The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis

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Jasmonic acid (JA) and its precursor 12-oxophytodienoic acid (OPDA) act as plant growth regulators and mediate responses to environmental cues. To investigate the role of these oxylipins in anther and pollen development, we characterized a T-DNA-tagged, male-sterile mutant of Arabidopsis, opr3. The opr3 mutant plants are sterile but can be rendered fertile by exogenous JA but not by OPDA. Cloning of the mutant locus indicates that it encodes an isozyme of 12-oxophytodienoate reductase, designated OPR3. All of the defects in opr3 are alleviated by transformation of the mutant with an OPR3 cDNA. Our results indicate that JA and not OPDA is the signaling molecule that induces and coordinates the elongation of the anther filament, the opening of the stomium at anthesis, and the production of viable pollen. Just as importantly, our data demonstrate that OPR3 is the only isozyme of OPR capable of reducing the correct stereoisomer of OPDA to produce JA required for male gametophyte development.

OPDA to JA. The first step in this conversion is catalyzed by 12-oxophytodienoic acid reductase (OPR) (15) (Fig. 1), and a cDNA encoding an OPR was first cloned from Arabidopsis, using sequence of the enzyme purified from Corydalis sempervirens (16). However, subsequent studies (17) showed that the Arabidopsis gene, OPR1, encodes a protein with almost no activity against 9S,13S-OPDA, which is believed to be the principal isomer in plants (18). At the same time, a second, separable OPR activity from C. sempervirens was shown to have the characteristic expected of an isozyme involved in JA synthesis in that it efficiently converted 9S,13S-OPDA to 3-oxo-2(Z)-pentenyl-1-octanoic acid (OPC:8) (17), which is hypothesized to be converted to 3R,7S-JA ([+]-7-isoo-JA) by three cycles of β-oxidation (19). A second OPR gene, OPR2, was identified in Arabidopsis (20). Originally, the observation by Biesgen and Weiler (21) that the OPR2 promoter directed β-glucuronidase expression in a late stage of pollen development led these authors to suggest that OPR2 might be the gene involved in producing JA during pollen development. More recently, biochemical studies performed on purified OPR1 and OPR2 showed that these two isoforms were able to reduce 9S,13S-OPDA only very poorly (21), whereas OPR3, a third isozyme identified as a cDNA representing a brassinosteroid-up-regulated gene (22), was highly effective in reducing 9S,13S-OPDA (21), the naturally occurring isomer in plants. These biochemical results suggest that OPR3 rather than OPR1 and OPR2 is the enzyme involved in JA biosynthesis in A. thaliana, although the contributions of both OPR1 and OPR2 cannot be ruled out.

In this paper, we report the cloning by T-DNA tagging of a gene encoding the third isozyme of OPR, OPR3. Characterization of the mutant phenotype indicates that JA but not OPDA acts to ensure pollen fertility and that the OPR3 gene product is the only isozyme of OPDA that can provide the JA in anther tissue required for pollen maturation and release.

Materials and Methods

Screening of the Arabidopsis Male-Sterile T-DNA Population. Twenty-eight male-sterile T-DNA mutagenized lines of Arabidopsis

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Abbreviations: AGI, Arabidopsis Genome Initiative; JA, jasmonic acid; 16:3, hexadecatrienoic acid; 18:3, linolenic acid; MeJA, methyl jasmonate; OPDA, 12-oxophytodienoic acid; OPR, 12-oxophytodienoate-10,11-reductase; RT-PCR, reverse transcription-PCR; WS, Wassilewskija.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AC006413, AAD19764, AAC33200, and AB010695).

