Identification of a Peroxisomal Acyl-activating Enzyme Involved in the Biosynthesis of Jasmonic Acid in Arabidopsis

Jasmonic acid (JA) is a lipid-derived signal that regulates a wide variety of developmental and defense-related processes in higher plants. JA is synthesized from linolenic acid via an enzymatic pathway that initiates in the plastid and terminates in peroxisomes. The C18 JA precursor 12-oxo-phytodienoic acid (OPDA) is converted in the peroxisome to 3-oxo-2-(2'-[2-pentenyl])cyclopentane-1-octanoic acid (OPC-8:0), which subsequently undergoes three rounds of β-oxidation to yield JA. Although most JA biosynthetic enzymes have been identified, several key steps in the pathway remain to be elucidated. To address this knowledge gap, we employed co-expression analysis to identify genes that are coordinately regulated with known JA biosynthetic components in Arabidopsis. Among the candidate genes uncovered by this approach was a 4-coumarate-CoA ligase-like member of the acyl-activating enzyme (AAE) gene family, which we have named OPC-8:0-CoA Ligase 1 (OPCL1). In response to wounding, opcl1 null mutants exhibited reduced levels of JA and hyperaccumulation of OPC-8:0. Recombinant OPCL1 was active against both OPDA and OPC-8:0, as well as medium-to-long straight-chain fatty acids. Subcellular localization studies with green fluorescent protein-tagged OPCL1 showed that the protein is targeted to peroxisomes. These findings establish a physiological role for OPCL1 in the activation of JA biosynthetic precursors in leaf peroxisomes, and further indicate that OPC-8:0 is a physiological substrate for the activation step. The results also demonstrate the utility of co-expression analysis for identification of factors that contribute to jasmonate homeostasis.

Oxylipins comprise a group of potent signaling molecules that are derived from oxidative metabolism of polyunsaturated fatty acids. In both animals and higher plants, oxylipins are synthesized in response to developmental and environmental cues and serve important roles in controlling diverse physiological processes. Members of the prostanoid family of lipid mediators have been studied extensively with respect to their synthesis from arachidonic acid and their function in the regulation of cell differentiation, immune responses, and homeostasis in animal systems (1). The study of plant oxylipins has been focused mainly on the jasmonate family of signaling compounds that includes jasmonic acid (JA)² and its bioactive precursors and derivatives. Genetic analysis has shown that endogenous jasmonates regulate a wide range of developmental processes including root growth, pollen maturation, anther dehiscence, seed production, and glandular trichome development (2–4). In addition, it is well established that jasmonates play a central role in regulating plant responses to biotic and abiotic stress (2, 5–9).

The biosynthesis of JA from linolenic acid (18:3) is initiated in plastids and terminated in peroxisomes (10). Lipases that release 18:3 from plastid lipids are thought to play an important role in regulating the pathway in response to environmental and developmental cues (11–13). The first step in the conversion of 18:3 to JA is catalyzed by 13-lipoxygenase. The resulting 13-hydroperoxy fatty acid is transformed to 12-oxo-phytodienoic acid (OPDA) by the sequential action of allene oxide synthase (AOS) and allene oxide cyclase (AOC). A parallel series of reactions converts hexadecatrienoic acid (16:3) to dinor-OPDA (dnOPDA), which is also a JA precursor (14). That large pools of OPDA/dnOPDA are esterified to chloroplast glycerolipids raises the possibility that JA synthesis is controlled, at least in part, by lipases that release these intermediates from plastid lipids (15, 16).

Very little is known about the mechanism of plastid-to-peroxisome trafficking of JA precursors. Evidence indicates that the ATP-binding cassette transporter PXA1 (also known as CTS1 and PED3) is involved in import of OPDA into peroxisomes (17). The peroxisomal enzyme OPDA reductase (OPR3)

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² The abbreviations used are: JA, jasmonic acid; dhJA, dihydrojasmonic acid; OPDA, 12-oxo-phytodienoic acid; dnOPDA, dinor-OPDA; AOS, allene oxide synthase; OPR3, 3-oxo-2-[(2'-[2-pentenyl])cyclopentane-1-octanoic acid; AAE, acyl-activating enzyme; WT, wild type; F, forward; R, reverse; GC-MS, gas chromatography-mass spectrometry; YFP, yellow fluorescent protein; GFP, green fluorescent protein; 4CL, 4-coumarate:CoA ligase; LACS, long-chain acyl-CoA synthetase; ACX, acyl-CoA oxidase.

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† This article was selected as a Paper of the Week.

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Figs. S1 and S2.

§ The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) EF014466.

