A Novel Acyl-CoA Oxidase That Can Oxidize Short-chain Acyl-CoA in Plant Peroxisomes*

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Short-chain acyl-CoA oxidases are β-oxidation enzymes that are active on short-chain acyl-CoAs and that appear to be present in higher plant peroxisomes and absent in mammalian peroxisomes. Therefore, plant peroxisomes are capable of performing complete β-oxidation of acyl-CoA chains, whereas mammalian peroxisomes can perform β-oxidation of only those acyl-CoA chains that are larger than octanoyl-CoA (C8). In this report, we have shown that a novel acyl-CoA oxidase can oxidize short-chain acyl-CoA in plant peroxisomes. A peroxisomal short-chain acyl-CoA oxidase from Arabidopsis was purified following the expression of the Arabidopsis cDNA in a baculovirus expression system. The purified enzyme was active on butyryl-CoA (C4), hexanoyl-CoA (C6), and octanoyl-CoA (C8). Cell fractionation and immunochemical analysis revealed that the short-chain acyl-CoA oxidase is localized in peroxisomes. The expression pattern of the short-chain acyl-CoA oxidase was similar to that of peroxisomal 3-ketoacyl-CoA thiolase, a marker enzyme of fatty acid β-oxidation, during post-germinative growth. Although the molecular structure and amino acid sequence of the enzyme is similar to those of mammalian mitochondrial acyl-CoA dehydrogenase, the purified enzyme has no activity as acyl-CoA dehydrogenase. These results indicate that the short-chain acyl-CoA oxidases function in fatty acid β-oxidation in plant peroxisomes, and that by the cooperative action of long- and short-chain acyl-CoA oxidases, plant peroxisomes are capable of performing the complete β-oxidation of acyl-CoA.

Glyoxysomes and leaf peroxisomes are members of a group of organelles called peroxisomes (2). In glyoxysomes, fatty acids are first activated to fatty acyl-CoA by fatty acyl-CoA synthetase (3). Fatty acyl-CoA is the substrate for fatty acid β-oxidation, which consists of four enzymatic reactions (4). The first reaction is catalyzed by acyl-CoA oxidase. The second and third enzymatic reactions are catalyzed by a single enzyme that possesses enoyl-CoA hydratase and β-hydroxyacyl-CoA dehydrogenase activities (5). The fourth reaction is catalyzed by 3-ketoacyl-CoA thiolase (6). Acetyl-CoA, an end product of fatty acid β-oxidation, is metabolized further to produce succinate by the glyoxylate cycle.

In mammalian cells, both peroxisomes and mitochondria contain a functional fatty acid β-oxidation system. In peroxisomes, the first enzyme of fatty acid β-oxidation, acyl-CoA oxidase, donates electrons to molecular oxygen, producing hydrogen peroxide (7). Mammalian peroxisomes oxidize long-chain fatty acids, but are inactive with fatty acids shorter than octanoic acid (C8). This is mainly the consequence of the exclusive presence of long-chain acyl-CoA oxidases and the absence of acyl-CoA oxidases that are active on short-chain acyl-CoAs. In contrast, mammalian mitochondria are capable of complete oxidation of fatty acids to acetyl-CoA (8); the first step of fatty acid β-oxidation is accomplished by long-, medium-, and short-chain acyl-CoA dehydrogenases, and electrons generated by the dehydrogenases are transferred to the mitochondrial respiratory chain. By analogy, Thomas and co-workers (9–11) have postulated the existence of plant mitochondrial β-oxidation, but the presence of acyl-CoA dehydrogenase was not investigated or not detected (12). In contrast, data reported by Gerhardt and co-workers (13–15) have suggested that glyoxysomes in plants can completely metabolize fatty acids to acetyl-CoA.

We have previously reported the existence of an acyl-CoA oxidase that is active on long-chain acyl-CoA in glyoxysomes (16). In the present study, we report evidence that glyoxysomes contain another acyl-CoA oxidase that can metabolize short-chain acyl-CoA. We also discuss the unique features of fatty acid β-oxidation accomplished by these acyl-CoA oxidases in plant cells.