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thaliana ecotype Wassilewskija (WS) (23) were obtained from the Arabidopsis Biological Resource Center at Ohio State University and screened for their response to JA. ABRC numbers CS2321–2329, CS2331, CS2332, CS2336–2338, CS2340–2343, CS2346, CS2347, CS2349, CS2350, CS2352, CS2811, CS2812, CS2817, CS2819, and CS2821 were germinated on soil and grown under a 12-h photoperiod (130 μmol quanta−1sec−1) at 22°C. After bolting, flower buds of sterile plants from each line were painted with a 100 μM solution of 7-epi JA (Cayman Chemicals, Ann Arbor, MI) in 0.1% Tween 20 sprayed every 2 days over a period of 2 weeks. Mutant plants were also tested for their response to a 0.1% solution of linolenic acid (Bio-Rad) every 2 days over a period of 2 weeks. Mutant plants from each line were painted with a 100 μM solution of OPDA (Cayman Chemicals) (both painted on flower buds), and a 450 μM solution of methyl jasmonate (MeJA) (left, right border rescue) or 0.1% Tween 20 sprayed on plants during flowering.

Seeds were collected from a single kanamycin-resistant fertile individual of line CS2338. To test for segregation of kanamycin resistance, seeds were sown in Petri dishes on Murashige and Skoog basal medium (GIBCO BRL) solidified with 0.8% agar and grown under a 12-h photoperiod (130 μmol quanta−1sec−1) at 22°C. After bolting, flower buds of sterile plants from each line were painted with a 100 μM solution of 7-epi JA (Cayman Chemicals, Ann Arbor, MI) in 0.1% Tween 20 sprayed every 2 days over a period of 2 weeks. Mutant plants were also tested for their response to a 0.1% solution of linolenic acid (18:3) soap (Nu Chek Prep, Elysian, MN), a 100 μM solution of OPDA (Cayman Chemicals) in 0.1% Tween 20 sprayed every 2 days over a period of 2 weeks. Mutant plants from each line were painted with a 100 μM solution of linolenic acid. The biosynthesis of jasmonic acid from linolenic acid. The projections shown represent the absolute stereoisomeric configuration of the side chains.

Plasmid Rescue. Genomic DNA from homozygous mutant plants was digested to completion with either EcoRI (right border rescue) or BamHI (left border rescue) restriction enzyme and allowed to religate at a dilute concentration (0.2 μg/ml) to promote self-ligation. The ligation products were used to transform Escherichia coli cells that were then subjected to ampicillin selection. No ampicillin-resistant colony was obtained from the experiment with EcoRI-digested DNA, but 22 colonies were identified from the plasmid rescue of BamHI-digested DNA. Based on the results of restriction analysis using BamHI/EcoRI double digestion, these 22 colonies included two different DNA constructs that contained the expected 14.2-kb portion of the T-DNA and one or more additional restriction fragments. Additional restriction fragments thus obtained were gel-purified (Prep-A-Gene; Bio-Rad), radiolabeled (Decaprime II; Ambion, Austin, TX), and used as probes in a Southern blot. The Southern blot was performed by digesting 5 μg of wild-type or mutant genomic DNA with BamHI and EcoRI, separation in 0.8% agarose gel, and transfer to Hybond N+ nylon membrane (Amersham Pharmacia). Hybridization and washes were carried out at 65°C. When the additional DNA fragments from one class of plasmids were used as probes in Southern analysis, hybridization only occurred with DNA from mutant plants and not with wild-type DNA, suggesting that these plasmids might be derived from internal rearrangements within multiple copies of the T-DNA at the insertion site. The second class of plasmids (represented by only a single clone) provided DNA fragments of 1.3 and 0.65 kb, which hybridized to DNA from both mutant and wild-type (ecotype WS) plants but labeled different-sized bands in the mutant relative to wild type (data not shown). This observation identified the fragments as plant DNA flanking the left border of the T-DNA insert.

These two 650-bp EcoRI–EcoRI and 1.3-kb EcoRI–BamHI fragments of plant sequence were subcloned into pBluescript SK (Stratagene) (designated pSK650 and pSK1300, respectively) and sequenced on both strands with an Applied Biosystem automated sequencer. Sequence analysis was performed using Genetic Computer Group (Madison, WI) software. Database searches were carried out (12/28/99) using the BLAST servers (24) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the Arabidopsis Information Resource (http://www.arabidopsis.org).

