and we could discern defense traits that are controlled by upstream or downstream signals.

Defense traits that require OPR3 and, by inference, JA/JA-Ile synthesis include type VI glandular trichomes and their terpene constituents. In *OPR3-RNAi* plants, monoterpenes and sesquiterpenes were found to be reduced, which is consistent with previous reports on reduced terpene production in the jasmonate-deficient tomato mutants *defenseless1* (Thaler et al., 2002; Ament et al., 2004; Degenhardt et al., 2010) and *suppressor of prosystemin-mediated response2* (*spr2*) (Sánchez-Hernández et al., 2006; Wei et al., 2013), as well as in the JA/JA-Ile-insensitive *jai1* mutant (Li et al., 2004). Likewise, a reduction in type VI glandular trichome density, similar to the approximately 65% reduction in *OPR3-RNAi* plants, was also reported for *jai1* (Li et al., 2004). These findings indicate that JA/JA-Ile is the relevant signal that acts through JAI1/COI1 to control trichome development and terpene synthesis. Consistent with this conclusion, trichome formation and terpene biosynthesis are both known to be induced in response to methyl jasmonate treatment in wild-type plants (Boughton et al., 2005; van Schie et al., 2007; Peiffer et al., 2009; Tian et al., 2012), but not in *jai1* mutants (Li et al., 2004) or *COI1*-silenced transgenic coyote tobacco (Heiling et al., 2010) mutants.

Despite the established function of type VI trichomes and trichome-born terpenes in insect resistance (Kennedy, 2003; Kang et al., 2010; Wei et al., 2013), increased levels of cis-3-hexenal rather than a reduction in terpene content were found to be responsible for the feeding preference of *M. sexta* larvae for *OPR3-RNAi* over wild-type leaves (Figs. 2 and 5). The accumulation of cis-3-hexenal in *OPR3-RNAi* leaves is likely to be an indirect effect of *OPR3* silencing, which blocks JA/JA-Ile

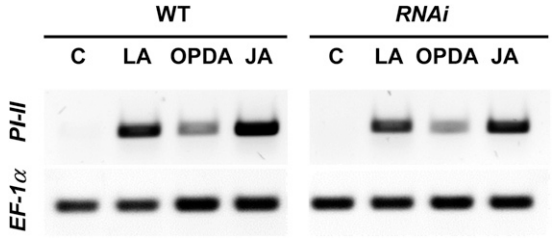

Figure 8. Conversion to JA is not required for wound-response gene induction by OPDA. The expression of the *PI-II* wound-response marker gene was analyzed in wild-type (WT) and *OPR3-RNAi* plants treated with linolenic acid (LA; 300 nmol/leaf), OPDA (6 nmol/leaf), JA (6 nmol/leaf), or the buffer control (C; 1% Tween 20 in 15 mM potassium phosphate buffer, pH 7.5). The compounds were applied as 5-μL droplets onto the surface of two leaves of 4-week-old plants. After 6 h, total RNA was isolated and used for RT-PCR expression analysis of *PI-II* and *EF-1α*, as a control for RNA integrity and cDNA synthesis. PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide.

biosynthesis in the AOS branch of oxylipin biosynthesis and may redirect metabolite flux into the hydroperoxide lyase (HPL) branch, resulting in an increase of green-leaf volatiles (GLVs), including cis-3-hexenal. Similarly, GLV production was found to be enhanced in coyote tobacco silenced for AOS expression (Halitschke et al., 2004), and substrate competition between the AOS- and HPL-branch pathways has also been reported for Arabidopsis and rice (*Oryza sativa*; Chehab et al., 2008; Tong et al., 2012).

