Identification and Functional Characterization of a Monofunctional Peroxisomal Enoyl-CoA Hydratase 2 That Participates in the Degradation of Even cis-Unsaturated Fatty Acids in Arabidopsis thaliana*

Received for publication, July 5, 2006, and in revised form, September 15, 2006. Published, JBC Papers in Press, September 18, 2006, DOI 10.1074/jbc.M606383200

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A gene, named AtECH2, has been identified in Arabidopsis thaliana to encode a monofunctional peroxisomal enoyl-CoA hydratase 2. Homologues of AtECH2 are present in several angiosperms belonging to the Monocotyledon and Dicotyledon classes, as well as in a gymnosperm. In vitro enzyme assays demonstrated that AtECH2 catalyzed the reversible conversion of 2E-enoyl-CoA to 3R-hydroxycarboxylic-CoA. AtECH2 was also demonstrated to have enoyl-CoA hydratase 2 activity in an in vivo assay relying on the synthesis of polyhydroxyalkanoate from the peroxisomes of Saccharomyces cerevisiae. AtECH2 contained a peroxisome targeting signal at the C-terminal end, was addressed to the peroxisome in S. cerevisiae, and a fusion protein between AtECH2 and a fluorescent protein was targeted to peroxisomes in onion cells. AtECH2 gene expression was strongest in tissues with high β-oxidation activity, such as germinating seeds and senescing leaves. The contribution of AtECH2 to the degradation of unsaturated fatty acids was assessed by analyzing the carbon flux through the β-oxidation cycle in plants that synthesize peroxisomal polyhydroxyalkanoate and that were over- or under-expressing the AtECH2 gene. These studies revealed that AtECH2 participates in vivo to the conversion of the intermediate 3R-hydroxyacyl-CoA, generated by the metabolism of fatty acids with a cis(2)-unsaturated bond on an even-numbered carbon, to the 2E-enoyl-CoA for further degradation through the core β-oxidation cycle.

The peroxisome is the site of numerous important biochemical reactions in plants, including photosynthesis, the β-oxidation cycle, and the glyoxylate cycle. Although several enzymes involved in these pathways have been identified, analysis of the plant proteome for proteins possessing putative peroxisome targeting sequences have identified numerous candidate peroxisomal proteins for which no functions have been assigned (1). Furthermore, prediction of peroxisomal proteins can be made difficult by the absence of recognizable signal peptide, particularly for peroxisomal membrane protein. Thus, our present knowledge of the complexity of the biochemical pathways present in the peroxisome is fragmented.

The peroxisomal β-oxidation cycle is of primary importance during seedling establishment following germination, because it is responsible for the breakdown of fatty acids into acetyl-CoA, which is subsequently converted to glucose via the glyoxylate cycle and gluconeogenesis (2). Although fatty acid β-oxidation is very active during germination in oleaginous seed and during senescence, this cycle is also present in mature photosynthetic tissues, such as leaves, as well as in developing seeds (3).

Degradation of saturated fatty acids in the peroxisome occurs via four enzyme activities located on three proteins that form the core β-oxidation pathway. The first step is mediated by an acyl-CoA oxidase, converting acyl-CoA to 2E-enoyl-CoA. This is followed by the hydration of the 2E-enoyl-CoA to 3-hydroxyacyl-CoA by an enoyl-CoA hydratase and the subsequent conversion to 3-oxoacyl-CoA by a 3-hydroxacyl-CoA dehydrogenase. The enoyl-CoA hydratase and 3-hydroxacyl-CoA dehydrogenase participating in β-oxidation are typically both present on a single protein, named multifunctional enzyme (MFE). Two forms of MFE have been found in various organisms. In bacteria, plants, and mammals, the type 1 MFE (MFE-1) mediates the conversion of 2E-enoyl-CoA to 3-oxoacyl-CoA via the isomer 3S-hydroxyacyl-CoA, whereas the type 2 MFE (MFE-2) found in mammals and fungi carries out the same reaction via the 3R-hydroxyacyl-CoA intermediate. Although MFE-1 and MFE-2 are catalyzing similar reactions, these two MFE have no sequence identity and are structurally different (4). A 3-ketohydroxylase completes the β-oxidation cycle by cleaving 3-oxoacyl-CoA to generate acetyl-CoA and an acyl-CoA. Several genes encoding the enzymes of the core β-oxidation cycle have been identified in Arabidopsis thaliana and other plants (5).

The core β-oxidation is not capable of completely degrading unsaturated fatty acids with cis(Z configuration) double bonds.
on an even-numbered carbon. For organisms possessing an MFE-1, this is due to the fact that hydration of 2Z-enoyl-CoA by the enoyl-CoA hydratase 1 generates the R-isomer of 3-hydroxyacyl-CoA, which is not a substrate for the 3S-hydroxyacyl-CoA dehydrogenase present in the MFE-1. Additional enzymes have thus been described that contribute to β-oxidation to avoid this metabolic block created by cis-un saturated bonds (6). Two pathways have been proposed by Schulz and Kunau (7) for the degradation of fatty acids having cis-double bonds at even-numbered carbons (see Fig. 1). In one pathway, named the reductase-isomerase pathway (Fig. 1, route 2), the unsaturated fatty acid is degraded by the core β-oxidation cycle to 2E,4-dienoyl-CoA, which is subsequently reduced to 3E-enoyl-CoA by the Δ_{2,4}-dienoyl-CoA reductase. The 3E-enoyl-CoA is then converted to 2E-enoyl-CoA by the enzyme Δ^{2,4}-dienoyl-CoA isomerase before returning to the core β-oxidation cycle. Peroxisomal Δ^{2,4}-dienoyl-CoA reductase and Δ^{2,4}-dienoyl-CoA isomerase have been identified and studied in both yeast and mammals (8, 9). In plants, genes encoding the same enzymes have been identified in *A. thaliana*³ and enzyme activities have been detected in germinating cucumber seedlings (10).