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converts OPDA to 3-oxo-2-(2’-Z-pentenyl)cyclopentane-1-octanoic acid (OPC-8:0) (18, 19). In the final steps of the pathway, three cycles of β-oxidation remove six carbons from the carboxyl side chain of OPC-8:0 to yield JA. In contrast to the way, three cycles of octanoic acid (OPC-8:0) (18, 19). In the final steps of the path-

EXPERIMENTAL PROCEDURES

Library of Insertion Mutations. Seed stock identifiers for the JA-modifying enzymes such as JA-amido synthetase ([i.e. JAR1] and JA methyltransferase play an important role in altering the biological activity of the hormone following its synthesis in peroxisomes (20, 21).

In addition to JA and its derivatives, C18 biosynthetic pre-
cursors such as OPDA exhibit potent biological activity. Among the physiological processes that are proposed to be regulated by endogenous OPDA are the tendril-coiling response of *Bryonia* (22), production of various secondary metabolites (23, 24), and defense against insects and pathogens (25). It has also been shown that exogenous OPDA and JA activate the expression of overlapping but distinct sets of target genes (25, 26). These observations have lead to the idea that diverse jasmonate-signalized responses are controlled by the relative abundance of multiple bioactive compounds including OPDA/dnOPDA, JA, and various derivatives of JA (14). Our current knowledge of the biochemical and cellular processes that determine this so-called oxylin signature is still in its infancy. Greater insight into this question will require identification of the complete repertoire of enzymes that promote jasmonate synthesis, as well as an understanding of how these components are regulated.

Co-expression analysis has emerged as a powerful tool in the search for plant genes that participate in complex biological processes (27–30). The fact that many JA biosynthetic genes are coordinately regulated in response to developmental and stress-related cues (31–33) indicates that this approach may be useful to identify uncharacterized genes that influence jasmonate homeostasis. Here, we report the use of data-mining tools to identify genes in Arabidopsis that are co-expressed with known JA biosynthetic components. Among the candidate genes singled out by this approach was a member of the large family of ATP-dependent acyl-activating enzymes (AAEs) that metabolize a wide range of carboxylic acid substrates. We provide a combination of genetic, biochemical, and cellular evidence indicating that the enzyme encoded by this gene, which was not previously implicated in JA synthesis, catalyzes the formation of CoA derivatives of jasmonate precursors in leaf peroxisomes. We also provide direct evidence that OPC-8:0 is the physiological substrate for the activation step that immediately precedes β-oxidation.

**EXPERIMENTAL PROCEDURES**

**Biological Material**—Arabidopsis plants were grown in growth chambers maintained at 22 °C with a photoperiod of 16 h light (100 μmol photons m−2 s−1) and 8 h dark. Columbia-0 (Col-0) was used as wild type (WT) for all experiments. All T-DNA insertion lines used in this study were identified from the Sequence-Indexed Library of Insertion Mutations. Seed stock identifiers for the T-DNA insertion lines were as follows: At4g05160, SALK_050214; At5g63380, SALK_003233; At1g20510, SALK_140659 (corresponds to opcl-1); and At1g20510, SALK_107614 (corresponds to opcl-2). Seed stocks were obtained from the Arabidopsis Biological Resource Center. Homozygous knock-out lines were selected by PCR-based screening for both the presence of T-DNA insertion and the absence of an intact endogenous gene. PCR reactions to detect the intact endogenous gene were performed with the following forward (F) and reverse (R) gene-specific primer sets: At4g05160, 5′-AGACACCGTGAAGATCTC-3′ (1F) and 5′-ATGTGTCCGGACGTGGAATC-3′ (1R); At5g63380, 5′-GGCTATCATTGCTTCTCAAGGC-3′ (2F) and 5′-GGAGCAGGCACCCGGAGAT-3′ (2R); and At1g20510 (opcl-1), 5′-TCCGTTACTGCTCCTACGAGTTG-3′ (3F) and 5′-GAGTCGAACGTCTAGCTTCC-3′ (3R); and At1g20510 (opcl-2), 5′-TCCGTTACTGCTCCTACGAGTTG-3′ (3F) and 5′-GAGTCGAACGTCTAGCTTCC-3′ (3R). PCR reactions to detect the T-DNA insert in each mutant line were performed with the T-DNA left border primer (Lb1, 5′-GCGTGGACGGCCTTGGCTGCAACT-3′) and the gene-specific primer indicated in supplemental Fig. S1. PCR analysis confirmed the homozygosity of the T-DNA insert in all mutant lines.