Although GLVs are primarily known for their roles in indirect defense (Shiojiri et al., 2006; Wei et al., 2007; Chehab et al., 2008; Allmann and Baldwin, 2010; Schuman et al., 2012) and the priming of defense responses (Engelberth et al., 2004; Frost et al., 2008), direct effects of GLVs on herbivore performance have also been reported. Aphid fecundity is enhanced on HPL-deficient potato plants (Vancanneyt et al., 2001), and GLVs were found to stimulate the feeding of lepidopteran larvae in coyote tobacco (Halitschke et al., 2004). We show here that cis-3-hexenal is a feeding stimulant for *M. sexta* in tomato (Fig. 5), adding to the suite of *M. sexta* feeding stimulants that also includes indioside D and related steroidal glucosides in potato and *Solanum surattense*, respectively (del Campo et al., 2001; Haribal et al., 2006).

In contrast with feeding stimulation by cis-3-hexenal [(*Z*)-3-hexenal], the trans-2-isomer [(*E*)-2-hexenal] was reported to inhibit feeding of *M. sexta* larvae on tobacco leaves (Avdiushko et al., 1997). Interestingly, the (*Z*)-3/(*E*)-2 ratio of GLVs is known to convey information to both *M. sexta* conspecifics and to predatory insects (Allmann and Baldwin, 2010; Allmann et al., 2013). Despite partial cis-trans isomerization, the predominant GLV products of the HPL pathway are (*Z*)-3-hexenal, the corresponding alcohol, and its acetate ester (Matsui, 2006). The GLV bouquet of mechanically damaged plants is thus characterized by a high (*Z*) to (*E*) ratio. However, upon *M. sexta* feeding, an enzymatic constituent of the insect's oral secretions causes a distinct change in the (*Z*) to (*E*) ratio, increasing the emission of (*E*) isomers with a corresponding decrease in (*Z*) isomers (Allmann and Baldwin, 2010). The lower GLV (*Z*) to (*E*) ratio attracts the generalist hemipteran predator *Geocoris* spp. and increases foraging efficiency on *M. sexta* eggs and early-instar larvae (Allmann and Baldwin, 2010). Gravid *M. sexta* females also perceive the change in isomer composition and use this signal to identify oviposition sites that pose a lower risk of predation (Allmann et al., 2013). The feeding stimulation we observed for cis-3-hexenal and the deterrent activity reported by Avdiushko et al. (1997) for the trans isomer indicate that the larvae of *M. sexta* are also able to distinguish between these isomers. Because a low (*Z*) to (*E*) ratio is associated with *M. sexta* feeding, preference for the (*Z*) isomer may allow conspecifics to choose sites with lower feeding competition.

Unlike the feeding preference of the larvae, oviposition preference of *M. sexta* moths for *OPR3-RNAi* plants could be attributed to their terpene deficiency (Fig. 3) and this is consistent with the importance of terpenes for insect resistance (Carter et al., 1989; Eigenbrode et al., 1994; Kang et al., 2010; Bleeker et al., 2012). Because the host for larval development is chosen by the female *M. sexta* moth during oviposition, the observed preference for terpene-deficient *OPR3-RNAi* plants implies a major impact of this JA/JA-Ile-controlled defense trait on insect resistance in a natural environment. Likewise, the terpene linalool was shown to deter *Manduca quinquemaculata* from oviposition on coyote tobacco in its natural habitat (Kessler and Baldwin, 2001). Consistent with the relevance of terpenes for host plant selection, the tomato *od2* mutant in which trichome density and trichome-born terpenes and flavonoids are reduced turned out to be hypersusceptible to insects in the field, attracting the Colorado potato beetle (*Leptinotarsa decemlineata*) that is no threat to wild-type tomato (Kang et al., 2010).