In the second pathway, named the hydratase-epimerase pathway (Fig. 1, route 1), the unsaturated fatty acid is degraded by the core β-oxidation enzymes to 3R-hydroxyacyl-CoA that is then converted to 3S-hydroxyacyl-CoA before rejoicing the core β-oxidation cycle. Conversion of 3R-hydroxyacyl-CoA to 3S-hydroxyacyl-CoA can be achieved either directly by a 3-hydroxyacyl-CoA epimerase or indirectly by the combined action of an enoyl-CoA hydratase 2, converting 3R-hydroxyacyl-CoA to 2E-enoyl-CoA, and an enoyl-CoA hydratase 1, converting 2E-enoyl-CoA to 3S-hydroxyacyl-CoA (2, 11–13). The MFE-1 of both *Escherichia coli* and cucumber has been shown to harbor a 3-hydroxyacyl-CoA epimerase activity (13–15). In both *Saccharomyces cerevisiae* and mammals, enoyl-CoA hydratase 2 is found as part of the MFE-2. In *S. cerevisiae*, MFE-2 is the only multifunctional enzyme present in the peroxisome, and thus acts as one of the enzyme of the core β-oxidation cycle for saturated and unsaturated fatty acids (9). In mammals, the MFE-2 has been found to contribute to bile acid synthesis, and the degradation of very long-chain fatty acids and branched-chain fatty acids, such as pristanic acid, but its involvement in the degradation of cis-un saturated fatty acids has not been studied (4). Monofunctional enoyl-CoA hydratase 2 has also been identified in several *Pseudomonas* species, as well as in *Aeromonas punctata*, and has been implicated in the synthesis of polyhydroxyalkanoates (PHA) (16, 17). No monofunctional enoyl-CoA hydratase 2 has been previously described in mammals or fungi, apart from proteolytic fragments of MFE-2 (4).

Partial purification of a protein showing enoyl-CoA hydratase 2 activity has previously been described in cucumber seedlings, but the corresponding gene had not been identified (11).

In this work, a gene from *A. thaliana*, named AtECH2, has been identified as encoding a peroxisomal monofunctional enoyl-CoA hydratase 2. Analysis of the carbon flux through the β-oxidation cycle in plants underexpressing the gene revealed the participation of AtECH2 in the degradation of unsaturated fatty acids.

**EXPERIMENTAL PROCEDURES**

**Sequence Analysis**—The Arabidopsis protein data base available on The Arabidopsis Information Resource (www.arabidopsis.org/Blast/) was investigated using the BLASTP algorithm for protein showing homology to the human and fungal enoyl-CoA hydratase 2 domain present in the MFE-2 from human (P51659) and *S. cerevisiae* (ScFox2p and Ykr0099p). Protein sequences were aligned using ClustalW (18). The Institute for Genomic Research plant data base (www.tigr.org/plantProjects.shtml) was investigated using the TBLASTN algorithm for cDNA fragments encoding homologous sequences to AtECH2. Expressed sequence tags were assembled into contigs using a contig assembly program (19), and the resulting contigs were translated into protein and aligned using ClustalW. Representative expressed sequence tags showing high homology to AtECH2 were: for *Zea mays*, BG842325.2, BG837223.1, and BE056943.1; for *Glycine max*, BI785998.1, BI787459.1, and AW349034.1; for *Lycopersicon esculentum*, BI921386.1 and BM409883.1; for *Hordeum vulgare*, BE59322.1, BE596452.1, BF657019.1, and BM329174.1; for *Triticum aestivum*, TC266049; for *Hordeum vulgare*, TC148035; for *Oryza sativa* sp. japonica cv nipponbare, LOC_Os09g37280; and for *Pinus taeda*, TC75425.

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³ S. Goepfert and Y. Poirier, unpublished observation.
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*E. coli* DH5α was used to maintain and propagate all plasmids, with the exception of Gateway® native plasmids (pB7GWIWGII (2), pMDC32, pDONR™/201, and pDONR™/207), which were maintained in *E. coli* DB3.1™ (Invitrogen). All Gateway® technology-related procedures were done according to the manufacturer’s instructions. Binary vectors containing T-DNA were electroproporated in *Agrobacterium tumefaciens* pGV3101 pM90.

Expressed sequence tag AY070763 was obtained from RIKEN Biological Resource Center (www.brc.riken.jp). The shuttle plasmid pYE352-AtECH2 was constructed by replacing the catalase A (CTA1) gene from pYE352-CTA (20) by AtECH2. This was achieved by PCR amplification of AtECH2 from the cDNA clone AY070763 using the primers 5'-TCTAATCTAGGAGTGTCGAGC-3' and 5'-AATATCTCGTGAGGACCTAACATT-3', followed by restriction with XbaI and Xhol restriction enzymes. For expression in plants, the AtECH2 cDNA sequence was amplified by PCR using primers AtECHcds-AttB1 5'-(AttB1)ATGGCCGACTCCAGAT-TCT-3' and AtECHcds-AttB2 5'-(AttB2)CTAAAGTGACGAC-AGATAA-3' and recombined into pDONR207. The resulting pDONR207-AtECH2 was further recombined with pMDC2 (21) to give the binary vector pMDC32-AtECH2, which was subsequently electroporated in *Agrobacterium tumefaciens*.

For the RNA interference construct, the gene-specific tag CATMA is formed in the yeast strain KFY5000 (22) and further amplified with the primers ECH2-AttB1 5'-(AttB1)ATGGCGAC-3' and AtECHcds-AttB1 5'-(AttB1)CGCGTATTGCGAGAGTA-3' to yield the plasmid pYE352-LeECH2, which was transformed into yeast strain BY4742 (23) to give the binary vector pMDC32-LeECH2. The LeECH2-pGEMT fragment was transferred from LeECH2-pGEMT in the XbaI site of pMDC32 generating pMDC32-LeECH2.