**RNA Blot Analysis**—Rosette leaves from 4-week-old plants were either wounded twice across the mid-vein with a hemostat or sprayed with JA (25 ml of a 50 μM solution per 24 plants). Tissue (1 g fresh weight) was collected at the indicated time points and frozen in liquid nitrogen. RNA extraction and Northern blot analysis were performed according to the procedure described by Li et al. (34). Gene-specific probes were prepared by PCR amplification of the corresponding cDNA clones for At1g20510 (OPCL1; U09223), At4g05160 (U19441), At5g-63380 (U24744), OPR3 (U13428), and Actin-8 (At1g49240). cDNA clones for OPCL1, At4g05160, At5g63380, and OPR3 were obtained from the Arabidopsis Biological Resource Center. For other probes, PCR amplification was performed with Arabidopsis genomic DNA as template. Forward and reverse PCR primers used to generate these probes were as follows: At4g05160, 5′-AATCCGGGCTACGGCAGAC-3′ (F) and 5′-TTCACACCTGGACAGCA-3′ (R); At5g63380, 5′-GAGCTCGGGCTTCTGATCA-3′ (F) and 5′-GGAGCAGGCAACCCGGAGAT-3′ (R); At1g20510, 5′-CTTCCCACAGGT-3′ (F2) and 5′-ACTGCCATAATCATTGCA-3′ (R2); At1g20480, 5′-AAGACGTGATCCGGTAGTGAAGCAGGGCT-3′ (F) and 5′-TCCAGCCACACAGTGGCACA-3′ (R); At1g20490, 5′-TGTTTGGATGACCTGAAAGG-3′ (F) and 5′-TGTTTGGATGACCTGAAAGG-3′ (R). PCR analysis confirmed the homozygosity of the T-DNA insert in all mutant lines.

**Jasmonate Measurements**—Rosette leaves were wounded and harvested for jasmonate determinations as described above. Fresh leaf tissue (100–200 mg) was transferred to a Fastprep tube (Qiogene, Carlsbad, CA) containing Zirnml beads (Saint-Gobain ZirPro, Mountainside, NJ) and 50 ng of dihydrojasmic acid (dhJA) as an internal standard. Tubes were
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capped, frozen in liquid nitrogen, and stored at -80 °C until extraction. Jasmonates were extracted and analyzed according to the vapor-phase extraction procedure (35), with minor modifications as described previously (34). Endogenous JA levels were quantified using a calibration curve for comparing detector responses of MeJA and dhMeJA (36). The combined peak area of the cis and trans isomers of each compound was used for quantification. Because recovery of the dhJA internal standard may not accurately reflect the recovery of OPDA and OPC-8:0 from plant tissue, levels of OPDA and OPC-8:0 were expressed as a relative quantity rather than as an absolute value. Specifically, the relative level of OPDA in extracts from WT and opcl1 plants was calculated by dividing the total GC peak area (cis and trans isomers) of OPDA by the total peak area of the dhJA internal standard. Similarly, the relative level of OPC-8:0 in the extracts was calculated by dividing the total GC peak area (cis and trans isomers) of OPC-8:0 by the peak area of the dhJA. The m/z of [M + H]+ ions and retention times of analyzed methyl esters were as follows: JA (m/z = 225), 7.62 and 8.17 min for trans and cis isomers, respectively; dhJA (m/z = 227), 7.68 and 8.20 min for trans and cis isomers, respectively; OPDA (m/z = 307), 34.69 and 35.22 min for trans and cis isomers, respectively; OPC-8:0 (m/z = 309), 33.45 and 34.17 min for trans and cis isomers, respectively. dhJA was synthesized from JA (Sigma) according to the platinum-catalyzed reduction method (36). dhJA isomers were chemically synthesized as described previously (37).