EXPERIMENTAL PROCEDURES

Plant Materials—Pumpkin seeds (Cucurbita sp. Kukawakara) were purchased from Aisan Seed Co. (Aichi, Japan). Pumpkin seeds were soaked in running tap water overnight and germinated in Rock-Fiber soil (66R, Nitto Boseki, Chiba, Japan) at 25 °C in darkness. Arabidopsis thaliana ecotype Landsberg erecta seeds were surface-sterilized in 2% NaClO and 0.02% Triton X-100 and grown on growth medium (2.3 mg/ml Murashige-Skoog salts (Wako, Osaka, Japan), 1% sucrose, 100 μg/ml mycinositol, 1 μg/ml thiamine HCl, 0.5 μg/ml pyri-

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doxine, 0.5 μg/ml nicotinic acid, 0.5 mg/ml Mes-1-KOH, pH 5.7, and 0.2% Gellan gum (Wako) in Petri dishes. Arabidopsis seeds were soaked in growth medium and germinated at 22 °C under continuous illumination or under darkness, and some of Arabidopsis seedlings were transferred to light after 4 days of growing in the dark. Some seedlings that were grown under continuous illumination or under darkness were grown under continuous illumination at 22 °C. Plasmids—The cDNA clone (GenBank™ accession number T46525) was obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH). DNA sequencing was performed by the service of Sanger et al. (117). DNA sequencing was performed using GeneWorks Release 2.5 computer software (IntelliGenetics, Mountain View, CA). The BLAST server was utilized for the analysis of homology among proteins. Alignment of several acyl-CoA oxidases and acyl-CoA dehydrogenases was performed using CLUSTAL W software (18).

Preparation of a Specific Antiserum—The Arabidopsis cDNA was inserted into pET32b vector (Novagen, Madison, WI). A fusion protein between short-chain acyl-CoA oxidase and a histidine tag was synthesized in Escherichia coli cells and purified by column chromatography on Ni²⁺ resin. The purified protein (~0.5 mg of protein) in 1 ml of sterilized water was emulsified with an equal volume of Freund’s complete adjuvant (Difco). The emulsion was injected subcutaneously into the back of a rabbit. Four weeks later, a booster injection (~0.25 mg of protein) was given similarly to the first injection. Blood was taken from a vein in the ear 7 days after the second booster injection. The serum was used for immunoblotting.

Expression of Recombinant Short-chain Acyl-CoA Oxidase from Insect Cells—Short-chain acyl-CoA oxidase was produced employing the baculovirus expression system from In vitrogen (San Diego, CA) following the manufacturer’s protocols. The system includes Spodoptera frugiperda (Sf9) as the insect cell line, pBlueBac 4.5 (19) as a transfer vector, and engineered baculoviral Autographa californica multiple polyhedrosis virus (Bac-N-Blue DNA) as an expression vector. In brief, the short-chain acyl-CoA oxidase cDNA was inserted into the pBlueBac 4.5 transfer vector and co-transfected together with linearized baculoviral Bac-N-Blue DNA in insect cells. Recombinant viruses were purified from the transfaction supernatant by plaque assay on medium containing 5-bromo-4-chloro-3-indolyl β-D-galactopuransidase, and recombinant plaques were verified by polymerase chain reaction. Afterward, a high-titer recombinant viral stock was generated, and following a time course of expression experiment, the optimal expression time was determined. The recombinant protein expression levels were optimized, and a large-scale expression of recombinant protein was performed.

Purification of Recombinant Short-chain Acyl-CoA Oxidase from Insect Cells—Log-phase growing Sf9 cells in 20 75-cm² flasks were infected with recombinant viral stock at a multiplicity of infection of 10. Four days after infection, the cells were dislodged from the flask walls and centrifuged at 500 × g for 5 min at 4 °C. The cell pellets were washed with phosphate-buffered saline, gently suspended in buffer A (50 mM sodium phosphate, pH 6.7, 10 mM NaCl, 100 μM phenylmethylsulfonyl fluoride, 10 μM FAD, and 10% glycerol), and lysed by three bursts of sonication (3 × 1 min at 30-min intervals on ice). After centrifugation of the sample at 15,000 × g for 30 min, the supernatant was dialyzed against buffer A and loaded on a Hitrap SP column (Amersham Pharmacia Biotech, Tokyo, Japan). Proteins were eluted with a gradient of 10–500 mM NaCl in buffer A, and fractions of 0.5 ml were collected. Fractions with high short-chain acyl-CoA oxidase activities were pooled and concentrated using Centricron 30 concentrators (Amicon Inc., Beverly, MA) and then loaded on a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech) equilibrated with buffer B (10 mM sodium phosphate, pH 7.2, 250 mM NaCl, 10 μM FAD, and 10% glycerol). Proteins were eluted with buffer B, and fractions of 0.5 ml were collected and analyzed for the presence of acyl-CoA oxidase activity.

