Molecular Identification and Characterization of the Arabidopsis Δ^{3,5},Δ^{2,4}-Dienoyl-Coenzyme A Isomerase, a Peroxisomal Enzyme Participating in the β-Oxidation Cycle of Unsaturated Fatty Acids

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Degradation of unsaturated fatty acids through the peroxisomal β-oxidation pathway requires the participation of auxiliary enzymes in addition to the enzymes of the core β-oxidation cycle. The auxiliary enzyme Δ^{3,5},Δ^{2,4}-dienoyl-coenzyme A (CoA) isomerase has been well studied in yeast (Saccharomyces cerevisiae) and mammals, but no plant homolog had been identified and characterized at the biochemical or molecular level. A candidate gene (At5g43280) was identified in Arabidopsis (Arabidopsis thaliana) encoding a protein showing homology to the rat (Rattus norvegicus) Δ^{3,5},Δ^{2,4}-dienoyl-CoA isomerase, and possessing an enoyl-CoA hydratase/isomerase fingerprint as well as aspartic and glutamic residues shown to be important for catalytic activity of the mammalian enzyme. The protein, named AtDCI1, contains a peroxisome targeting sequence at the C terminus, and fusion of a fluorescent protein to AtDCI1 directed the chimeric protein to the peroxisome in onion (Allium cepa) cells. AtDCI1 expressed in Escherichia coli was shown to have Δ^{3,5},Δ^{2,4}-dienoyl-CoA isomerase activity in vitro. Furthermore, using the synthesis of polyhydroxyalkanoate in yeast peroxisomes as an analytical tool to study the β-oxidation cycle, expression of AtDCI1 was shown to complement the yeast mutant deficient in the Δ^{3,5},Δ^{2,4}-dienoyl-CoA isomerase, thus showing that AtDCI1 is also appropriately targeted to the peroxisome in yeast and has Δ^{3,5},Δ^{2,4}-dienoyl-CoA isomerase activity in vivo. The AtDCI1 gene is expressed constitutively in several tissues, but expression is particularly induced during seed germination. Proteins showing high homology with AtDCI1 are found in gymnosperms as well as angiosperms belonging to the Monocotyledon and Dicotyledon classes.

Catabolism of fatty acids occurs primarily through the β-oxidation cycle. In plants, β-oxidation is located in peroxisomes, while in animal cells, it occurs in both peroxisomes and mitochondria (for review, see Gerhardt, 1992; Graham and Eastmond, 2002; Hooks, 2002). β-Oxidation is of primary importance for seedling establishment following germination since it allows the breakdown of fatty acids stored in triacylglycerides into acetyl-CoA, which is subsequently converted to Glc via the glyoxylate cycle and gluconeogenesis (Hayashi et al., 1998; Germain et al., 2001). Although β-oxidation is very active during germination, this cycle is also present in mature photosynthetic tissues, as well as in developing seeds (Poirier et al., 1999; Graham and Eastmond, 2002).

The peroxisomal core β-oxidation cycle in plants is composed of four enzymatic activities located on three proteins. The first enzyme is acetyl-CoA oxidase, converting acetyl-CoA into 2-trans-enoyl-CoA. The second is a multifunctional enzyme that harbors two activities in a single polypeptide, namely, an enoyl-CoA hydratase and a 3-hydroxyacyl-CoA dehydrogenase, catalyzing the successive conversion of 2-trans-enoyl-CoA into 3-hydroxyacyl-CoA and 3-ketoacyl-CoA, respectively. The final enzyme is the 3-ketothiolase and is responsible for cleavage of 3-ketoacyl-CoA to form acetyl-CoA and an acyl-CoA that is two carbons shorter and that can reenter the β-oxidation spiral. In Arabidopsis (Arabidopsis thaliana), at least four genes have been characterized to encode for acyl-CoA oxidases with different chain-length specificities, two genes encode for multifunctional enzymes, and four genes encode 3-ketothiolases (Graham and Eastmond, 2002).