Expression and Purification of Recombinant OPCL1 — A full-length OPCL1 cDNA clone was PCR-amplified with the primer set of BamHI (5'-CGCGGATCCATGGCTTCAGTGAATTCT-3') and SalI (5'-CAAGATGGCTTCAGTGAATTCT-3') restriction sites for cloning. The resulting 1641-bp PCR product was ligated into the single BamHI site, which places the fusion gene under the control of the cauliflower mosaic virus 35S promoter. This modified pBI121 vector was constructed as follows. Two pairs of restriction enzyme sites for cloning were obtained from E. coli cells expressing the empty pQE30 vector were subject to the nickel affinity chromatography procedure purified to yield 0.1% Triton X-100 and <0.01% ethanol. Reaction mixtures (700 μl) contained 0.1 M Tris-HCl (pH 7.5), 2 mM dithiothreitol, 5 mM ATP, 10 mM MgCl2, 0.5 mM CoA, 0.1 to 0.4 mM NADH, 100 μM fatty acyl substrate, 1 mM phosphoenolpyruvate, and 10 units each of myokinase, pyruvate kinase, and lactate dehydrogenase. The reaction was initiated by adding ~10 μg of purified OPCL1. Oxidation of NADH was monitored at 340 nm with a Beckman DU530 spectrophotometer (Fullerton, CA). The assay was validated with a commercial acyl-CoA synthetase (Sigma) from Pseudomonas and 14:0 as a substrate. Heat-killed enzyme was used to determine the rate of non-enzymatic NADH oxidation. Alternatively, total lyase obtained from E. coli cells expressing the empty pQE30 vector was used as a mock-enzyme treatment. Pyruvate kinase (from rabbit muscle), myokinase (from chicken muscle), l-lactate dehydrogenase (from rabbit muscle), and fatty acyl substrates were obtained from Sigma. 12-OPDA and OPC-8:0 were chemically synthesized as described previously (37) and were >97% pure. GC-MS analysis showed that the ratio of cis to trans-OPDA isomers was ~25:1. The ratio of cis- to trans-OPC-8:0 isomers was ~1.5:1.

Construction of Transgenic Plants Expressing Yellow Fluorescent Protein (YFP)-tagged OPCL1 — To determine the subcellular localization of OPCL1, we constructed a transgenic line of Arabidopsis expressing a derivative of the yellow fluorescent protein that is fused to the N terminus of OPCL1. The open reading frame of YFP was PCR-amplified from the 5'-CGCGGATCCATGGTGAGCAAGGGCGAG-3' (SalI site underlined) and 5'-CTTGTACAGCTCGTCATGAGAGAGGGAAGAGGCGAGGAGA-3' (BamHI site underlined) primers and ligated into the BamHI site of the pBI121 vector. The resulting open reading frame was PCR-amplified from the full-length cDNA with the primer set of 5'-GACCAGGCTGAAGATGGAATTCT-3' and 5'-GGATGCCTAAAGCTTGGATGTTGAGA-3' (an overhang encoding overlapping sequence with YFP is underlined) and 5'-GGATGCCTAAAGCTTGGATGTTGAGA-3' (BamHI site underlined). The fusion between YFP and OPC-8:0 was created in a second PCR in which eqimolar amounts of the two products from the first round of PCR were used as template with the primer set of 5'-CGCGGATCCATGGTGAGCAAGGGCGAGGAGA-3' and 5'-GGATGCCTAAAGCTTGGATGTTGAGA-3' (BamHI site underlined). The resulting product was cloned into the BamHI restriction site of a modified pBII121 vector containing a single BamHI site, which places the fusion gene under the control of the cauliflower mosaic virus 35S promoter. This modified pBII121 vector was constructed as follows. Two pairs of complementary primers (pair 1: 5'-GATCCACTAGTCTCGAG-3' and 5'-ACGTGCTCGAGACTAGTG-3'; pair 2: 5'-CAGCGGTGCTCAGGATCAGCTGACTAGTG-3' and 5'-GCCTCGAGCTACGCA-3') were annealed and ligated to a pBII121 vector that was digested with BamHI and SalI. This manipulation removed the GLU gene and introduced a new set of restriction enzyme sites for cloning.

The GFP-OPR3 fusion gene was constructed as follows. The open reading frame of OPR3 was PCR amplified from the cDNA template with the primer set of 5'-GGGCTCGAGATGACG-
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GGGGCACAAGGGAA-3’ (XhoI site underlined) and 5’-CC-
CACCAGATTGACCAGGCGGAAAGGAG-3’ (SphI site
underlined). The resulting product was cloned into the XhoI and
SpeI restriction sites of vector pTA7002. A GFP open reading
frame containing 5’ and 3’ XhoI overhangs was amplified from
plasmid pEGAD (CD3-389, Arabidopsis Biological
Resource Center) with the primer set of 5’-AAACTCGAGAT-
GTTGACGAGCGGCCAGGA-3’ and 5’-AAGCTTCTCGAG-
GCCGGGAATTCGG-3’ (XhoI sites underlined). This
product was cloned into the XhoI site of the OPR3-containing
pTA7002 vector described above. The YFP-OPCL1 and GFP-
OPR3 constructs were transformed into Agrobacterium tume-
faciens strain C58C1. The floral dip method (40) was used for
Agrobacterium-mediated transformation of Arabidopsis Col-0.
T1 generation plants expressing YFP-OPCL1 and T2 genera-
tion plants expressing green fluorescent protein (GFP)-OPR3
were used for confocal imaging experiments.