Subcellular Fractionation—Four-day-old pumpkin etiolated cotyledons (15 g, fresh weight) were homogenized in a Petri dish by chopping with a razor blade for 5 min in 10 ml of a medium that contained 150 mM Tricine-KOH, pH 7.5, 1 mM EDTA, and 0.5 mM sucrose. The homogenate was filtered through four layers of cheesecloth. Three hundred mg of filtrate was layered onto a sucrose gradient that consisted of a 1-ml cushion of 60% (w/v) sucrose and 11 ml of a linear sucrose gradient from 60 to 30% without buffer. The gradient was centrifuged at 21,000 rpm for 3 h in a Beckman SW 28.1 rotor in a Beckman Model XL-90 ultracentrifuge. After centrifugation, fractions of 0.5 ml were collected with a gradient fractionator (Model 185, Isco Inc., Lincoln, NE). All procedures were carried out at 4 °C. Subcellular fractionation of Arabidopsis etiolated cotyledons was performed as follows. One-hundred mg of seeds (~5000 seeds) was grown on growth medium for 5 days in darkness at 22 °C. Etiolated cotyledons were harvested and chopped with a razor blade in a Petri dish with 2 ml of chopping buffer (150 mM Tricine-KOH, pH 7.5, 1 mM EDTA, 0.5 mM sucrose, and 1% bovine serum albumin). The extract was then filtered with a cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ). Two ml of the homogenate was layered directly on top of a 16-ml linear sucrose density gradient (30–60%, w/v) that contained 1 ml EDTA. Centrifugation was performed in the SW 28.1 rotor at 25,000 rpm for 2.5 h at 4 °C. Fractions of 0.5 ml were collected with the gradient fractionator.

Immunoelectron Microscopy—Arabidopsis etiolated cotyledons were harvested after 3 days in darkness. The samples were fixed, dehydrated, and embedded in LR white resin (London Resin, Basingstoke, United Kingdom) as described previously (20, 21). Ultrathin sections were cut on a Reichert ultracrotome (Leica, Heidelberg, Germany) with a diamond knife and mounted on uncoated nickel grids. The protein A-gold labeling procedure was essentially the same as that described (20, 21). Ultrathin sections were incubated at 4 °C overnight with a 20-fold diluted suspension of catalase (15 nm for short-chain acyl-CoA oxidase from Arabidopsis; Amersham Pharmacia Biotech) at room temperature for 30 min. The sections were examined with a transmission electron microscope (1200EX, Joel, Tokyo) at 80 kV.

Enzyme Assay and Isoelectric Focusing—Enzyme activities were measured at 25 °C in 1 ml of reaction mixture and monitored with a Beckman DU-7500 spectrophotometer. Acyl-CoA oxidase (EC 1.3.3.8) was assayed according to the method of Gerhardt (22), with the concentration of acyl-CoA substrates reduced to 25 μM. Acyl-CoA dehydrogenase (EC 1.3.99.3) was assayed according to Dommes and Kunau (23) and Furuta et al. (24). Catalase (EC 1.11.1.6) was assayed according to Aebi (25). Cytoschrome c oxidase (EC 1.9.3.1) was assayed according to Hodges and Leonard (26). Isoelectric focusing was performed at 15 °C using a Multiphor II electrophoresis system and Immobiline Dry Strip (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

Western Blot Hybridization—Arabidopsis and pumpkin cotyledons were homogenized in extraction buffer (0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.1% SDS); the homogenate was centrifuged at 15,000 × g for 20 min; and the supernatant was subjected to SDS-polycrylamide gel electrophoresis. Immunoblot analysis was then performed essentially following the method of Towbin et al. (27). Immunologic reactions were detected by monitoring horseradish peroxidase activity (ECL system, Amersham Pharmacia Biotech). Thiolase (28), castor bean isocitrate lyase (29), and pumpkin catalase (30) antisera were prepared as described previously. Protein was quantitated with a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo).