In contrast to the degradation of saturated fatty acids, which can be mediated completely by the four enzymatic activities of the core β-oxidation cycle, the degradation of several types of unsaturated fatty acids has been shown to require the presence of auxiliary enzymes (Kunau et al., 1995; Hiltunen et al., 2003; van Roermund et al., 2003). This is because the core β-oxidation functions through a 2-trans-enoyl-CoA intermediate while many fatty acids have unsaturated bonds either on an odd-numbered carbon or in the...
cis-configuration, leading to the generation of enoyl-CoA intermediates that cannot be directly metabolized via the core \( \beta \)-oxidation enzymes. Three auxiliary enzymes have thus been described to be involved in the metabolism of unsaturated fatty acids. The \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase converts a 3-cis- or 3-trans-enoyl-CoA into 2-trans-enoyl-CoA. In many organisms, a \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase activity has been found to be associated with the multifunctional protein of the core \( \beta \)-oxidation cycle as well as with monofunctional enzymes. The 2,4-dienoyl-CoA reductase catalyzes, in eukaryotes, the conversion of either 2-trans,4-cis-dienoyl or 2-trans,4-trans-dienoyl-CoA to 3-trans-enoyl-CoA. The \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase activities have been found to be essential in yeast (Saccharomyces cerevisiae) for the metabolism of fatty acids having cis-double bonds on even-chain carbons, such as petroselinic acid (6-cis-octadecenoic acid), catalyzing the stepwise conversion of the intermediate 2-trans,4-cis-dienoyl-CoA to 3-trans-enoyl-CoA and 2-trans-enoyl-CoA, the latter intermediate being a normal substrate for the core \( \beta \)-oxidation cycle. Both \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase activities have been detected in plants, but the corresponding genes have not been characterized (Behrends et al., 1988; Engeland and Kindl, 1991).

The third auxiliary enzyme is the \( \Delta^{3,5}, \Delta^{2,4} \)-dienoyl-CoA isomerase catalyzing the conversion of 3,5-dienoyl-CoA to 2,4-dienoyl-CoA. This enzyme is thought to contribute to the degradation of conjugated fatty acids, such as 9-cis-11-trans-octadecadienoic acid, in animal cells (Ren and Schulz, 2003). It also has been implicated in one of the alternative pathways for the degradation of fatty acids with cis-double bonds on odd-numbered carbons (Shoukry and Schultz, 1998; Gurvitz et al., 1999). For example, degradation of oleic acid (9-cis-octadecenoic acid) generates the intermediate 2-trans,5-cis-dienoyl-CoA, which can be metabolized to the conjugated intermediate 3-trans,5-cis-dienoyl-CoA by the \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase, and subsequently converted to 2-trans,4-trans-dienoyl-CoA by the \( \Delta^{3,5}, \Delta^{2,4} \)-dienoyl-CoA isomerase. This latter intermediate is further metabolized to 2-trans-enoyl-CoA by the combined action of the 2,4-dienoyl-CoA reductase and the \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase. Although the \( \Delta^{3,5}, \Delta^{2,4} \)-dienoyl-CoA isomerase has been characterized and the corresponding gene cloned in yeast and mammals, little information is available on this enzyme in plants at the biochemical and molecular level. In this work, we present the identification and the molecular characterization of the \( \Delta^{3,5}, \Delta^{2,4} \)-dienoyl-CoA isomerase from Arabidopsis.