Confocal Microscopy—Leaf and root tissue of 15–20-day-old
transgenic Arabidopsis plants expressing the fluorescent pro-
teins were hand-sectioned with a razor blade and mounted in
distilled water between a slide and coverslip. Confocal fluores-
cence images of the specimens were taken with a Zeiss LSM5
Pascal laser-scanning confocal microscope (Carl Zeiss, Jena,
Germany) equipped with an argon laser. Chloroplast autofluo-
rescence was excited with a 488-nm argon laser and was
detected after passage through a long-pass 650-nm emission
filter. GFP and GFP fluorescence was excited with a 488-nm
laser and was detected after passage through a band pass 505–
530-nm emission filter. MitoTracker Orange CM-H$_2$TMRos
(Molecular Probes Inc.) was used to stain mitochondria accord-
ing to the manufacturer’s instructions. Fluorescence from
MitoTracker-treated root tissue was detected with a 543-nm
helium neon laser and a band pass 560–615-nm filter.

RESULTS

Identification of an AAE Gene That Is Co-regulated with
Other JA Biosynthetic Genes—To facilitate the discovery of new
genes involved in jasmonate biosynthesis, we took advantage of the
fact that many components of the pathway are coordinately
regulated in Arabidopsis (30, 32, 41, 42). The data-mining tool
Expression Angler uses publicly available Affymetrix Arabi-
dopsis GeneChip data to compare the expression pattern of a
given query gene to all other elements in the data set (27). Query
sets, stress and pathogen-related data sets, and hormone appli-
cation experiments (supplemental Table S2; data not shown).
Based on the results presented below, we propose the trivial
name OPC-8: CoA Ligase I (OPCL1) to describe the biochemical
function of At1g20510.

Co-expression of OPCL1 with OPR3 in wound- and
JA-treated Arabidopsis leaves was verified by RNA blot analysis
(Fig. 2). OPCL1 and OPR3 transcript levels peaked 1 h after
wounding or JA treatment and declined steadily thereafter.
OPCL1 and OPR3 were not induced in wounded or JA treated
leaves of the coi1-1 mutant that is defective in JA signaling (46).
Rather, a low basal level of expression of both genes was
detected in coi1 leaves (Fig. 2). These results demonstrate that
the expression of OPCL1 is tightly coordinated with that of
OPR3 via the jasmonate signaling pathway.

Physiological Role of OPCL1 in Jasmonate Biosynthesis—To
determine whether OPCL1 has a physiological role in JA syn-
thesis, a mutant line (designated opcl1-1) for this gene was
obtained from the collection of sequence-indexed T-DNA
insertions (47). We also characterized insertion mutants for the
two above-mentioned 4CL-like genes (At4g05160 and
At5g63380) that were previously implicated in JA biosynthesis
(39). PCR analysis confirmed the homozygosity of the T-DNA
insert in all three mutant lines (see “Experimental Procedures”
and supplemental Fig. S1). None of these lines exhibited obvi-
ous defects in growth, development, or reproduction (data not
shown). Using RNA blot analysis with gene-specific probes for

![FIGURE 1. Phylogeny of the 4CL and 4CL-like subfamily of AAEs in Arabidopsis. The deduced full-length amino acid sequences were aligned by ClustalW and displayed as an unrooted neighbor-joining phylogenetic tree constructed as described previously (34). To test branching order reliability, 500 neighbor-joining bootstrap replicates were analyzed. Bootstrap values are displayed for each branching point. The phylogeny illustrates the separation of 4CL and 4CL-like sequences into two distinct groups, designated clade IV and V, respectively (44). 4CL-like members that were characterized by Schneider et al. (39) are underlined. Numbers in parentheses give the percent amino acid sequence identity/similarity of each 4CL-like member in comparison to At1g20510 (OPCL1; boxed).](image-url)
OPCL1, At4g05160, and At5g63380, we found no transcripts in the corresponding mutant line, indicating that we were assessing the null phenotype for each mutant (Fig. 3). These experiments also showed that At4g05160 and At5g63380 are expressed at a basal level in unwounded leaves of WT plants. In contrast to OPCL1, however, these two genes were not induced by mechanical wounding.

To assess the effect of the insertion mutations on JA production, we used GC-MS to measure the endogenous levels of JA in mutant and WT plants grown under identical conditions. JA levels in control and wounded leaves of insertion lines corresponding to At4g05160 and At5g63380 were not significantly different from WT levels (Fig. 4). We also found that a double mutant defective in both At4g05160 and At5g63380 produced normal levels of JA in response to wounding (data not shown). In contrast to these mutant lines, opcl1-1 plants showed a significant reduction in wound-induced JA accumulation (Fig. 4). The level of JA in wounded opcl1-1 leaves at each time point (30, 60, and 90 min) tested was 50–60% of that observed in WT leaves. This pattern of JA deficiency was observed in three independent experiments.