RESULTS

Identification of a Short-chain Acyl-CoA Oxidase cDNA—As result of a similarity search with a long-chain acyl-CoA oxidase (16) in a DNA data base, we found a putative Arabidopsis acyl-CoA dehydrogenase cDNA and the availability of another homologous cDNA clone (EBI/GenBank™ accession number T46525, AB017643) in the Arabidopsis Expressed Sequence Tag data base. We received the latter from the Arabidopsis Biological Resource Center and fully sequenced it. The expressed sequence tag clone contained an insert of 1.6 kilobases. The open reading frame encodes a polypeptide of 436 amino acids, which corresponds to a molecular mass of ~47 kDa (Fig. 1). Because mammalian acyl-CoA dehydrogenase is a mito-

1 The abbreviations used are: Mes, 4-morpholinoethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PTS, peroxisomal targeting signal; PS, protein signature.

2 Grellet, F., Gabier, P., Wu, H.-J., Laudie, M., Berger, C., and Delseny, M. (1996) EBI/GenBank™ accession number U72905.
chondrial enzyme, this putative acyl-CoA dehydrogenase was thought to be localized in plant mitochondria. However, we failed to find a mitochondrial targeting signal in the amino acid sequence. Instead, a typical peroxisomal targeting signal (PTS1) was present at the carboxyl terminus (SRL) (Fig. 1, boxed). Therefore, we postulated that this cDNA encodes a short-chain acyl-CoA oxidase. The GenBank™ accession number for Arabidopsis short-chain acyl-CoA oxidase is AB017843.

As shown in Fig. 3, the purified protein showed oxidase activity toward acyl-CoAs from butyryl-CoA (C4) to octanoyl-CoA (C8). The maximum activity was observed when hexanoyl-CoA (C6) was used for the substrate. The activity was monitored employing various acyl-CoAs as substrates at a concentration of 25 μM. CoA (C6) was used for the substrate. The $K_m$ value for hexanoyl-CoA was estimated at 8.3 μM (Table II). No activity was observed employing crotonoyl-CoA (C4,1), an unsaturated carboxylic ester) or glutaryl-CoA (a dicarboxylic ester). The enzyme was active on isobutyryl-CoA at a concentration of 67 μM (2.5 units/mg). Furthermore, we detected no acyl-CoA dehydrogenase activity when hexanoyl-CoA (C6), decanoyl-CoA (C10), and palmitoyl-CoA (C16) were used as substrates. These data indicated that this Arabidopsis cDNA encodes a short-chain acyl-CoA oxidase.

Gel-filtration chromatography of the short-chain acyl-CoA oxidase on a Superose 12 HR 10/30 column indicated a native molecular mass of ~180 kDa (Table II). Because the subunit molecular mass of the short-chain acyl-CoA oxidase is 47 kDa,
the purified enzyme must be a homotetramer. The highest activity was observed between pH 8.5 and 9.0. Table II summarizes the characteristics of the short-chain acyl-CoA oxidase.

Interestingly, the alignment of the conserved regions of acyl-CoA oxidases and acyl-CoA dehydrogenases revealed that the short- and long-chain acyl-CoA oxidases have conserved signatures for mammalian acyl-CoA dehydrogenase (PS1, (G/A/C)-L/I/V/M/F/Y), short- and long-chain acyl-CoA oxidases have conserved signatures for mammalian medium-chain (Glut 376) and short-chain (Glut 368) acyl-CoA dehydrogenases serve as the α-proton-abstracting base (33–36). Both the Arabidopsis short-chain and pumpkin long-chain acyl-CoA oxidases contain a glutamic acid residue in a corresponding position (Fig. 4A, asterisk). To analyze the similarity between acyl-CoA oxidases and acyl-CoA dehydrogenases, we compared amino acid sequences of plant acyl-CoA oxidases with human acyl-CoA oxidases and acyl-CoA dehydrogenases. A phylogenetic tree indicates that the plant short-chain acyl-CoA oxidase is clustered together with mitochondrial acyl-CoA dehydrogenases, whereas it is relatively far from other peroxisomal acyl-CoA oxidases (Fig. 4B).

**Subcellular Localization of Short-chain Acyl-CoA Oxidase**—To investigate the subcellular localization of the short-chain acyl-CoA oxidase, homogenates from 5-day-old Arabidopsis etiolated cotyledons were subjected to sucrose density centrifugation. Fractions thus obtained were analyzed using an immunoblot technique with antibodies raised against short-chain acyl-CoA oxidase and catalase. Catalase was used as a glyoxysomal marker enzyme. As shown in Fig. 5A, short-chain acyl-CoA oxidase and catalase were present together in fractions 21–23.