**Microscopy**—The plasmids pCAT-ECFP-MDH and pCAT-EYFP-Not have previously been described (24). In-frame fusion of AtECH2 with the enhanced yellow fluorescent protein (EYFP) was achieved by amplifying AtECH2 from the cDNA clone AY070763 with the primers AtECH2-EagI-5'- AGAGCAGGCTTGTTAGGATTTAATGGCAGGGTCT-GGA-3' and LeECH2-Xhol 5'-CAGGCGGCTCGAGAAACAC-TCACAG-GTA-3'. The PCR product was subcloned in pGEMT-Easy (Promega, Madison, WI). LeECH2 was subsequently excised by XbaI-Xhol and replaced the CTA1 gene in pYE352-CTA1 to yield the plasmid pYE352-LeECH2, which was transfected into yeast strain PHAC1. Similarly, a SpeI-XbaI fragment was transferred from LeECH2-pGEMT in the XbaI site of pMDC2 generating pMDC2-LeECH2.

**Enzymatic Assays and PHA Production in Yeast**—Wild-type *S. cerevisiae* strain BY4742 ( mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0) and the isoxygenic fox2Δ mutant (Ykr009c::kanMX4) were obtained from EUROSCARF (www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). Plasmids were transformed into *S. cerevisiae* strains by the lithium acetate procedure (26). Yeast strains were routinely propagated on selective media composed of 0.67% yeast nitrogen base without amino acids (Difco, Detroit, MI), 0.5% ammonium sulfate, 2% glucose, and appropriate drop-out supplement (Clontech, Palo Alto, CA). For protein extraction and enzymatic assays, pYE352-AtECH2 was transformed in yeast strains fox2Δ PHAC1. Crude protein extract was obtained from cells grown in selective media containing 0.1% glucose, 2% Pluronic-127, and 0.1% oleic acid (Sigma). Assays were performed as described in a previous study (27).

PHA-synthesizing strains were generated with the yeast shuttle plasmid Yiplac111-PHA containing the PHAC1 synthase gene from *Pseudomonas aeruginosa* modified for peroxisomal targeting by the addition, at the carboxyl end of the encoded protein, of the last 34 amino acids of the *Brassica napus* isocitrate lyase (28). For experiments analyzing PHA synthesis on selective media containing 2% glucose was harvested by centrifugation, and cells were washed once in water and resuspended at a 1:10 dilution in fresh selective media containing 0.1% (w/v) glucose, 2% Pluronic-127 (w/v) (Sigma), and 0.1-0.01% (v/v) fatty acid. Cells were grown for an additional 3–4 days before harvesting them for PHA analysis as previously described (29). Fatty acids were purchased from Nu-Check-Prep (Elysian, MN).

**Plant Culture, RNA Extraction, and Northern Blot Analysis**—Seeds of *A. thaliana*, accession Columbia (Col-0), were surface-sterilized and plated on media containing half-strength Murashige and Skoog (MS) media, 1% sucrose, and 0.8% agar. Plates were left in the dark at 4 °C for 48 h before placing them under constant illumination (70 μE.m⁻².s⁻¹) at 21 °C (defined as day 0 after imbibition (DAI)). After 15 DAI, plants were transplanted into soil under the same light and temperature conditions. Whole plants were collected from plates from 0 to 15 DAI. Green leaves, senescent leaves (30–50% chlorotic), stems, and flower buds were collected from plants grown in pots 40 DAI. Roots were harvested 7 DAI from plants grown in liquid half-strength MS medium supplemented with 2% sucrose. Transgenic plants over- and underexpressing *AtECH2* were grown on half-strength MS media, 1% sucrose, 0.8% agar and harvested at 14 DAI. Total RNA was extracted with hot borate protocol (30) for seedlings from 0 to 5 DAI and with the LiCl protocol (www2.unil.ch/ibpv/WWPR/Docs/rna_preparation.htm) for all other samples. Northern blots were made with 20 μg of total RNA according to Sambrook and Russell (31). [32P]dCTP-labeled probe was made by random priming using Prime-a-gene® labeling system (Promega) and purified on the ProbeQuant® kit (Amersham Biosciences). Probe template is a PCR fragment corresponding to the 5' part of the cDNA of *AtECH2*. Prehybridization and hybridization were carried in sodium phosphate buffer 25 mM, 7% SDS, 1% bovine serum albumin, 0.372 g/liter EDTA at 65 °C. Washes were done at 65 °C, twice for 10 min with 2× SSC 0.1% SDS, and then twice for 10 min in 1× SSC 0.1% SDS.
Production and Analysis of Transgenic Plants for PHA Accumulation—Wild-type *A. thaliana* and transgenic line PHA C3.3 (32) were transformed by the flower dip method (33). Selection of seeds was carried on half-strength MS medium containing 1% sucrose, 0.8% agar, and either the herbicide Basta (15 μg/ml), kanamycin (50 μg/ml), or hygromycin (30 μg/ml).

For PHA analysis, seeds were surface-sterilized and plated on media containing half-strength MS, 1% sucrose, and 0.8% agar. Plates were first placed at 4 °C for 48 h before placing them under constant illumination (70 μE·m⁻²) at 21 °C. After 7 DAI, 8–10 seedlings were transferred in liquid half-strength MS, 1% sucrose, 2% Pluronic-127, and 0.025% fatty acid and grown for an additional 7 days with constant agitation (90 rpm) under the same light and temperature conditions before harvest. PHA was analyzed by gas chromatography-mass spectrometry as previously described (34).