Isolation of cDNA and Genomic Clones Spanning the Site of T-DNA Insertion. Nucleotides 1–1087 from pSK1300 showed 98% similarity to nucleotides 28,326–29,414 from A. thaliana chromosome II (accession number AC006413), which lie within intron 3 of a putative OPDA reductase gene. Gene-specific primers were designed to allow amplification by reverse transcription–PCR (RT-PCR) of the entire coding sequence for the putative reductase.

Primer 1 (5′-CCCCGGGTCTAGATTTCTATGACGCGGCCAAGGGGAACCT-3′) encompassed the translational start ATG and nucleotides 29,727–29,749 of AC006413, flanked by an EcoRI restriction site (underlined), and primer 2 (5′-CCCCGGATCGTATTCAGGCAGGGAAGGAGCGCACG-3′) encompassed the sequence complementary to the stop codon and nucleotides 27,018–27,042 of AC006413, flanked by a ClaI restriction site (underlined). RT-PCR on 500 ng of wild-type WS anther total RNA was performed using the Superscript 1 step RT-PCR system (GIBCO/BRL). After an initial incubation at 50°C for 30 min, 30 cycles of PCR (95°C, 20 sec; 63°C, 40 sec; 72°C, 80 sec) were performed.

Using primers 1 and 2, a cDNA ampiclon of 1.18 kb was obtained, digested with EcoRI and ClaI, cloned into pART7 (25) as pART7-OPR3, and sequenced. The WS sequence differed by four nucleotides from the Columbia allele, which has recently been designated OPR3 (22). Primers 1 and 2 were also used to generate a genomic fragment by PCR from wild-type WS DNA in a 500-μl PCR reaction containing 50 ng of DNA as a template. After 38 cycles (95°C, 20 sec; 63°C, 40 sec; 72°C, 225 sec), the PCR reaction was separated on a 0.8% agarose gel, and a predominant 4-kb fragment was gel purified and ethanol precipitated. Direct sequencing of this fragment was performed using 60 ng of DNA for each sequencing reaction. Finally, primer 3 (5′-TTGTAACATTGATGACATGCGACC-3′), encompassing nucleotides 27,390–27,413 of AC006413, and primer 4 (5′-GCTTTGAAATGGGATTCAGGCTG-3′), encompassing nucleotides 27,657–27,680 of AC006413, were used in combination with primers T3 or T4 to screen by PCR a flower cDNA library (CD4–6, ABRC). A 650-bp and a 800-bp ampiclon, correspond-

Fig. 1. The biosynthesis of jasmonic acid from linolenic acid. The projections shown represent the absolute stereoisomeric configuration of the side chains.
ing, respectively, to the 5' and the 3' ends of the OPR3 cDNA, were cloned into TOPO TA (Invitrogen) and sequenced.

RNA Extraction and Gel-Blot Analysis. Total RNA was obtained (26) from whole flowers or anthers harvested at stage 12 (27) or rosette stage leaves from both wild-type WS and mutant CS2338. Poly(A) RNA was purified from flower and leaf total RNA (PolyATtract; Promega), following the manufacturer's protocol. RNA samples were separated on a 1.2% agarose/formaldehyde denaturing gel, transferred to HyBond N+, and probed with 32P-labeled OPR3 cDNA. Dashes were performed at 65°C and 0.2× SSC.

Gene Constructs and Plant Transformation. The 1.18-kb OPR3 cDNA cloned in pART7-OPR3 is under the control of the cauliflower mosaic virus 35S promoter and the transcriptional termination region of the octopine synthase gene. The entire expression cassette was released with NotI and inserted into the binary vector pBART, a derivative of pART27 (25) in which the neomycin phosphotransferase gene has been replaced by the bialaphos resistance gene (bar) coding for phosphinotricinic acid transferase to create pBART-35SOPR3. Agrobacterium tumefaciens GV3101 transfected with pBART-35SOPR3 by electroporation were used to transform 2-month-old homozygous mutant plants by the floral dip method (28). After transformation, T0 plants were sprayed every second day with MeJA to allow seed set, and the resulting T1 seeds were germinated on potting mix wetted with 25 mg/liter of glufosinate ammonium (Finale) to select for transformants.