In addition to type VI trichome formation and terpene production, JA/JA-Ile was also found to be indispensable for the induction of a systemic defense response. In reciprocal grafting experiments between tomato wild-type and *OPR3-RNAi* plants, we found that functional OPR3 is required in the wounded root stock for systemic signaling, whereas the activation of the defense gene expression in the unwounded scion is independent of OPR3 (Fig. 7). Hence, the generation of a systemic wound signal requires JA/JA-Ile biosynthesis only in the wounded but not in systemic tissues. This result is fully consistent with similar grafting experiments by Li et al. (2002), who demonstrated that the systemic defense response requires jasmonate biosynthesis locally, whereas distant tissues rely on jasmonate signaling for defense gene activation. Using the trienoic acid-deficient *spr2* mutant, Li et al. (2002) could not distinguish between a local requirement for OPDA or JA/JA-Ile. Our grafting experiments with *OPR3-RNAi* plants and others utilizing a β-oxidation mutant (Li et al., 2005) indicate that the entire octadecanoid pathway, and by inference the formation of JA/JA-Ile, are necessary to initiate systemic signaling. In coyote tobacco, the activity of JAR4 and JAR6 was also found to be required for the induction of systemic defense (Wang et al., 2008). Recent radiotracer studies support JA-Ile itself as the systemically mobile signal (Sato et al., 2011; Matsuura et al., 2012). Alternatively, a yet-to-be-identified signal molecule is formed in response to JA/JA-Ile accumulation after wounding (Wang et al., 2008).

As opposed to the induction of the PI-II wound-response marker that did not require OPR3 activity in the unwounded scion (Fig. 7), Koo et al. (2009) observed the very rapid (<5 min after wounding) conversion of OPDA to JA/JA-Ile in systemic leaves in Arabidopsis obviously requiring OPR3 activity in these tissues. Electrical signaling by membrane depolarization and wound-induced surface potential changes are likely to be responsible for the very rapid formation of JA/JA-Ile and subsequent defense gene activation (Wildon et al., 1992; Schaller and Frasson, 2001; Mousavi et al., 2013). The different long-distance signaling systems utilizing JA/JA-Ile-dependent signaling molecules and physical signals may interact to modulate systemic

responses to wounding and harmonize them in a spatial and temporal manner (Koo et al., 2009).

Whereas systemic signaling for the induction of the wound-response marker was impaired in *OPR3*-silenced plants, the local induction of defense gene expression was not. *PI-II* transcripts accumulated in wounded leaves but not in unwounded, systemic tissues (Fig. 7). Hence, the local induction of wound-response genes is independent of OPR3 activity, suggesting that either OPDA may substitute for JA/JA-Ile as a signal for gene expression or that cis-3-hexenal, which accumulates to higher levels in *OPR3*-silenced plants, is responsible for defense gene induction. Excluding the latter possibility and consistent with OPDA being the active signal, we found that cis-3-hexenal treatment failed to induce *PI-II* expression in *OPR3-RNAi* plants (Supplemental Fig. S6). On the other hand, OPDA as well as its precursor linolenic acid induced the expression of the wound-response marker in *OPR3-RNAi* plants (Fig. 8). OPR3-independent induction of defense is also apparent from the rate of larval development on the different tomato genotypes (Fig. 6). On the *jai1* mutant lacking COI1-dependent induction of defense, larvae developed much faster and reached the wandering stage much earlier than on the wild type. By contrast, on *OPR3-RNAi* plants, larval weight gain and the rate of development were indistinguishable from the wild type (Fig. 6). Therefore, under these experimental conditions, the OPDA-mediated local induction of herbivore defense appears to be sufficient to provide wild-type levels of resistance against *M. sexta*.

Support for OPDA as a genuine defense signal is also derived from a comparison of insect resistance in *OPR3-RNAi* plants and the tomato *acx1* mutant, which is defective in acyl-CoA oxidase (ACX1A), the enzyme catalyzing the first step in peroxisomal β-oxidation. In contrast with *OPR3-RNAi* plants that maintain wild-type levels of resistance against *M. sexta* (Fig. 6), *acx1* is much more susceptible (Li et al., 2005) and in fact resembles the JA/JA-Ile-insensitive *jai1* mutant (Fig. 6). It was concluded that β-oxidation is required for induced resistance against this insect and that resistance is mediated by JA rather than OPDA (Li et al., 2005). However, the loss of resistance in *acx1*, which is still able to convert OPDA to 3-oxo-2-[2′(Z)-pentenyl]-cyclopentane-1-octanoic acid (OPC-8:0), compared with intact resistance of *OPR3-RNAi* plants in which conversion of OPDA is blocked, is consistent with our conclusion that OPDA may serve as a signal for induced resistance in absence of JA/JA-Ile.