To test the hypothesis that OPCL1 functions as a CoA ligase for C18 precursors of JA, we measured the relative levels of OPDA, OPC-8:0, and JA in WT and opcl1-1 plants in response to wounding. OPDA and OPC-8:0 levels in WT plants increased within 30 min of wounding and remained steady during the remainder of the time course (Fig. 5, A and B). OPC-8:0 levels in wounded opcl1-1 leaves were significantly greater (p < 0.01, Student’s unpaired t test) than that in the WT at both the 30- and 60-min time points. This wound-induced hyperaccumulation of OPC-8:0 was associated with decreased amounts of JA in the same extracts (Fig. 5C). The OPDA content in wounded and control opcl1-1 leaves was not significantly different.
different from that in WT plants. To exclude the possibility that the altered JA and OPC-8:0 content of opcl1-1 leaves resulted from a secondary mutation unrelated to the T-DNA insertion, we compared the oxylipin profile in WT and opcl1-1 plants to that in an allelic mutant (designated opcl1-2) that is homozygous for an independent T-DNA insertion (supplemental Fig. S1). The results showed that the wound-induced pattern of OPDA, OPC-8:0, and JA accumulation in opcl1-2 plants exactly matched that of opcl1-1 plants (supplemental Fig. S2). These findings indicate that OPCL1 has a physiological role in wound-induced JA synthesis and further support the idea that OPC-8:0 is an endogenous substrate for the activation step.

OPCL1 Metabolizes C18 Precursors of JA—To confirm that the altered levels of JA and OPC-8:0 in wounded opcl1 leaves result directly from a defect in the CoA activation step, it was necessary to demonstrate that OPCL1 protein has catalytic activity toward JA precursors. To address this question, we expressed recombinant OPCL1 with an N-terminal histidine tag in E. coli and purified the enzyme by nickel affinity chromatography (Fig. 6A). The apparent molecular weight of the purified protein as determined by SDS-polyacrylamide gel electrophoresis was in good agreement with the calculated molecular weight of 59,375. A spectrophotometric assay in which AAE-catalyzed AMP formation is enzymatically coupled to NADH oxidation (38, 39) was used to study the in vitro substrate specificity of purified OPCL1. See “Experimental Procedures” for details. M, molecular weight standards. B, activity of OPCL1 with various substrates. Reaction rates obtained with the empty vector control were subtracted from the rate obtained with 20 μg of recombinant OPCL1. Data for 14:0, 18:1, OPDA, and OPC-8:0 represent the mean ± S.E. of triplicate assays. Data for 6:0 and 8:0 substrates are the average of two independent measurements.
similar to $K_m$ values reported for other 4CL-like enzymes that utilize JA precursors in vitro (39). These results demonstrate that OPCL1 has the capacity to activate OPDA and OPC-8:0 precursors of JA in vitro.

**OPCL1 Is Targeted to Peroxisomes**—Acyl-activating enzymes that supply JA precursors for $\beta$-oxidation are expected to reside in the peroxisome. The predicted sequence of OPCL1 contains a consensus peroxisomal targeting signal type 1 motif (-SKL) at the C terminus (44, 45). To determine whether OPCL1 is targeted to peroxisomes in planta, we constructed a chimeric gene that encodes a protein in which the YFP is fused to the N terminus of OPCL1. This gene was cloned into a binary vector under the control of the cauliflower mosaic virus 35S promoter and subsequently introduced into Arabidopsis by Agrobacterium-mediated transformation. As a control for these experiments, we also analyzed a transgenic line expressing a GFP-OPCL1 fusion construct that is known to be targeted to peroxisomes (19). Confocal imaging of leaf tissue from 35S::YFP-OPCL1 plants showed a punctate pattern of expression that is typical for peroxisome-targeted proteins (49–51) (Fig. 7, C and F). The size and shape of the fluorescent signal in 35S::YFP-OPCL1 leaves was identical to that observed in the 35S::GFP-OPR3 line (Fig. 7, B and E). Staining of root tissue with Mito-Tracker Orange showed that the GFP/YFP-fluorescing structures are distinct from mitochondria (Fig. 7, D–F). These results indicate that the YFP-OPCL1 fusion protein is targeted to peroxisomes.