Pollen Germination. Pollen germination tests were carried out according to the method of Preuss et al. (29). In the case of the mutant, for which dehiscence of the anther did not occur at anthesis, the locule was opened manually to release pollen. Pollen was incubated for 24 h at room temperature and then analyzed for pollen tube formation. In the representative experiment reported herein, a total of 2,900 pollen grains from untreated mutant flowers in 60 microscope fields were scored along with 1195 wild-type pollen (32 microscope fields) and 1193 pollen (28 microscope fields) from mutant flowers that had been treated with MeJA.

Flower Development and JA Responsiveness. To determine the stage of flower development at which JA is responsible for the complementation of the male-sterile phenotype in the mutant, opened flowers (beyond anthesis and JA responsiveness) were removed from 63 inflorescences belonging to 24 plants. The remaining young flower buds were sprayed and left in contact with MeJA in a confined growth chamber for 2 h, then transferred to a growth chamber without MeJA. Simultaneously, buds on 12 inflorescences were staged according to the method of Smyth et al. (27). After 5 days, nonelongated followed by elongated siliques from JA-treated mutant plants were counted and averaged and compared with the average of the cumulative number of flowers at each stage of development along the flower bud.

Light Microscopy. Bright-field photographs of individual flowers were taken using a dissecting microscope (Model Orthoplan; Leitz). Photographs of germinating pollen grains were taken with a compound microscope (model B202; Olympus, New Hyde Park, NY).

Results

Identification of a Jasmonate-Responsive Male-Sterile Mutant. The original population of Arabidopsis lines in which mutations have been created by T-DNA insertion (23) contain 28 lines that have been characterized as male sterile (see Materials and Methods). A total of 30–90 plants from each line were grown in the course of five experiments, and male-sterile segregants (identified by their inability to set seed) were treated by painting flower buds with either 100 µM JA in 0.1% aqueous Tween-20 or with a control detergent solution without JA. One line, CS2338, segregated male-sterile plants that consistently produced progeny seed after treatment with JA but remained completely sterile after treatment with the control solution.

The absence of progeny from male-sterile plants would have resulted in a progressive loss of the mutation during propagation of the CS2338 line before we obtained it. For this reason, seeds were germinated on agar containing 100 µg/ml kanamycin, and surviving seedlings were transferred to soil. At maturity, a single fertile plant was identified, and seeds from this plant were harvested. When 153 of these seeds were germinated on kanamycin-containing media, 118 were kanamycin resistant and 35 were sensitive. These data are a good approximation to a 3:1 ratio expected for a single T-DNA insertion. A further 365 progeny were germinated on soil and grown to maturity, and 94 of these were sterile. This proportion of sterile plants is a good fit to the 3:1 hypothesis (χ² = 0.094; P > 0.5), indicating that sterility is caused by a single, recessive nuclear mutation. For reasons described below, the mutant locus was designated opr3. After confirmation of the sterile phenotype, plants were sprayed with a 450 µM methyl jasmonate (MeJA) solution to render them fertile, and seeds were harvested. Approximately 60–100 seeds from each plant were germinated on agar containing 100 µg/ml kanamycin. All progeny from all 94 sterile plants rescued with MeJA were kanamycin resistant, indicating that these plants were also homozygous for the T-DNA insert. This experiment indicated cosegregation of the opr3 mutation with the T-DNA insert.