**RESULTS**

Identification of a Candidate Gene Encoding an Enoyl-CoA Hydratase—Searches using the BLASTP program available on The Arabidopsis Information Resource (www.arabidopsis.org) for *A. thaliana* proteins showing significant similarity to the enoyl-CoA hydratase domain of the MFE-2 of human (HsMFE2) and of *S. cerevisiae* (ScFox2p) revealed only one protein encoded by the gene At1g76150. This protein, named AtECH2, showed 40, 34, and 38% amino acid identity to the enoyl-CoA hydratase 2 domain of the MFE-2 of human and *S. cerevisiae*, and the monofunctional enoyl-CoA hydratase 2 from *Pseudomonas aeruginosa*, respectively. AtECH2 was predicted to encode a protein of 309 amino acids with a pI of 7.6 and an estimated molecular mass of 34 kDa. Fig. 2A presents the alignment of AtECH2 with the monofunctional enoyl-CoA hydratase 2 from *A. punctata* (ApPHAJ) and *P. aeruginosa* (PaPHAJ2), and the hydratase domain of the MFE-2 from human (HsMFE2), *S. cerevisiae* (ScFox2p), and *Candida tropicalis* (CtMFE2). Multiple sequence alignment of eukaryotic enoyl-CoA hydratase 2 has previously revealed a conserved region showing a motif (Y/F)X₁₋₂(L/V/I/G)(S/T/G/C)GDX-NP(L/I/V)HX₅(AS) (35), called the hydratase 2 motif. This motif was found in AtECH2 between amino acids 203 and 219 (upper bracket in Fig. 2A and B). Crystallization of the enoyl-CoA hydratase domain of the *C. tropicalis* MFE-2 identified four amino acids interacting with the CoA moiety that are highly conserved among eukaryotic hydratase 2 (arrows 2 in Fig. 2A) as well as five amino acids forming the active site (arrows 1 in Fig. 2A) (36). All these nine amino acids were conserved in the AtECH2. Another notable feature of AtECH2 is the
C-terminal tripeptide Ser-Ser-Leu (arrows 3 in Fig. 2A). Although this terminal tripeptide was not considered as a peroxisomal targeting signal in targeting prediction algorithms, a recent study aimed at identifying plant peroxisomal targeting signals described Ser-Ser-Leu as a minor peroxisomal targeting signal (37).

AtECH2 protein sequence was used to identify putative enoyl-CoA hydratase 2 in various plant species. Alignment of these plant enoyl-CoA hydratase 2 is shown in Fig. 2B. There is a high degree of conservation found throughout the proteins for ECH2 of plants belonging to the Monocotyledon (H. vulgare, T. aestivum, S. bicolor, Z. mays, and O. sativa) and Dicotyledons (G. max and L. esculentum) classes as well as in one member of the Coniferophyta division (P. taeda). All residues forming the catalytic sites were conserved, and, of the four conserved residues (arrows 2 in Fig. 2B) interacting with the CoA moiety, only the aromatic amino acid Phe-153 of AtECH2 was replaced in various plants by the aromatic amino acid Tyr. Plant enoyl-CoA hydratase 2 showed predominance for the C-terminal tripeptide Ser-Ser-Leu (arrows 3 in Fig. 2B), whereas the pine homologous protein ended with the tripeptide Ser-Ala-Leu.

Localization of AtECH2 in Peroxisomes—To assess the peroxisomal addressing of AtECH2, a fusion protein between an EYFP at the N terminus and AtECH2 at the C terminus was constructed and expressed under the control of a double cauliflower mosaic virus (CaMV) 35 S viral promoter to allow transient expression of the fusion protein in onion cells following biolistic bombardment. As a control, a second plasmid carrying a fusion gene between an enhanced cyan fluorescent protein (ECFP) and the peroxisomal malate dehydrogenase from cucumber (MDH) was used. The EYFP:AtECH2 and ECFP:MDH constructs were transformed together in onion epidermal cells, and the fluorescence was examined by confocal microscopy after 12 h. Control experiments have determined that fluorescence of the EYFP and ECFP could be detected without cross-interference when expressed in the same cells (data not shown). Cells expressing the EYFP:AtECH2 have a punctuate fluorescence pattern that was expected for proteins located in the peroxisomes (Fig. 3A). The fluorescence pattern observed with the EYFP:AtECH2 matched precisely the pattern observed with the ECFP:MDH, revealing that AtECH2 is a peroxisomal protein (Fig. 3B and C).

In Vivo Complementation of the Enoyl-CoA Hydratase 2 Activity in S. cerevisiae—The in vivo enoyl-CoA hydratase 2 activity of AtECH2 was first assessed through the synthesis of PHA in transgenic S. cerevisiae. PHA is synthesized in bacteria from the polymerization of 3R-hydroxyacyl-CoA by a PHA synthase (38). Previous studies have shown that accumulation of PHA in yeast expressing a P. aeruginosa PHA synthase in the peroxisome was dependent on the β-oxidation of fatty acids and on the generation of 3R-hydroxyacyl-CoA from the MFE-2 encoded by the FOX2 gene (28). Fig. 4 presents the gas chromatography-mass spectrometry profiles of monomers present in PHA produced in S. cerevisiae strains grown in media containing 0.1% (v/v) tridecanoic acid. Fig. 4A displays the profile obtained from a wild-type strain having an intact β-oxidation cycle. The 3-hydroxy acid monomers are identified with the prefix H, followed by the number of carbons in the chain and the number of unsaturated bonds. The PHA produced is mainly composed of odd-chain 3-hydroxy acids corresponding to the 3-hydroxyacyl-CoA intermediates generated by the β-oxidation of tridecanoic acid, namely 3-hydroxytridecanedioic acid (H13:0), 3-hydroxyundecanedioic acid (H11:0), 3-hydroxyoctanedioic acid (H9:0), and 3-hydroxyheptanedioic acid (H7:0). Fig. 4B shows that, in a fox2Δ strain devoid of MFE-2 activity, no PHA is accumulated. Fig. 4C shows that expression of AtECH2 in the fox2Δ strain enabled the synthesis of PHA that contained only H13:0 as an odd-chain monomer, indicating that, although the 3R-hydroxytridecanedioyl-CoA was generated, this intermediate was not further converted to 3-oxo-tridecanoyl-CoA to complete the β-oxidation cycle. These results indicated that AtECH2 encoded an enoyl-CoA hydratase 2 activity but was devoid of 3-hydroxyacyl-CoA dehydrogenase activity normally
TABLE 1
Catalytic properties of AtECH2 in vitro