Although these enzymes were detected in the first few fractions (top of the gradient), this may be due to disruption of the glyoxysomes during homogenization and subsequent cell fractionation. We confirmed this result using 5-day-old pumpkin etiolated cotyledons. As is the case with Arabidopsis, a short-chain acyl-CoA oxidase was detected in fractions 21–23 by the immunoblot technique (Fig. 5B). These fractions had short-chain acyl-CoA oxidase as well as catalase activities. In contrast, no short-chain acyl-CoA oxidase activity was detected in fractions 8–13, which correspond to the activity of a mitochondrial marker enzyme, cytochrome c oxidase.

Fig. 6 shows an immunoelectron microscopic observation of short-chain acyl-CoA oxidase and catalase in cotyledon cells of Arabidopsis etiolated seedlings. Double staining by polyclonal antibodies against Arabidopsis short-chain acyl-CoA oxidase (arrow) and pumpkin catalase (arrowhead) revealed that both enzymes are co-localized in glyoxysomes. No signal was detected on other organelles. These results clearly indicated that the short-chain acyl-CoA oxidase is exclusively localized in glyoxysomes.

**Table II**

Properties of Arabidopsis short-chain acyl-CoA oxidase

| Subunit molecular mass | 47 kDa |
| Native molecular mass | 180 kDa |
| pH | 9.5 |
| $K_m$ | 8.3 μM |
| Optimal pH | 8.5–9.0 |
| Acyl-CoA dehydrogenase activity | None |
| Subcellular localization | Peroxisomes |

![Figure 4](image4.png)

**Fig. 4. Partial alignment of acyl-CoA oxidases and acyl-CoA dehydrogenases (A) and phylogenetic tree of acyl-CoA oxidases and acyl-CoA dehydrogenases (B).** White letters indicate corresponding PS1 and PS2 amino acids. The PS1 and PS2 regions are underlined. PS1 has the form (G/A/C)-L/I/V/M/F/Y-D/E/N-T/R/K (D/E) (32). Multiple sequence alignments of the protein sequences were performed using the CLUSTAL W program. The phylogenetic tree was constructed according to the NJPLOT program. AtSACOX, Arabidopsis short-chain acyl-CoA oxidase (GenBank® accession number AB017643); PumLACOX, pumpkin long-chain acyl-CoA oxidase (accession number AF002016); PhaAcoX, Phalaenopsis acyl-CoA oxidase (accession number U66299); HumAcoX, human acyl-CoA oxidase (accession number S69198); HumBACO, human branched-chain acyl-CoA oxidase (accession number X95190); HumVLACDH, human very long-chain acyl-CoA dehydrogenase (accession number D43682); HumLACDH, human long-chain acyl-CoA dehydrogenase (accession number M74096); HumMACDH, human medium-chain acyl-CoA dehydrogenase (accession number M18527); HumSACDH, human short-chain acyl-CoA dehydrogenase (accession number M26393); HumSBCADH, human short-branched-chain acyl-CoA dehydrogenase (accession number U12778).

**Developmental Changes in the Level of Short-chain Acyl-CoA Oxidase**—Fig. 7 shows changes in the levels of short-chain acyl-CoA oxidase during the post-germinative growth of the Arabidopsis seedlings. An immunoblot analysis of Arabidopsis seedlings grown in the dark showed that short-chain acyl-CoA oxidase as well as thiolase, another enzyme for fatty acid β-oxidation, reached a maximum level after 5–7 days of growth. These enzymes were still present in the seedlings after 9 days of growth in the dark. After illumination of the seedlings was started, the amount of these enzymes decreased, but faint bands were still detectable after 5 days of illumination (Fig. 7, 4D5L). Instead, isocitrate lyase, an enzyme of the glyoxylate cycle, reached a maximum level earlier than short-chain acyl-CoA oxidase (3 days after germination) and completely disappeared after 9 days in the dark.