Our previous investigation of the JA-responsive, male-sterile mutant fad3-2 fad7-2 fad8 (8) identified three characteristics of the male-sterile phenotype, and each of these characteristics is also observed in the opr3 mutant. (i) Floral organs develop normally within the closed bud, but the anther filaments do not elongate sufficiently to position the locules above the stigma at anthesis (Fig. 2A and B). (ii) The anther locules do not dehisce at the time of flower opening (Fig. 2B) (although limited dehiscence occurs later). (iii) Even though pollen on mutant plants develops to the trinucletic stage, as determined by staining with 4',6-diamino-2-phenylindole (8) (data not shown), the pollen grains are predominantly inviable (Fig. 2C). Irrespective of the stage at which pollen was taken from opr3 plants, germination of the pollen was <4%, compared with 97.6% for mature pollen from wild-type plants. Application of JA to flower buds corrected all three of these defects in opr3 plants (Fig. 2B), resulting in rates of pollen germination equivalent to wild type (97.2%) in in vitro tests (Fig. 2C) and abundant seed set on treated plants. By staging flowers (27) and monitoring seed production after a single application of JA, we established that only flower buds corresponding to stage 12 in floral development (27) responded to JA; flowers at earlier and later stages of development could not be rendered fertile by JA treatment. Stage 12 corresponds to the final stage before the bud opens; it starts when the petals reach the top of the long stamens (Fig. 2A) and ends when the sepals open. At this stage, the second mitotic division occurs in the anthers.

In all of these respects, the phenotype of the opr3 mutants was similar to those of fad3-2 fad7-2 fad8 plants that are deficient in 18:3 and 16:3 fatty acids, which are the precursors for JA synthesis (8). However, opr3 plants contained normal levels of 18:3 and 16:3 fatty acids, and application of 18:3 did not restore fertility to this mutant. These results suggest that the opr3 mutation blocks conversion of 18:3 and 16:3 fatty acids to oxylin(s) (Fig. 1) that initiate the processes of anther and pollen maturation. To more closely define the mutant lesion within the pathway of JA synthesis, we treated opr3 plants and
Cloning of a cDNA Encoding Isoform 3 of 12-Oxophytodienoic Acid Reductase. The modified T-DNA used to generate the mutant population contains the origin of replication and the ampicillin resistance gene of plasmid pBR322 (23). This feature allows recovery of T-DNA/plant DNA junction fragments by the method of plasmid rescue (30). Left border plasmid rescue (see Materials and Methods) identified a 1.9-kb plant DNA fragment subeloned as pSK 650 and pSK 1300.

Comparison of the plant-derived DNA in pSK650 and pSK1300 with the database available from the Arabidopsis Genome Initiative (AGI) showed that the sequence from 16 to 771 bp (relative to the left border/plant DNA junction) is a rigorous match (BLAST score 1.499) to a sequence on overlapping cosmids TSE21 and F10B6 at 23.6 cM on chromosome I. The sequence on chromosome I does not appear to be part of an active gene but is homologous to sequences scattered throughout the genome that are related to the tandem repeat of the putative nonautonomous transposable element Tnat1 (31). In contrast, the sequence from 766 to 1852 bp corresponds closely to the sequence from 28,326 to 29,414 of cosmid F5K7 on chromosome II. This insert is 99% identical to AGI cosmids TSE21 and F10B6 on chromosome I and shows lower sequence identity to other, shorter Tnat1 sequences (31). The difference between Col-O and WS at the opr3 locus can most easily be explained by a transposition event occurring after divergence of the Col-O and WS ecotypes.

Interestingly, the 15 bp immediately adjacent to the left border of the T-DNA (Fig. 3) is not present in either the Col-O or WS allele of opr3. A possible explanation for these observations is that the T-DNA first integrated elsewhere in the genome and was then excised together with 15 bp or more of plant DNA adjacent to the left border. This plant DNA then acted as a de facto left border (32) during integration into chromosome II. There is not a perfect match to this 15-bp sequence in the current AGI database.