| Substrate                           | Activity (units/mg) | +/− |
|-------------------------------------|--------------------|-----|
| 2E-Hexenoyl-CoA                     | 3.5                | 0.2 |
| 2E-Decenoyl-CoA                     | 30                 | 1   |
| 2E-Hexadecenoyl-CoA                 | 1.2                |     |
| 3R-Hydroxydecanoyl-CoA              | 12.4               | 0.6 |

associated with peroxisomal MFE-2. Similar results were obtained when fox2Δ strain expressing AtECH2 was grown in media containing fatty acids ranging from 6 to 14 carbon lengths, namely that the PHA formed contained primarily one monomer of the same carbon length as the external fatty acid used, indicating that AtECH2 has an affinity for a broad range of enoyl-CoAs (data not shown). Similar results were obtained when fox2Δ strain expressed the cDNA encoding the homologous protein from tomato (LeECH2) (data not shown).

Enzymatic Activity in Vitro—Enzymatic assays were performed as described by Palosaari et al. (39) by monitoring the production of 3-oxoacyl-CoA from 2E-enoyl-CoA in the presence of either a 3R- or 3S-hydroxyacyl-CoA dehydrogenase (see “Experimental Procedures”). Conversion of 2E-enoyl-CoA to 3-oxoacyl-CoA in the presence of the 3R-hydroxyacyl-CoA dehydrogenase fragment of the S. cerevisiae MFE-2 was strictly dependent on extracts derived from fox2Δ-expressing AtECH2. No conversion was detectable by using protein extract from fox2Δ or by using a 3S-hydroxyacyl-CoA dehydrogenase derived from the rat MFE-1 (data not shown). Substrates used were 2E-hexenoyl-CoA, 2E-decenoyl-CoA, and 2E-hexadecenoyl-CoA. Table 1 presents the maximal specific activities measured in the linear range of initial speed. AtECH2 catalyzed in vitro the conversion of 2E-hexenoyl-CoA, 2E-decenoyl-CoA, and 2E-hexadecenoyl-CoA to the corresponding 3R-hydroxyacyl-CoA, with a maximal activity measured for 2E-decenoyl-CoA. Reverse activity from 3R-hydroxyacyl-CoA to 2E-enoyl-CoA was monitored as previously described by Hiltunen et al. (12) using a racemic mixture of 3-hydroxydecanoyl-CoA. Protein extract from fox2Δ-expressing AtECH2 but not that of the parental fox2 strain was capable of catalyzing the conversion of 3R-hydroxyacyl-CoA to 2E-enoyl-CoA (Table 1). These data demonstrated that AtECH2 is a bidirectional enzyme converting 2E-enoyl-CoA to 3R-hydroxyacyl-CoA and vice versa.

Gene Expression in Wild-type and Transgenic Plants—Transcript abundance of AtECH2 at various stages of development and in different organs of wild-type plants was monitored by Northern blot analysis (Fig. 5A). AtECH2 was expressed in all plant organs. Peaks of expression can be seen in 1- to 2-day-old germinating seedlings and during senescence of leaves.

To assess the effect of increasing and decreasing the expression of AtECH2 on the β-oxidation cycle, transgenic plants were created that either contained AtECH2 under the control of the strong constitutive CaMV35S promoter, or an RNA interference construct (RNAi) under the CaMV35S promoter for induction of gene silencing (no T-DNA insertion mutants were available from the public collections for AtECH2). Transgenic plants were generated in both the wild-type Col-0 accession or in a transgenic line that was stably transformed and expressed a P. aeruginosa PHA synthase gene modified for localization into the peroxisomes. This later line, named PHAC3.3, has previously been shown to accumulate PHA in the peroxisome from the polymerization of the 3-hydroxyacyl-CoA intermediates of the β-oxidation cycle (34). Several independent lines were generated in each genetic background, and plants homozygous for the transgenes were selected. Northern blot analysis was performed to isolate lines that either over- or underexpressed AtECH2. Fig. 5B shows the expression pattern of two selected underexpressing lines derived from the RNAi expression and two selected lines overexpressing AtECH2, all in the PHAC3.3 background. Similar levels of under- and overexpression were also achieved in wild-type lines without PHA synthase (data not shown). Quantification of the signal intensities indicated an estimated down-regulation of AtECH2 by 10- and 5-fold in RNAi line 1 and RNAi line 2 compared with the PHAC3.3 control, respectively, and a 7- to 8-fold overexpression in the 35 S line 2 over the PHAC 3.3 control, respectively.

All plants tested that either over- or underexpressed the AtECH2 gene had the same physical appearance, development rate, germination rate in the absence of sucrose, level of hypocotyl elongation in seedlings grown in the dark in the absence of sucrose, and sensitivity to the auxin analogue 2,4-dichlorophenoxybutyric acid (data not shown).