**Presence of Short-chain Acyl-CoA Oxidase in Various Organs**—Short-chain acyl-CoA oxidase was particularly abundant in 5-day-old Arabidopsis etiolated cotyledons (Fig. 8, upper panel, lane 1). This enzyme was also present in flowers, roots, and siliques (lanes 4, 5, and 7), whereas it was present at very low levels or not at all in 7-day-old green cotyledons, rosette leaves, and stems (lanes 2, 3, and 6). The expression pattern of the thiolase was essentially similar to that of short-chain acyl-CoA oxidase, except that a band was detected at certain levels in 7-day-old green cotyledons, rosette leaves, and...
In contrast, isocitrate lyase was detected only in extracts from etiolated cotyledons (Fig. 8, lower panel).

**DISCUSSION**

In higher plants with fatty seeds such as pumpkin, the triacylglycerols are stored in lipid bodies. During germination, the fatty acids are liberated by lipase and then degraded by the \( \beta \)-oxidation system in the glyoxysomes, and the resulting acetyl-CoA is further metabolized by the glyoxylate cycle. Thus, fatty acids serve as the main source for energy and carbon compounds. Therefore, fatty acid \( \beta \)-oxidation plays an important role in metabolism until the etiolated cotyledons turn green during late germination. To use storage lipids efficiently, fatty acids need to be completely converted from acyl-CoA to acetyl-CoA by fatty acid \( \beta \)-oxidation. Because most storage lipids are long-chain molecules (C\(_{16}\)–C\(_{18}\)) in higher plants, the first step in fatty acid \( \beta \)-oxidation begins with long-chain acyl-CoA oxidase, and for the shorter acyl-CoAs, short-chain acyl-CoA oxidase.

**FIG. 5.** Subcellular localization of short-chain acyl-CoA oxidase in *Arabidopsis* (A) and pumpkin (B) etiolated cotyledons. Both extracts from 5-day-old etiolated cotyledons were fractionated by sucrose density gradient centrifugation. The arrowheads indicate the bands corresponding to the short-chain acyl-CoA oxidase. A, immunological detection of *Arabidopsis* short-chain acyl-CoA oxidase (SACOX) and catalase; B, immunological detection of pumpkin short-chain acyl-CoA oxidase and enzyme activities. \( \text{C} \), short-chain acyl-CoA oxidase; \( \text{A} \), catalase; \( \text{F} \), cytochrome c oxidase; \( \text{M} \), sucrose concentration (w/w). Twenty \( \mu l \) (Arabidopsis short-chain acyl-CoA oxidase) and 5 \( \mu l \) (pumpkin short-chain acyl-CoA oxidase and catalase) of samples from each odd-numbered fraction were subjected to SDS-polyacrylamide gel electrophoresis (10% acrylamide) and immunoblotting.

**FIG. 6.** Immunoelectron microscopic analysis of the localization of *Arabidopsis* cotyledons of 3-day-old dark-grown seedlings using polyclonal antibodies against *Arabidopsis* short-chain acyl-CoA oxidase and pumpkin catalase. Mt, mitochondria; G, glyoxysome; L, lipid body. The arrows indicate short-chain acyl-CoA oxidase (15-nm gold particles), and the arrowheads indicate catalase (10-nm gold particles). Bar = 1 \( \mu m \).

**FIG. 7.** Developmental changes in the levels of short-chain acyl-CoA oxidase, thiolase, and isocitrate lyase in *Arabidopsis* cotyledons. \( D \) indicates the days of growth in the dark. \( L \) indicates the days of continuous illumination following 4 days in the dark. Each lane was loaded with 10 \( \mu g \) (short-chain acyl-CoA oxidase) or 5 \( \mu g \) (thiolate, isocitrate lyase) of total proteins extracted from *Arabidopsis* cotyledons. Electrophoresed proteins were blotted on a nylon membrane, and then the membrane was allowed to hybridize with polyclonal antibodies raised against recombinant *Arabidopsis* short-chain acyl-CoA oxidase (SACOX; A), pumpkin thiolase (B), and castor bean isocitrate lyase (ICL; C).

**FIG. 8.** Short-chain acyl-CoA oxidase, thiolase, and isocitrate lyase expression in various *Arabidopsis* tissues. Each lane was loaded with 10 \( \mu g \) of total proteins. The tissues indicated below were excised from 5-week-old plants, except cotyledons, which were excised 5 or 7 days after sowing. SACOX, short-chain acyl-CoA oxidase; thi, thiolase; ICL, isocitrate lyase. Lane 1,* Arabidopsis* etiolated cotyledons from plants grown in the dark for 5 days; lane 2, green *Arabidopsis* cotyledons from plants grown in the dark for 4 days, followed by 3 days in the light; lane 3, rosette leaves; lane 4, flowers; lane 5, roots; lane 6, stems; lane 7, siliques.