Using information from the AGI sequence, we designed oligonucleotide primers and amplified two independent RT-PCR products, using anther RNA from WS wild type as a template. We also isolated a cDNA clone from a library derived from the Landsberg erecta wild type. RT-PCR products and the cDNA provided identical sequences, with the exception of a single base pair change; the peptide sequence predicted for the Col-O product was 4.0 kb, and sequencing of this DNA confirmed that the WS predicted intronic sequence contains a 1,284-bp insert (relative to the AGI Col-O), which corresponds to Tnat1 (Fig. 3). Over its entire length, this insert is 99% identical to AGI cosmids TSE21 and F10B6 on chromosome I and shows lower sequence identity to other, shorter Tnat1 sequences (31). The difference between Col-O and WS at the opr3 locus can most easily be explained by a transposition event occurring after divergence of the Col-O and WS ecotypes.

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Amino acid alignment of the A. thaliana OPR1 (GenBank accession number Y10617), OPR2 (U92460) and OPR3 (AF293653). Identical residues in at least two of three sequences are indicated in reverse print. The peroxisomal targeting signal in OPR3 is underlined.

Expression of OPR3 Restores Fertility to opr3 Plants. The identification of OPR3 as one of the loci disrupted by T-DNA insertion in line CS2338 provided a persuasive explanation of the male-sterile phenotype and of the results obtained in our attempts to restore fertility to opr3 plants, with OPDA in place of JA. However, in light of the complex structure of the opr3 locus, it was particularly important to demonstrate that expression of the OPR3 protein could complement the mutant phenotype. We transformed opr3 mutant plants with a cDNA expressed under control of the cauliflower mosaic virus 35S promoter. Treatment of T₀ plants with MeJA during flowering allowed for abundant seed set. When 50,000 T₁ seeds were germinated in the presence of glufosinate ammonium, a total of 123 resistant plants were identified and allowed to grow to maturity. All but one of these plants were partially or fully fertile. Twelve of the lines were chosen for further analysis, and a single leaf from each of these was used to prepare genomic DNA. When tested by PCR using appropriate primers, all 12 plants were positive for both the T-DNA allele and expression of the corresponding transcript, whereas the sterile plants showed no detectable transcript (data not shown). These results confirm that OPR3 expression is required for fertility in Arabidopsis.

Discussion

The production of the male gametophyte in angiosperm plants is a complex series of developmental processes that has been studied by genetic techniques for several decades (33, 34). The discovery that a jasmonate-deficient mutant of Arabidopsis was male sterile and was rendered fertile by applications of JA (8) provided new insight into the chemical regulation of some of the later processes in both pollen and anther development. The stringent requirement for JA signaling potentially has great practical significance, since male sterility and the ability to restore fertility are prerequisites for the development of hybrid breeding strategies in many crop plants (34). opr3 was isolated as a JA-responsive male-sterile mutant in which a distinctive member of the OPR gene family, designated OPR3, is disrupted by a T-DNA insertion. The phenotype of the opr3 mutant is strikingly similar to that described for the Arabidopsis triple mutant fad3-2 fad7-2 fad8 (8) in several different respects. The anther filaments on opr3 plants do not elongate before anthesis, and anther locules do not dehisce at anthesis, so that mutant stigmas typically remain unpollinated. Just as importantly, pollen taken from opr3 anthers at the time of anthesis showed less than 4% germination compared with more than 97% germination for pollen from either wild-type or JA-treated opr3 plants. Mutant analysis suggests that pollen maturation and anther dehiscence are uncoupled processes (8, 35), so our characterization of the opr3 mutant confirms that JA independently activates at least two separate maturation processes in anthers. The fad3-2 fad7-2 fad8 mutant lacks 18:3 and 16:3 fatty acids that are precursors for JA synthesis but is assumed to be wild type for all of the enzymes of the JA pathway. Accordingly, JA, OPDA, or 18:3 restored fertility to fad3-2 fad7-2 fad8 plants when applied. Application of 18:3 to unopened flower buds of triple-mutant plants measurably increased 18:3 levels in sepal and petals, but this fatty acid could not be detected in the anthers after application. This result was interpreted as indicating that JA (or other oxylipins) produced in the petals and sepals could meet the requirements for JA signaling in the anthers (8). In contrast, only JA, not OPDA or 18:3, could render opr3 plants fertile. This result demonstrates that OPDA, which is more abundant than JA in vegetative tissues of Arabidopsis (2, 12), cannot replace JA as the chemical signal triggering anther and pollen maturation.