Modulation of the Carbon Flux through β-Oxidation in Transgenic Plants Over- or Underexpressing AtECH2—To determine whether AtECH2 participated in the degradation of fatty acids with a cis-unsaturated bond on an even-numbered carbon, PHA synthesized in lines overexpressing or underexpressing AtECH2 in the PHAC3.3 background was analyzed.
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Fig. 6 presents the 3-hydroxyacyl-CoAs that are generated by the two alternative pathways portrayed in Fig. 1 that are involved in the degradation of fatty acids with a cis- or trans-unsaturated bond on an even-numbered carbon. The model shows all 3-hydroxyacyl-CoAs until seven carbons that are generated by the degradation of 10Z-heptadecenoic acid and 10E-heptadecenoic acid and is used for the interpretation of the PHA monomer composition obtained in plants grown in media containing these two fatty acids (Fig. 7). The PHA synthase from P. aeruginosa is efficient at polymerizing 3R-hydroxyacyl-CoAs from 6 to 14 carbons in length. Furthermore, 3-hydroxyacyl-CoA containing a double bond on carbon 4 has been reported to be a poor substrate for the PHA synthase (shown in parentheses in Fig. 6) and are typically not found in PHA (34, 40, 41).

Because of the presence of a MFE-1, the core β-oxidation cycle in plants generates the S stereoisomer of 3-hydroxyacyl-CoA. Therefore, there are one or more steps required to change these 3S-hydroxyacyl-CoA intermediates into the corresponding R stereoisomers that are substrates for the PHA synthase. Such conversion could be generated by the 3-hydroxyacyl-CoA epimerase activity of the plant MFE-1 or the activity of the enoyl-CoA hydratase 2 on 2E-enoyl-CoA intermediates. Degradation of 10Z-heptadecenoic acid via the reductase-isomerase pathway or the hydratase-epimerase pathway (see Fig. 1) can be distinguished by the presence of different 3-hydroxyacyl-CoAs (Fig. 6). Thus, whereas 3S-hydroxynonanoyl-CoA is only generated by the reductase-isomerase pathway and could be included into PHA after its conversion to 3R-hydroxynonanoyl-CoA, the hydratase-epimerase pathway generates the intermediate 3R-hydroxy-4Z-undecenoyl-CoA, which is not a substrate for the PHA synthase. Furthermore, although the reductase-isomerase pathway generates a 3S-hydroxynonanoyl-CoA, the hydratase-epimerase pathway generates directly a 3R-hydroxynonanoyl-CoA (indicated in bold in Fig. 6) that can be directly incorporated into PHA. Similarly, degradation by the hydratase-epimerase pathway of the endogenous fatty acids linoleic acid (9Z,12Z-octadecadienoic acid) and linolenic acid (9Z,12Z,15Z-octadecatrienoic acid), the two major fatty acids found in triacylglycerides of A. thaliana seeds, would generate 3R-hydroxystearic acid and 3S,5Z-hydroxyoctadecenoic acid, respectively, whereas all other 3-hydroxyacyl-CoA intermediates would be 3S-hydroxyacyl-CoA. In contrast to 10Z-heptadecenoic acid, the degradation of 10E-heptadecenoic acid occurs via the intermediate 2E,4E-undecenoyl-CoA, which can either be metabolized directly by the MFE-1 to generate 3S-hydroxy-4E-undecenoyl-CoA, which is not a substrate to the PHA synthase (41), or by the successive reaction of the Δ2,4-dienoyl-CoA isomerase, Δ3,Δ5-enoic-CoA isomerase and MFE-1, to generate the intermediate 3S-hydroxyundecanoyl-CoA. Thus, although the degradation of 10E-heptadecenoic acid would be mediated by pathways generating only 3S-hydroxyacyl-CoAs, the degradation of 10Z-heptadecenoic acid via the hydratase-epimerase pathway would generate a 3R-hydroxynonanoyl-CoA, whereas all other 3-hydroxyacyl-CoAs would be generated in the S conformation.

Fig. 7 presents the monomer composition of PHA generated in transgenic plants from the degradation of even-chain endogenous fatty acids and odd-chain exogenous fatty acids added to the growth media. Unsaturated monomers present at <0.1 μmol/g of dry weight in PHA are difficult to reproducibly quantify in unpurified PHA samples from plants and are thus not included in this analysis.

The PHA composition obtained from plant lines 1 and 2 overexpressing AtECH2 in the PHAC3.3 background and grown in media containing 10Z-heptadecenoic acid is presented in Fig. 7A. The parental line PHAC3.3 produced a polymer containing the monomers H13:1, H11:0, H9:0, and H7:0 generated from the degradation 10Z-heptadecenoic acid and the monomers H14:0, H12:0, H10:0, H8:1, H8:0, and H6:0 generated from the degradation of the endogenous even-chain fatty acids. Overexpression of AtECH2 led to a small but statistically significant decrease in the H9 monomer in lines 1 and 2, as well as a small but significant decrease in H8:1 and H8:0 only in line 1. The abundance of all other monomers did not change to a statistically significant degree, and the overall amount of PHA in the overexpressing lines compared with control was not statistically different.

Effects of the down-regulation of AtECH2 on monomer composition of the PHA are presented in Fig. 7, B and C. The control line used is a segregant of RNAi line 1 that does not
contain the RNA interference construct. In all experiments, the quantity and monomer composition of the PHA produced in this line were not significantly different from the PHA produced in the parental line PHAC3.3 as well as to segregating lines from RNAi line 2 that did not contain the RNA interference construct (data not shown). Fig. 7B shows the effect of growth in medium containing 10Z-heptadecenoic acid on PHA composition for plants. Analysis of the odd-chain monomers generated by the degradation of the external fatty acids revealed a 4.8- and 3.6-fold increase in the H9:0 monomer for the RNAi lines 1 and 2 grown in 10Z-heptadecenoic acid, respectively. For even-chain monomers, the main changes observed were a 6.4- and 4.4-fold increase in the H8:1 monomer, and a 3.4- and 3-fold increase in the H8:0 monomer for the RNAi lines 1 and 2, respectively. Compared with control, the amounts of PHA in RNAi lines 1 and 2 increased by 3.5- and 2.8-fold.