On the other hand, the observation that OPDA levels in the *acx1* mutant are similar to the wild type (Li et al., 2005) is difficult to reconcile with the apparent loss of resistance in *acx1* compared with intact resistance in *OPR3-RNAi* plants. As a tentative explanation, we might suggest that subcellular compartmentalization of the OPDA pool may differ between *acx1* and *OPR3-RNAi* mutants and that subcellular localization may be relevant for OPDA signaling. Uptake of OPDA (and other long chain fatty acids) from the cytoplasm into peroxisomes is mediated by COMATOSE, an ATP-binding cassette transporter protein (Theodoulou et al., 2005). Activated acyl-CoA esters rather than free fatty acids are substrates of COMATOSE (Fulda et al., 2004), which are cleaved by its intrinsic thioesterase activity that is required for the delivery of free fatty acids into peroxisomes (De Marcos Lousa et al., 2013). Vectorial transport was further shown to require reactivation of the fatty acid by peroxisomal acyl-CoA synthetases (Fulda et al., 2004; De Marcos Lousa et al., 2013). It thus seems that the imported fatty acid needs to be metabolized to make uptake efficient. In the case of OPDA, OPR3 activity would be required to produce OPC-8:0, the suggested physiological substrate of the acyl-CoA synthetase OPC-8:0 CoA Ligase1 (Koo et al., 2006). It is thus conceivable that the uptake of OPDA into peroxisomes depends on OPR3 to form OPC-8:0 as the substrate for OPC-8:0 CoA Ligase1. In this scenario, transport of OPDA into peroxisomes would be impaired in *OPR3-RNAi* plants but not in the *acx1* mutant. As a further implication of this model, one might expect that OPDA exerts its signaling function in the cytosol. The questions of where OPDA is localized and the relevance of OPR3 activity for transport into peroxisomes remain to be investigated.

Similar to the high level of resistance that OPR3-deficient tomato plants show against *M. sexta* larvae, resistance against *Bradysia impatiens* and *Alternaria brassicicola* is not compromised in the *opr3* mutant in Arabidopsis (Stintzi et al., 2001; Zhang and Turner, 2008). It was suggested that OPDA can substitute for JA/JA-Ile as a signal for defense gene induction in this system, and indeed, an overlapping but distinct set of defense genes was found to be activated in *opr3* in response to OPDA compared with JA treatment (Stintzi et al., 2001; Taki et al., 2005). However, these findings were recently questioned, because under certain conditions, Arabidopsis *opr3* does not seem to be a true null mutant (Chehab et al., 2011). The findings reported here, including OPR3-independent activation of local defense, *M. sexta* resistance in absence of JA/JA-Ile, and induction of defense gene expression by OPDA in tomato, lend indirect support to the conclusions that were drawn for Arabidopsis, and confirm OPDA as a bona fide defense signaling molecule. In addition to its role in defense signaling, there is an increasing body of evidence implying OPDA as the signal for tendril coiling (Stelmach et al., 1998), phytochrome A signaling and hypocotyl growth (Brüx et al., 2008), fertility (Stumpe et al., 2010; Goetz et al., 2012), seed germination (Dave et al., 2011), redox homeostasis (Park et al., 2013), and gene regulation (Taki et al., 2005; Mueller et al., 2008; Ribot et al., 2008).