These experiments on chemical complementation of the opr3 male-sterile phenotype, together with measurements of OPR3 transcript levels in mutant and wild-type plants and the successful molecular complementation of opr3 by transgenic expression of an OPR3 cDNA, indicate that OPR3 encodes the only OPR isozyme capable of producing the JA required for pollen maturation and release. This conclusion is supported by the recent finding by Schaller et al. (21) that the recombinant Arabidopsis OPR3 protein reduces 9,13,15-OPDA as efficiently as it does the 9R,13R isomer. The previously described OPR1 and OPR2 genes are strongly expressed in at least some flower tissues, as determined by transcript analysis and promoter–β-glucuronidase fusions (20). The fact that 18:3 applied to fad3-2 fad7-2 fad8 flower buds restores fertility apparently without penetrating to the anthers suggests that the failure of OPR1 and OPR2 to maintain fertility in opr3 plants is not likely to be due to insufficient expression in a particular organ or tissue of

Fig. 4. Amino acid alignment of the A. thaliana OPR1 (GenBank accession number Y10617), OPR2 (U92460) and OPR3 (AF293653). Identical residues in at least two of three sequences are indicated in reverse print. The peroxisomal targeting signal in OPR3 is underlined.

Fig. 5. Expression of OPR3 in anthers, flowers, and leaves of wild-type and opr3 plants. Gel-blot analysis was performed, using 10 μg of total RNA from anthers and 5 μg of poly(A) RNA from flowers and leaves, with an OPR3 cDNA as a probe. Ethidium bromide staining of the 18S rRNA band was used to confirm equal loading.
the flower. The critical determinant may instead be the substrate specificity of the OPR1 and OPR2 gene products. Characterization of the recombinant OPR1 and OPR2 proteins has determined that they show little activity against 9S,13S-OPDA (21), the principal isomer in plants and the isomer believed to be involved in JA synthesis. Instead, the recombinant enzymes reduced mostly other OPDA isomers and thus correspond to the biochemical activity designated OPR1 (17). A distinct OPR1I activity from OPRI (17). A distinct OPRII activity from OPR2, and thus correspond to the biochemical activity designated recombinant enzymes reduced mostly other OPDA isomers as well as other isomers (17). Our results are consistent with OPR3 encoding an OPR1I activity in anthers and other tissues of Arabidopsis. They also suggest that OPR1 and OPR2 may not be involved in the synthesis of biologically active JA. The physiological function of the additional OPR genes and enzymes remains to be elucidated. While this suggestion is substantially consistent with the biochemical data discussed above, it will require extensive additional studies of the OPR genes and proteins to determine whether this is the case.

OPR3 is a quite distant relative of OPR1 and OPR2, showing 53% identity with both of these proteins (Fig. 4). Two other OPR genes are identified in the Arabidopsis database (GenBank accession numbers AAC33200 and ABO10695). The putative coding sequence of ABO10695 (located on P1 clone MDK4 of chromosome 5) predicts a protein somewhat shorter than other OPR isozymes and may represent a pseudogene. Regardless of this possibility, both protein sequences are more than other OPR isozymes and may represent a pseudogene. However, several steps in the biosynthesis of JA require further investigation, and additional evidence for this model of pathway elucidation, and additional evidence for this model of pathway organization is also necessary. Further studies of the opr3 mutant would provide useful information in this respect and help to elucidate the respective roles of OPDA and JA in other oxylipin responses.

**Note Added in Proof.** A paper describing some aspects of the opr3 mutant was published recently by Sanders et al. (40).

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