Fig. 7C presents the composition of the PHA extracted from plants grown in media containing 10Z-heptadecenoic acid. Analysis revealed that the proportion of odd-chain monomers derived from 10Z-heptadecenoic acid degradation in the AtECH2-underexpressing lines was not significantly different from the control plants. In contrast, an increase in the H8:1 and H8:0 monomers in the two AtECH2-underexpressing lines were still observed. No significant changes in PHA quantity were observed among the different lines under these conditions.

To further strengthen the link between reduced level of AtECH2 expression and changes in PHA monomer composition and PHA quantity, the effect expressing the tomato ECH2 (LeECH2) in an AtECH2-underexpressing line was analyzed. The AtECH2-underexpressing line RNAi line 2 was transformed with a T-DNA carrying a LeECH2 cDNA under the control of a double CaMV35S sequence promoter or with a control T-DNA vector without insert, and homozygous plants for either construct were selected. Fig. 7D presents the composition of PHA accumulated in plants growing in media containing 10Z-heptadecenoic acid. Expression of LeECH2 in the AtECH2-underexpressing line RNAi line 2 resulted in a reduction in the amount of PHA as well as of the level of the H9:0, H8:0, and H8:1 monomers back to levels comparable to the control line expressing the endogenous AtECH2.

DISCUSSION

AtECH2 has been demonstrated to be a peroxisomal protein having enoyl-CoA hydratase 2 activity toward a broad range of 2E-enoyl-CoA. Engelard and Kindl (11) had previously reported the purification from germinating cucumber seedlings of a homodimeric peroxisomal protein of 65 kDa having enoyl-CoA hydratase 2 activity. The identified protein was shown to catalyze the reversible conversion of 2E-decenoyl-CoA to 3R-hydroxydecanoyl-CoA, and to have greater activity toward 2E-decenoyl-CoA compared with 2E-butenoyl-CoA. These characteristics of the cucumber protein are similar to the A. thaliana ECH2.

Most eukaryotic proteins identified to date possessing an enoyl-CoA hydratase 2 activity are multifunctional enzymes of ~75–80 kDa that harbor at least one additional domain encod-
Monofunctional Enoyl-CoA Hydratase 2 from Arabidopsis

...ing a 3-hydroxyacyl-CoA dehydrogenase (4). Mammalian peroxisomal MFE-2 has, in addition to a 3-hydroxyacyl-CoA dehydrogenase domain, a third domain homologous to sterol carrier protein. In contrast to these eukaryotic MFE-2, AtECH2 is a smaller protein (~34 kDa) and has no other domains with homology to either 3R-hydroxyacyl-CoA dehydrogenase or sterol carrier protein. Several examples of monofunctional enoyl-CoA hydratase 2 have been studied in bacteria (16, 17). There are also uncharacterized predicted proteins that appear to be monofunctional enoyl-CoA hydratase 2 in the genome of Caenorhabditis elegans and Dictyostelium discoideum (4). However, whereas in these later organisms, predicted monofunctional proteins homologous to the 3R-hydroxyacyl-CoA dehydrogenase domain of the mammalian and fungal MFE-2 are also present in the genome, no such protein could be found by searching the Arabidopsis data base.

The AtECH2 gene was expressed in a broad range of tissues but was found particularly enhanced during the first 2 days of germination. Germination is a period of high β-oxidation requirement in oleaginous plants, such as A. thaliana (5). Furthermore, β-oxidation is activated during senescence, and AtECH2 expression was also enhanced in senescent leaves compared with green leaves. Thus, the similarity between the expression pattern of AtECH2 and that of other genes coding for peroxisomal proteins involved in β-oxidation, such as the AtPED1 encoding a peroxisomal 3-oxoacyl-CoA thiolase, support the notion that AtECH2 is involved in fatty acid catabolism (42).

In S. cerevisiae, the Δ2,4-dienoyl-CoA reductase and a Δ3,Δ2-enoyl-CoA isomerase have been found to be essential for the degradation of fatty acids having a cis double bond on an even-numbered carbon (41, 43, 44). These results, combined with the absence of an enoyl-CoA hydratase I and 3-hydroxyacyl-CoA epimerase activity in S. cerevisiae indicate that the reductase-isomerase pathway is the only functional pathway in this organism. In mammals, significant epimerase activity has been found associated with the peroxisome but not the mitochondria (45). Furthermore, the mitochondrial enoyl-CoA hydratase was found unable to act on 2E,4Z-decadienoyl-CoA, and the mitochondrial thiolase was inhibited by 3-oxo,4Z-enoyl-CoA (46, 47). Thus, the reductase-isomerase pathway appears also to be the only functional pathway in mammalian mitochondria. In mammalian peroxisomes, epimerase activity was shown to occur via the combined action of two specific hydratases: the enoyl-CoA hydratase I of the MFE-1 and an enoyl-CoA hydratase 2 activity that was subsequently found to be associated with the MFE-2 (12, 48). Epimerase activity has been found to be associated with the MFE-1 of both E. coli and cucumber (13, 15). Experiments with purified peroxisomal enzymes from cucumber have shown that the acyl-CoA oxidase, MFE-1, and 3-ketothiolase can convert 4Z-decenoyl-CoA to 2Z-oc-tenoyl-CoA. Furthermore, conversion of 3R-hydroxydecanoyl-CoA to octanoyl-CoA by the combination of peroxisomal MFE-1, 3-ketothiolase, and monofunctional enoyl-CoA hydratase 2 from cucumber seedlings was also demonstrated (11). These results, combined with the presence of both Δ2,4-dienoyl-CoA reductase and a Δ3,Δ2-enoyl-CoA isomerase activities in plant peroxisomes, indicate that degradation of fatty acids having a cis double bond on an even-numbered carbon could occur via two distinct pathways in plants and that conversion of 3R-hydroxyacetyl-CoA to 3S-hydroxyacetyl-CoA could occur via the epimerase activity of the MFE-1 or the participation of the monofunctional enoyl-CoA hydratase 2. The contribution of AtECH2 to the degradation of such unsaturated fatty acids was assessed by the use of PHA analysis. PHA biosynthesis in the peroxisome of plants and yeasts has previously been shown to be a useful tool to study the in vivo carbon flux through the β-oxidation cycle (28, 32, 34, 40, 41).