MATERIALS AND METHODS

Silencing of *Solanum lycopersicum OPR3* by RNAi

For gene silencing, a hairpin construct was generated (sequences of all PCR primers are given in Supplemental Methods S1) comprising 408 bp of the tomato (*Solanum lycopersicum*) *OPR3* complementary DNA (cDNA; nucleotides 411–819; accession no. AJ278332) in sense and antisense orientations separated by the first intron of *FATTY ACID DESATURASE2* (At3g12120) in the plant transformation vector pRTL2 (Restrepo et al., 1990; Stoutjesdijk et al.,

2002; Supplemental Fig. S1A). As a regulatory element, pRTL2 contains the *Cauliflower mosaic virus* 35S promoter with a duplicated enhancer and the *Cauliflower mosaic virus* 35S polyadenylation signal (Restrepo et al., 1990). The construct was transformed into *Agrobacterium tumefaciens* strain LBA4404. Transgenic tomato (cv UC82B; Royal Sluis) plants were generated according to Fillatti et al. (1987) with the following modifications. Cotyledons from etiolated tomato seedlings were used as explants for *A. tumefaciens*-mediated transformation, and feeder cells were omitted. Tissue culture was done on Murashige and Skoog basal salts medium with minimal organics (M-6899; Sigma-Aldrich) containing thiamine (9.6 mg/L), pyridoxine (1 mg/L), and niacin (1 mg/L), supplemented with trans-zeatin (1 mg/L) and timenten (250 mg/L) during selection, or indole acetic acid (0.1 mg/L) and vancomycin (500 mg/L) for rooting, respectively. Kanamycin was used for selection at increasing concentrations ranging from 35 to 100 μg/mL.

More than 30 putative transgenics were regenerated from independent transformation events. Those that tested positive for the presence of the sense and antisense parts of the silencing construct turned out to be sterile, but selfing was facilitated by repeated spraying of flower buds with 0.003% (v/v) methyl jasmonate, 0.01% (v/v) Tween 20 in water, and squeezing of the anther cone. All experimental plants were grown from T1 seeds and genotyped by PCR. Silencing of *OPR3* expression was confirmed by western-blot analysis using leaf extracts from plants that had been wounded 2 h before (Supplemental Fig. S1B). To confirm specificity of the silencing construct, we retrieved all *OPR* and *OPR*-like sequences form the tomato genome database (http://solgenomics.net). The His and Tyr residues that are essential for catalysis in positions 185, 188, and 190 (tomato OPR3 numbering) and a prerequisite for functional enone reductases (Breithaupt et al., 2009; Schaller and Stintzi, 2009) were present in only 5 of 10 retrieved sequences. A phylogenetic tree was generated for these five putative *OPR* genes after multiple sequence alignment (ClustalX) showing that *OPR3* (Solyc07g007870) is the most distantly related member of the tomato *OPR* family (Supplemental Fig. S2A). Pairwise sequence comparisons indicated that *OPR3* is most closely related to *OPR1* (Solyc10g08220; Strassner et al., 2002) with 57% identity (Supplemental Fig. S2B). *OPR1* expression was analyzed at the transcript and protein levels, revealing no obvious differences between *OPR3-RNAi* and wild-type plants (Supplemental Fig. S2C) and thus confirming specificity of silencing for *OPR3*.

Selection of *jai1-1* Mutants

Segregating F2 seeds of the *jai1* mutant in the cv Castlemart background (Li et al., 2004) were kindly provided by Gregg Howe (Michigan State University). Homozygous mutants were identified by PCR. Specific primer pairs were used to distinguish between the mutant (JAI-1-F/Jai-1-R) and the wild-type allele (JAI-1-F/JAI -1-R), yielding amplicons of 777 bp and 525 bp, respectively.

Growth of Tomato Plants, Grafting, and Wounding

To minimize the risk of *Tobamovirus* infection, dry tomato seeds were incubated overnight at 70°C, sterilized in 70% ethanol for 5 min, rinsed in water, incubated in 10% (w/v) trisodium phosphate for 3 h, and rinsed again in five changes of water for 5 min each. Plants were grown in the greenhouse with supplemental light with a 16-h photoperiod and a 26°C/18°C day/night temperature regime. Plants were fertilized at weekly intervals. Experimental plants, as opposed to those that were grown for seed propagation, were not subjected to phytosanitary procedures.