**SACOX**

**Thi**

**ICL**

**mt**

**G**

**L**

**Mt**

**DISCUSSION**

In higher plants with fatty seeds such as pumpkin, the triacylglycerols are stored in lipid bodies. During germination, the fatty acids are liberated by lipase and then degraded by the \( \beta \)-oxidation system in the glyoxysomes, and the resulting acetyl-CoA is further metabolized by the glyoxylate cycle. Thus, fatty acids serve as the main source for energy and carbon compounds. Therefore, fatty acid \( \beta \)-oxidation plays an important role in metabolism until the etiolated cotyledons turn green during late germination. To use storage lipids efficiently, fatty acids need to be completely converted from acyl-CoA to acetyl-CoA by fatty acid \( \beta \)-oxidation. Because most storage lipids are long-chain molecules (C\(_{16}\)–C\(_{18}\)) in higher plants, the first step in fatty acid \( \beta \)-oxidation begins with long-chain acyl-CoA oxidase, and for the shorter acyl-CoAs, short-chain acyl-
CoA oxidase takes the place of long-chain acyl-CoA oxidase. Thus, higher plants make efficient use of storage lipids to produce carbon and energy sources. In this study, we characterized an *Arabidopsis* peroxisomal short-chain acyl-CoA oxidase and its cDNA. The presence of a peroxisomal short-chain acyl-CoA oxidase explains how higher plant peroxisomes are able to completely oxidize fatty acids by a β-oxidation system.

In mammalian cells, fatty acid β-oxidation is localized both in peroxisomes and in mitochondria. The presence of a short-chain acyl-CoA oxidase distinguishes the peroxisomal β-oxidation of higher plants from that of mammals. In fact, mammalian peroxisomes contain three acyl-CoA oxidase isoforms that act on CoA derivatives of fatty acids with chain lengths from C₉ to C₁₈ and that are inactive in oxidizing acyl-CoA esters with carbon chains shorter than 8 carbons. Short-chain fatty acids (C₇-C₉) that could not be oxidized by these peroxisomal acyl-CoA oxidases are transported to mitochondria (7). The mitochondrial β-oxidation system is able to completely degrade fatty acids from long- to short-chain fatty acids (37).

Common features of the amino acid sequences of the *Arabidopsis* short-chain acyl-CoA oxidase and the mammalian mitochondrial short-chain acyl-CoA dehydrogenase are shown in Fig. 4 and can be summarized as follow: (a) the presence of the two acyl-CoA dehydrogenase protein signatures (PS1 and PS2) in both enzymes; (b) a 35% identity between acyl-CoA oxidase and acyl-CoA dehydrogenase; and (c) similar subunit molecular masses. However, the short-chain acyl-CoA oxidase differs from pumpkin long-chain acyl-CoA oxidase (16), not considering the subunit specificity, as follows: (a) a subunit molecular mass of 47 versus 77 kDa (precursor subunit), (b) the presence of a C-terminal peroxisomal targeting signal (PTS1) versus an N-terminal cleavable targeting signal (PTS2), (c) a total identity of only ~18%, and (d) a dimeric structure versus a dimeric one. A phylogenetic tree (Fig. 4B) including some representative acyl-CoA dehydrogenases and acyl-CoA oxidases from mammals and higher plants clearly summarizes the data presented above: the short-chain acyl-CoA oxidase of *Arabidopsis* is relatively unrelated to the other peroxisomal acyl-CoA oxidases, whereas it is clustered together with mitochondrial acyl-CoA dehydrogenases. The low homology to other acyl-CoA oxidases might suggest that the short-chain acyl-CoA oxidase shares a common ancestor with acyl-CoA dehydrogenases. Short-chain acyl-CoA oxidase could have arisen from a mitochondrial acyl-CoA dehydrogenase that acquired the peroxisomal targeting signal and the new intracellular location during evolution. That allowed plant peroxisomes to host a novel acyl-CoA oxidase ability that distinguishes plant organelles from mammalian peroxisomes.