**Identification of OPCL1-related Genes That Are Induced by Wounding and JA**—The capacity of opr1 mutants to accumulate $\sim$50% of WT levels of JA in wounded leaves (Figs. 4 and 5 and supplemental Fig. S2) indicates that additional enzymes are involved in the activation of JA precursors. Other 4CL-like genes (Fig. 1) that have an expression pattern similar to OPCL1 can be considered good candidates for this function. Results presented in Fig. 3 show that two 4CL-like genes (At4g05160 and At5g63380) are not induced by mechanical wounding. We used RNA blot analysis and gene-specific probes to assess the expression pattern of four additional members of this group, namely At1g20480, At1g20490, At1g20500, and At5g38120. At1g20480 and At1g20500 transcripts were not detected in either undamaged or wounded leaves of WT plants (data not shown). In contrast, At1g20490 and At5g38120 mRNA accumulation was induced both by wounding and JA treatment (Fig. 8A). The time course of expression of these two inducible genes was similar to that of OPCL1 (see Fig. 2).

In summary, these results indicate that three members (OPCL1, At1g20490, and At5g38120) of the 4CL-like gene family are expressed in leaves in response to wounding and JA treatment.

**DISCUSSION**

Metabolic pathways that utilize carboxylic acids often involve enzymes that activate these substrates to their corresponding CoA derivatives. Plant genomes encode a large family of AAEs that catalyze this reaction. The AAE gene superfamily of Arabidopsis thaliana, for example, is comprised of 63 members that have a known or postulated role in the activation of various carboxylic acid substrates (44). Included among these are genes encoding long-chain acyl-CoA synthetases (LACSs) (50), 4-CLs (39, 48, 52), and adenylating enzymes that metabolize phytohormones (53). The large number of AAEs in higher plants presumably reflects the diversity of carboxylic acid-containing compounds involved in primary and secondary metabolism. By analogy to enzymes involved in the $\beta$-oxidation of bulk fatty acids, it has been generally assumed that biosynthetic precursors of JA are converted to their CoA esters prior to entering the peroxisomal $\beta$-oxidation cycle (54, 55). Recent experimental evidence provides support for this idea. An acyl-CoA oxidase (ACX) involved in JA production in tomato leaves metabolizes OPC-8:0-CoA and OPDA-CoA but not the corresponding unesterified compounds (34). It was also reported recently that two 4CL-like AAEs from Arabidopsis have the capacity to activate C18 and C16 precursors of JA in vitro (39). Despite this evidence, enzymes that catalyze the activation of JA precursors in vivo have not been discovered until now.

Data-mining tools allowed us to identify OPCL1 (At1g20510) as a candidate for the peroxisomal CoA activation step of the pathway. Follow-up studies provided several independent lines of evidence to support this hypothesis. First, wound-induced
accumulation of OPCL1 and OPR3 transcripts was co-regulated by the jasmonate signaling pathway. Second, wounded leaves of opcl1-1 and opcl1-2 knock-out mutants exhibited reduced levels of JA and increased levels of OPC-8:0. Third, OPCL1 protein showed robust activity against C18 precursors of JA in vitro. Finally, the predicted peroxisomal targeting of the activating enzyme was verified by localization of a YFP-tagged derivative of OPCL1. We thus conclude that OPCL1 has a physiological role in the activation of JA precursors in leaf peroxisomes. This finding demonstrates the utility of co-expression analysis for identification of uncharacterized genes that contribute to JA synthesis and suggest that this approach may be useful for identifying additional components in the pathway.

Several observations indicate that enzymes other than OPCL1 contribute to the activation of JA precursors. For example, opcl1-1 and opcl1-2 mutants retain the capacity to accumulate ~50% of WT levels of JA in wounded leaves. We also observed that the basal level of JA in undamaged opcl1 leaves was similar to that in WT leaves. Moreover, opcl1 mutants did not exhibit male sterility or other hallmark phenotypes of JA deficiency. It can be hypothesized that functional redundancy in the activation step involves other members of the peroxisome-targeted group of 4CL-like proteins. The wound- and JA-inducible expression pattern of At1g20490 and At5g38120 (Fig. 8A) indicates that these genes are good candidates for such a function. Additional support for this hypothesis comes from the finding that At1g20490 and At5g38120 are co-expressed with other JA biosynthetic genes such as AOS and OPR3 in Affymetrix GeneChip data sets. A potential role for At5g38120 in JA biosynthesis is also consistent with recent work by Costa et al. (48) showing that the enzyme encoded by this gene does not have significant activity against hydroxycinnamoyl CoA intermediates in the phenylpropanoid pathway. Measurement of jasmonate levels in mutants that are defective in At1g20490 and At5g38120 is needed to rigorously test the role of these genes in JA production. To address the question of functional redundancy, it will be informative to analyze mutants that are defective in multiple members of the 4CL-like gene family. In this context, it is noteworthy that genes encoding four of the eight members of this family, including OPCL1, are clustered in a ~12-kilobase region on chromosome 1 (Fig. 8B).