Grafting was performed on 4-week-old plants as described by Li et al. (2002) with minor modifications. A V-shaped incision was made in the middle of the root stock stem, to accommodate the scion that was trimmed to the shape of a wedge. The graft junction was fixed with water-soaked paper towels and parafilm, and plants were kept in a foil tunnel and sprayed with water every day to maintain 100% relative humidity for 1 week. Three weeks after grafting, plants were wounded using a hemostat to crush the individual leaflets of two opposing leaves of the root stock across the midvein. Two h later, a second wound was placed basipetally to the first, and 8 h later, the wounded leaves from the root stock and unwounded leaves from the scion were harvested for analysis.

Extraction and Quantification of Jasmonates

The analysis of JA, JA-Ile, and 12-OH-JA at 40 min after wounding was done by liquid chromatography-mass spectrometry after solid-phase extraction of methanolic extracts of 50 mg of leaf tissue as described by Balcke et al. (2012). Three biological replicates were performed on three independent *OPR3-RNAi* lines. For the analysis of OPDA, JA, and 12-OH-JA by GC-MS at 2 h after wounding, about 0.5 g of leaf tissue was used and extracted with 5 mL 80% (v/v) methanol. [$^2H_6$]-JA, [$^2H_5$]-OPDA, 11-[$^2H_3$]OAc-JA, and 12-[$^2H_3$]OAc-JA were added as internal standards. Samples were further purified and fractionated (Stenzel et al., 2003), and GC-MS analysis was performed as described (Hause et al., 2000; Miersch et al., 2008). Eight biological replicates (eight independent RNAi lines) were analyzed after wounding, and four independent RNAi lines and wild-type plants were used for controls. SigmaPlot 10.0 (Systat Software) was used for statistical analysis of the data, utilizing either the Student's *t* test or the Wilcoxon signed-rank test depending on whether data were normally distributed.

Insect Feeding Bioassays

Leaf palatability was analyzed in no-choice bioassays with 500 mg of leaf material from the different tomato genotypes (including four independent *OPR3-RNAi* lines) offered to forth-instar *Manduca sexta* larvae that were starved for 35 min before the experiments. After 30 min of feeding, the remaining leaf material was weighed, the result was corrected for the weight loss by evaporation, and the consumed leaf mass was determined. For the analysis of feeding preference, dual-choice tests were performed using 2-cm leaf discs, three from each of the two genotypes, placed alternately at the circumference of a 9-cm petri dish. Three third-instar *M. sexta* larvae starved for 1 h were placed in the center, and the consumed leaf area was determined after 4 h of feeding. To assess the effect of induced defenses, plants were wounded mechanically 24 h before the experiment. A hemostat was used to crush a leaflet at its tip across the midvein as well as the base at the left and right edge. Leaf discs were excised the following day with a cork borer from the unwounded area in the center.

To assess the effect of terpenes and cis-3-hexenal on feeding preference, dual-choice tests were performed with an artificial diet (Gipsy Moth Wheat Germ Diet; MP Biomedicals) to which the test compounds were added. A cork borer was used to punch out 2.4-cm discs from a 0.5-cm sheet of the artificial diet, the test compounds or solvent control were added, and the discs were placed in two rows of three on opposing sides of a covered 24 × 18 cm plastic dish. Three forth-instar *M. sexta* larvae were put in the center and allowed to feed for 16 h. The consumed mass was determined as the weight difference before and after feeding, corrected for the weight loss by evaporation. Of the terpenes that were identified in trichome extracts (Fig. 4C), those that are commercially available (α-pinene, 2-carene, α-phellandrene, β-caryophyllene, α-humulene, and limonene; Sigma-Aldrich) were diluted in hexane in a ratio reflecting the terpene composition of wild-type and *OPR3-RNAi* trichomes, respectively (Supplemental Table S1). Forty μL of the terpene mix (corresponding to the terpene content of 0.8 g of leaf tissue) was added to each leaf disc. In the same way, cis-3-hexenal was applied in 40 μL of water in the concentration reflecting the hexenal content of wild-type and *OPR3-RNAi* plants, respectively.