The presence of the H11:0 monomer in PHA derived from the degradation of 10Z-heptadecenoic acid or 10E-heptadecenoic acid directly supports the presence of a functional reductase-isomerase pathway in plants (Figs. 6 and 7) (40). Although the presence of the monomer 3-hydroxy,4Z-undecenoic acid in PHA derived from the degradation of 10Z-heptadecenoic acid would have provided direct evidence for the hydratase-epimerase pathway, 3-hydroxyacyl-CoA monomers having an unsaturated bond between the third and fourth carbon are not substrates to the PHA synthase (34, 40, 41). However, the increase in the proportion of the H9:0 monomer relative to H7:0 and H11:0 in PHA derived from the degradation of 10Z-heptadecenoic acid compared with PHA derived from 10E-heptadecenoic acid (Figs. 6 and 7) (40) can be explained by the presence of the hydratase-epimerase pathway that generates directly the 3R-hydroxyynonanoyl-CoA intermediate that is a substrate for the PHA synthase, instead of the 3S-hydroxyynonanoyl-CoA intermediates generated by the reductase-isomerase pathway, which must first be converted to the R-isomer before being captured by the PHA synthase.

Overexpression of AtECH2 in 35S:AtECH2 lines 1 and 2 synthesizing PHA and grown in the presence of 10Z-heptadecenoic acid resulted in a modest but significant decrease in the H9:0 monomer in two independent lines tested, a significant decrease in the H8:1 and H8:0 in line 1, while the proportion of all other monomers remained unchanged (Fig. 7A). The decrease in H9:0, H8:0, and H8:1 indicates the lower availability of the corresponding 3R-hydroxyacyl-CoAs for the PHA synthase. This would be explained by the increased conversion of the intermediates 3R-hydroxyynonanoyl-CoA, 3R-hydroxyoctanoyl-CoA, and 3R-hydroxy-5Z-octenoyl-CoA generated by the degradation of the exogenous 10Z-heptadecenoic acid and the endogenous linoleic and linolenic acid, respectively, to the corresponding 3S-hydroxyacyl-CoA via the hydratase-epimerase pathway. The increase conversion to the S-isomer would make the corresponding R-isomer less available to the PHA synthase. Conversely, the reduction in AtECH2 expression in the two RNAi lines used is shown to lead to a relative large increase in the proportion of the same monomers H9:0, H8:0, and H8:1 in plants grown under the same conditions, which would be explained by the decreased conversion of the intermediates 3R-hydroxyynonanoyl-CoA, 3R-hydroxyoctanoyl-CoA, and 3R-hydroxy-5Z-octenoyl-CoA to the corresponding 3S-hydroxyacyl-CoAs, making the 3R-hydroxyacyl-CoA intermediates more available to the PHA synthase (Fig. 7B). This interpretation has been further tested by the analysis of PHA in the RNAi lines 1 and 2 grown in media containing 10E-heptadecenoic acid (Fig. 7C). Degradation of 10E-heptadecenoic acid via either the core β-oxidation cycle or a pathway involving the Δ2,4-dienoyl-CoA reductase and a Δ3,Δ2-enoyl-CoA isomer-
ase would directly only the S-isomer of 3-hydroxy-
nonanoyl-CoA, and thus down-regulation of \textit{AtECH2} would be
expected to have no influence on the H9:0 monomer. In agree-
ment with this, no significant changes in the relative abundance of
the H9:0 monomer was observed between the control and RNAi
lines in plants grown in media containing 1O\textsuperscript{E}-heptadecenoic acid,
whereas there was again a significant increase in the two RNAi
lines compared with the proportion of the H8:0 and
H8:1 monomers that are derived from the degradation of the
endogenous linoleic acid and linolenic acid.

The correlation between \textit{AtECH2} underexpression and the
shift in the H9:0, H8:0, and H8:1 monomers observed for plants
grown in media containing 1O\textsuperscript{Z}-heptadecenoic acid was further
strengthened by complementing the RNAi line 2 with the expres-
sion of the tomato ECH2 homologue under the control of the
CaMV\textsc{35S} (Fig. 7D). The effects of \textit{AtECH2} overexpression on PHA
monomer composition, although significant, was more
modest compared with the effects of \textit{AtECH2} underexpression.
This indicates that \textit{AtECH2} expression in control plants is likely to
be at near saturation level, thus overexpression of the gene has
relatively smaller effects compared with its down-regulation.
In plants underexpressing \textit{AtECH2}, the increase in H9:0, H8:0, and
H8:1 monomer was sufficiently high to impact the total amount of
PHA accumulating in these lines, with the amount of PHA in the
RNAi lines being 2- to 3-fold higher than the control lines.
Complementation of the RNAi line 2 with the tomato ECH2 gene
had little or no impact on the availability of monomers other then H9:0, H8:1, and
H8:0 to the PHA synthase. Altogether, these results reveal that
the primary effect of \textit{AtECH2} is to metabolize the 3R-hydroxyacyl-
CoAs that are directly generated by the degradation of fatty acid
with cis-unsaturated bonds on even-numbered carbon via the
hydratase-epimerase pathway and that it has little effect on the
overall conversion of the 3S-hydroxyacyl-CoA that would be
generated by the MFE-1 of the core $\beta$-oxidation cycle to the R-isomer.

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