Our results indicate that two of these genes (OPCL1 and At1g20490) are induced by wounding and JA treatment, whereas the other two genes (At1g20480 and At1g20500) in the cluster are either not expressed or are expressed at very low levels in leaves. The close proximity of OPCL1 and At1g20490 may hinder construction of the corresponding double mutant by traditional genetic approaches.

A recent study (39) provided in vitro evidence that 4CL-like members At4g05160 and At5g63380 may have a role in JA biosynthesis. We did not observe a significant JA-deficient phenotype in mutants that are defective in either of these genes singly or both genes in combination. Although results obtained with Expression Angler (27) indicate that At4g05160 and At5g63380 are co-expressed with various genes involved in lipid metabolism, known JA biosynthetic components were not among these co-expressed genes. It thus seems likely that At4g05160 and At5g63380 do not play a significant role in wound-induced JA production but rather may function in other aspects of lipid metabolism. Given the ability of these enzymes to activate JA precursors in vitro (39), however, it is important to consider scenarios in which these isozymes may contribute to JA production in more subtle ways. For example, a JA-deficient phenotype may go undetected in our assays if At4g05160 and At5g63380 contribute to JA synthesis in a specific cell type. That At4g05160 has in vitro activity against OPC-6:0 (39) raises an alternative possibility, namely that the enzyme participates in the conversion of dnOPDA to JA via an OPC-6:0 intermediate. Assuming that the contribution of dnOPDA to total JA production is relatively minor in comparison to JA derived from OPDA (14), a specific defect in the formation of OPC-6:0-CoA may have a subtle impact on total JA content. Detailed analysis of oxylipin levels in well defined mutants should help to address this possibility.

It is generally assumed that OPR3-catalyzed reduction of OPDA to OPC-8:0 occurs prior to the CoA activation step that supplies substrates for β-oxidation (13, 34, 55). This notion is consistent with the fact that OPR3 readily metabolizes unesterified OPDA in vitro (18, 19) and that free OPC-8:0 can be detected in plant tissues (10) (this study). Nevertheless, the substrate for the activation step had not been previously identified. Our analysis of oxylipin levels in opcl1-1 and opcl1-2 leaves provides direct evidence bearing on this question. Specifically, we found that loss of OPCL1 function results in wound-induced hyperaccumulation of OPC-8:0 and reduced JA accumulation but does not significantly affect OPDA levels. The elevated level of OPC-8:0 in wounded opcl1 leaves argues against the possibility that OPCL1 acts at the surface of the peroxisome to supply a pool of OPDA-CoA that is imported into the peroxisome matrix. Rather, we propose that the increased pool of OPC-8:0 is caused by the reduced capacity of opcl1 plants to generate OPC-8:0-CoA. That is, OPC-8:0 is a physiological substrate for OPCL1. Our data thus support a biosynthetic

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scheme in which the order of initial reactions in leaf peroxisomes is OP3 → OPCL1 → ACX (i.e. β-oxidation).

Recombinant OPCL1 showed a similar level of activity with OPDA and OPC-8:0. The lack of strict substrate specificity is supported by the observation that OPCL1 also metabolizes medium-to-long straight-chain fatty acids. This feature of OPCL1 is similar to other 4CL-like enzymes that metabolize JA precursors in vitro (39) and raises the possibility that OPCL1 may perform additional roles in lipid metabolism. These results also indicate that the double bond in the cyclopentane ring of OPDA is not a critical determinant of the in vitro substrate selectivity of OPCL1. A similar result was reported for the tomato JA biosynthetic enzyme ACX1A, which metabolizes both OPDA-CoA and OPC-8:0-CoA in vitro (34).

The inability of OPCL1 to discriminate between OPDA and OPC-8:0 suggests that specificity in the biosynthetic pathway is achieved by other mechanisms. For example, peroxisomal components of JA synthesis may be organized in a manner that channels OPDA across the peroxisomal membrane to OPR3. Substrate channeling through multienzyme complexes is a well established theme in plant metabolism (56). In this context, it is interesting to note that OPDA is excluded from entering the peroxisome as a free acid or as an esterified derivative will achieve by other mechanisms. For example, peroxisomal co-transporters OP3 might perform additional roles in lipid metabolism. These results provide much needed insight into the mechanism by which OPCL1 supplies JA precursors for β-oxidation.

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