For the analysis of oviposition preference, a two-channel olfactometer was used consisting of a central box (100 × 74 × 95 cm) connected by plexiglass tubes to two opposing BugDorm-2 insect tents (75 × 75 × 115 cm; MegaView Science) housing the tomato plants. A pair of adult moths was placed in the center for mating, and the female was allowed to make its choice for oviposition. Naïve plants were used for each new pair of moths, and were placed randomly on the left or right side of the olfactometer to exclude positional effects.

For a comparison of larval development, 3-d-old *M. sexta* larvae that were all about the same size were distributed on the different tomato genotypes (a pool of four independent *OPR3-RNAi* lines with cv UC82B, and the *jai1-1*mutant with cv Castlemart as the respective controls; 75–80 larvae per genotype). The host plants were about 10 weeks old with a height of 60 cm and were exchanged as needed before all of the leaf material had been consumed. Larvae on *OPR3-RNAi*/'UC82B' were weighed collectively when they were 3 d old, and individually at age 11, 13, 15, 16, and 17 d; those on *jai1-1*/'Castlemart' developed faster and were weighed at age 7, 11, 12, 13, and 14 d. The experiments were terminated when the larvae entered the wandering stage.

Carbon, Nitrogen, Sugar, and Starch Content

A Variomax V5.2 analyzer (Elementar Analysensysteme) was used to analyze total nitrogen content and bound carbon in 200 mg of lyophilized and pulverized tomato leaves. After Dumas combustion, oxidation, and reduction

of the samples, N_2 and CO_2 were separated by gas chromatography with helium as the carrier gas and were quantified on the basis of peak height using a temperature conductivity detector (Walch-Liu et al., 2000). For determination of starch and soluble sugars, leaf material was shock-frozen in liquid N_2, ground to a fine powder, and lyophilized. Twenty mg of dry leaf powder was extracted twice in 2.5 mL of 70% ethanol. Soluble sugars and starch content were assayed according to Blakeney and Mutton (1980) in the combined extracts and the solid residue, respectively. Briefly, reducing sugars were derivatized with *p*-hydroxybenzoic acid hydrazide and quantified as Glc equivalents at 415 nm. For the determination of total sugars, extracts were first digested with invertase. The starch in the remaining solid residue was hydrolyzed with glucoamylase and quantified with respect to a Glc standard curve as described (Blakeney and Matheson, 1984).