At least five isoforms of acyl-CoA dehydrogenases are present in mammalian mitochondria: very long-, long-, medium-, short-, and short/branched-chain acyl-CoA dehydrogenases. Except for the very long-chain acyl-CoA dehydrogenase, all the other isoforms are tetrameric enzymes with a subunit of ~45 kDa. Very long-chain acyl-CoA dehydrogenase appears to be a dimer of ~75 kDa (8). In conclusion, both acyl-CoA dehydrogenases and acyl-CoA oxidases are tetramers or dimers of ~45 or 75 kDa. Our analysis revealed the presence of a short-chain acyl-CoA oxidase in plant peroxisomes that shares high homology with mitochondrial acyl-CoA dehydrogenases in mammals. The alignment of the conserved regions of acyl-CoA oxidases and acyl-CoA dehydrogenases (Fig. 4A) revealed that the short- as well as long-chain acyl-CoA oxidases contain amino acids of the typical mammalian acyl-CoA dehydrogenase protein signatures (PS1 and PS2). PS1 has the form (G/A/C)/(L/I/V/M)/(S/T)EX₁₆(G/S)/A/N/GSDX₄(G/S)/A, and PS2 has the form (Q/E)X₁₀(G/S)/X₃(G/L/I/V/M/F/Y/X₂(D/E/N/X₄(K/R)/X₃(D/E)) (32). The amino acid sequence of pumpkin long-chain acyl-CoA oxidase also contains 7 of the 9 amino acids of PS1 and 6 of the 8 amino acids of PS2 (Fig. 4A). Therefore, PS1 and PS2 might be unrelated to the functions of the dehydrogenase and the oxidase.

The purified short-chain acyl-CoA oxidase was active exclusively against short-chain acyl-CoA (C₇-C₉) substrates and had a reduced affinity for octanoyl-CoA (C₈) and a very low activity for branched-chain substrates. This substrate specificity resembles the characteristics of the maize short-chain acyl-CoA oxidase as indicated by Hooks et al. (38). The *Kₘ* value of 8.3 µM is close to the value reported for the maize enzyme (6 µM). The optimum pH of 8.5–9.0 is similar to that of the maize enzyme (pH 8.3–8.5). Hooks et al. have reported the purification of medium- and short-chain acyl-CoA oxidases from maize. The former was a monomeric enzyme of 62 kDa, and the latter was a homotetrameric enzyme of 15-kDa subunits. The 15-kDa subunit has one-third of the subunit mass (47 kDa) of the *Arabidopsis* short-chain acyl-CoA oxidase. Since the maize short-chain acyl-CoA oxidase was not yet cloned, the discrepancy in the subunit molecular mass needs to be further investigated to determine whether there are different families of acyl-CoA oxidases.

Regulation of the expression of short-chain acyl-CoA oxidase seems to be similar to that of other β-oxidation enzymes such as thiolase (Figs. 7 and 8). A similar regulatory mechanism was reported for the expression of pumpkin long-chain acyl-CoA oxidase (16). On the contrary, isocitrate lyase, a marker enzyme of the glyoxylate cycle, is differently regulated. This enzyme disappeared very quickly compared with short-chain acyl-CoA oxidase and thiolase. Additionally, the organ-specific expression of short-chain acyl-CoA oxidase and thiolase does not appear to be coordinated with the expression of isocitrate lyase. These results suggest that β-oxidation enzymes are present in a wider range of organs than enzymes of the glyoxylate cycle such as isocitrate lyase. Particularly, it seems that β-oxidation enzymes are present in significant amounts in roots, siliques (Fig. 8), leaves (4, 5, and 7). Our data further support the hypothesis that the β-oxidation pathway plays an important role not only during the degradation of stored lipids, but also in normal lipid turnover and senescence (28) and in jasmonic acid synthesis (39). This hypothesis is also supported by the finding that the cDNA of an acyl-CoA oxidase of *Phaenops* (which is probably a long-chain acyl-CoA oxidase) was isolated by a search for flower senescence-related genes (40). Recent additional evidence has indicated that the expression of a gene for medium-chain acyl-CoA oxidase was induced when a lauroylacetyl carrier protein thioesterase was overexpressed in *Brassica* (41), indicating that expression of the acyl-CoA oxidase gene is regulated by fatty acid biosynthesis or by the amount of fatty acids that are present in the cells. Thus, acyl-CoA oxidase isoforms might have a fundamental role in the control of fatty acid homeostasis in higher plants.

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