Analysis of Type VI Trichomes and Trichome Volatiles

Trichome density was determined on 2 cm-long terminal leaflets, 5 to 26 for each of the different genotypes. Type VI trichomes were counted on six leaf discs punched out from each leaflet using a 0.4-cm cork borer. For trichome isolation according to Yerger et al. (1992), 20 g of leaf material was shock-frozen in liquid nitrogen in a 50-mL tube, and 2 to 3 cm^3 of crushed dry ice was added and vortexed for 1 min. Trichomes were sieved into 10 mL of hexane and the suspension was filtered through a 500-μm mesh. Extracts were sonicated (5 × 1 min) and cleared by centrifugation (5 min, 3,200*g*, 4°C). For identification and quantification of trichome volatiles, the hexane extracts (1 mL) were dried on anhydrous $MgSO_4$. Toluene was added as the internal standard at a final concentration of 8.665 ng μL^{-1}. The samples were then analyzed on a Hewlett-Packard 6890 series gas chromatograph connected to a Hewlett-Packard 5973 quadrupole mass selective detector. An ionization potential of 70 eV, a scan range of 50 to 350 atomic mass units, a transfer line temperature of 230°C, ion source temperature of 230°C, and quadrupole temperature of 150°C were used as parameters for electron impact ionization and mass analyses. Separation was performed on a DB-5ms column of 30 m × 0.25 mm × 0.25 μm film thickness (Agilent Technologies), using helium as the carrier gas at a flow rate of 1 mL min^{-1}. Samples (1 μL) were injected splitless onto a column held at 40°C. After 3 min, the column temperature was increased to 80°C at a rate of 2°C per minute, followed by an increase of 5°C per minute to 160°C, and then 60°C per minute to 300°C. Compounds were identified by comparing the mass spectra and retention times with those of external standards (Supplemental Table S2). Where external standards were not available, compounds were identified by comparing mass spectra and retention times with those in the commercially available mass spectra libraries NIST98, Wiley275, and Adams2205. Individual compounds were quantified by calculating the peak area relative to the internal standard peak area. Quantification was based on the integration of specified fragment ion peak areas and the amount of compound was calculated based on external calibration curves of authentic standards (Supplemental Table S3). The fragment ions corresponding to a mass-to-charge ratio (*m/z*) of 93 were used for quantification of most compounds, with fragment ions of *m/z* 80, *m/z* 91, and *m/z* 119 being used for quantification of cis-3-hexenal, toluene, and the unknown compound, respectively.

Reverse Transcription-PCR Analysis of PI-II Gene Expression

Tomato leaf tissue samples (0.5 g) were flash-frozen in liquid nitrogen, ground to a fine powder, and extracted in 2 volumes of 25 mM Tris/HCl, pH 8, 75 mM NaCl, 25 mM EDTA, 1% SDS, and 1 M β-mercapto ethanol for reverse transcription (RT)-PCR analysis. The cleared supernatant was extracted twice with phenol:chloroform (2:1), followed by chloroform extraction. RNA was precipitated by the addition of one-fourth volume of 10 M LiCl overnight at 4°C. After centrifugation, the precipitate was resuspended in water and subjected to ethanol precipitation. Finally, RNA was resuspended in water and its concentration determined spectrophotometrically at 260 nm. To remove residual genomic DNA, 4 μg of total RNA was treated with DNase I according to the manufacturer's instructions (Thermo Fisher Scientific). The reaction was terminated by the addition of EDTA (12.5 mM) and heating (65°C, 15 min). For oligo(dT)-primed first-strand cDNA synthesis, the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) was utilized according to the supplied protocol. The cDNA was then used as template in a 25-μL PCR using 5 units of Taq polymerase to detect expression of *PI-II* (Solyc03g020050) and *elongation factor1α* (*ef1α*; accession no. BT013246) by use of specific primer pairs (0.2 μM forward and reverse primers; Supplemental Methods S1). PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining after 25, 30, and 35 cycles (30 s at 95°C, 45 s at 59°C, and 50 s at 72°C) to identify the exponential phase of amplification.

Supplemental Data

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

Supplemental Figure S1. Silencing of *OPR3* expression by RNAi.

Supplemental Figure S2. Specificity of silencing.

Supplemental Figure S3. Total ion chromatograms for trichome extracts from wild-type and *OPR3-RNAi* plants.

Supplemental Figure S4. Fragmentation spectrum of the unknown compound detected in *OPR3-RNAi* trichome extracts.

Supplemental Figure S5. Jasmonate levels induced by *M. sexta* feeding in wild-type and *OPR3-RNAi* plants.

Supplemental Figure S6. Quantitative PCR analysis of cis-3-hexenal-induced *PI-II* expression in *OPR3-RNAi* and wild-type plants.

Supplemental Table S1. Volatile blends used in dual-choice feeding assays.

Supplemental Table S2. Identification of trichome volatiles.

Supplemental Table S3. Quantification of trichome volatiles.

Supplemental Methods S1. Oligonucleotide primer sequences.

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