**RESULTS**

**Identification of an Arabidopsis Gene Coding for a Putative \( \Delta^{3,5}, \Delta^{2,4} \)-Dienoyl-CoA Isomerase**

The search for a \( \Delta^{3,5}, \Delta^{2,4} \)-dienoyl-CoA isomerase in Arabidopsis was first performed through homology searches using the BLASTP program available on The Arabidopsis Information Resource (TAIR) database (www.arabidopsis.org) with protein sequences for the \( \Delta^{3,5}, \Delta^{2,4} \)-dienoyl-CoA isomerase from yeast and rat (Rattus norvegicus). The Arabidopsis genome contained only one protein of unknown function, encoded by At4g16210, showing low, but significant, homology to the yeast Dci1p (E = 2 × 10^{-5}). In contrast, analysis with the rat protein sequence revealed the existence of seven Arabidopsis proteins with E-values higher than 5 × 10^{-10}. These proteins are, in decreasing order, At5g43280 (E = 1 × 10^{-20}), At4g16800 (E = 1 × 10^{-19}), At4g16210 (E = 3 × 10^{-16}), At1g60550 (E = 1 × 10^{-12}), At3g06860 (E = 2 × 10^{-11}), At2g30650 (E = 3 × 10^{-11}), and At4g29010 (E = 8 × 10^{-10}). All these proteins contain regions with homology to the enoyl-CoA hydratase/isomerase fingerprint (Interpro domain IPR001753; www.ebi.ac.uk/InterProScan). Genes At3g06860 and At4g29010 encode the previously characterized peroxisomal multifunctional enzymes, while At2g30650 codes for a peroxisomal hydroxybutyryl-CoA hydratase (Richmond and Bleecker, 1999; Eastmond and Graham, 2000; Zolman et al., 2001). Of the remaining proteins, only At4g16210 and At5g43280 possess a peroxisomal targeting sequence (PTS), namely a PTS1 at the C terminus, indicating their potential localization in the peroxisome, and only At5g43280 showed correct alignment with the rat \( \Delta^{3,5}, \Delta^{2,4} \)-dienoyl-CoA isomerase with regard to the Asp and Glu residues defined to be important for catalytic activity (Modis et al., 1998; Zhang et al., 2001; Fig. 1). The protein encoded by At5g43280 was thus assigned the name AtDCI1 and analyzed in further detail.

AtDCI1 encodes a protein of 278 amino acids with a predicted pI of 7.6 and a calculated M<sub>r</sub> of 29,920. Figure 1 shows the alignment of the Arabidopsis protein with human (Homo sapiens), rat, and yeast \( \Delta^{3,5}, \Delta^{2,4} \)-dienoyl-CoA isomerase. Based on the Blosum62 matrix, AtDCI1 exhibits 39%, 37%, and 18% amino acid identity to rat, human, and yeast \( \Delta^{3,5}, \Delta^{2,4} \)-dienoyl-CoA isomerase, respectively. The Arabidopsis protein is thus more closely related to the mammalian enzyme than to the yeast counterpart. AtDCI1 shares with other \( \Delta^{3,5}, \Delta^{2,4} \)-dienoyl-CoA isomerases the hydratase/isomerase fingerprint found between amino acids 118 and 138, but does not contain a predictable mitochondrial transit peptide as found in the mammalian homologs.

**Localization of Enhanced Yellow Fluorescent Protein Fusion Protein**

The protein AtDCI1 contains the tripeptide Ala-Lys-Leu at the carboxy end, a major PTS1 in plants conforming to the consensus sequence Ser-Lys-Leu (Reumann et al., 2004). In order to confirm the peroxisomal nature of AtDCI1, the localization of a fusion protein between an enhanced yellow fluorescent protein (EYFP) at the N terminus and AtDCI1 protein at the C terminus was analyzed by a transient...
transformation assay of onion (Allium cepa) epidermal cells with the corresponding gene fusion. As a control, a second plasmid carrying a fusion gene between an enhanced cyan fluorescent protein (ECFP) and the peroxisomal malate dehydrogenase (MDH) from cucumber (Cucumis sativus) was used. The EYFP:AtDCI1 and ECFP:MDH gene constructs were transformed either individually or 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:AtDCI1 have a punctate fluorescence pattern that was expected for proteins located in the peroxisomes (Fig. 2A). In cells cotransformed with the EYFP:AtDCI1 and ECFP:MDH gene constructs, the fluorescence pattern observed with the EYFP:AtDCI1 matched precisely the pattern observed with the ECFP:MDH, revealing that AtDCI1 is a peroxisomal protein (Fig. 2, B and C).

**Bacterial Expression and Enzymatic Assay of the Recombinant Protein**

The ability of the AtDCI1 protein to catalyze the conversion of Δ^{3,5}-dienoyl-CoA to Δ^{2,4}-dienoyl-CoA in an in vitro assay was tested. The AtDCI1 gene was modified to add a His-tag at the N terminus of the protein and the final construct cloned in an Escherichia coli vector allowing gene expression and protein synthesis under the control of a T7 promoter (see “Materials and Methods”). Enzyme assays were performed either with crude extracts from cells expressing AtDCI1, with AtDCI1 protein purified from the bacterial extract on a nickel affinity column, or from crude extracts from cells transformed with the expression plasmid without the AtDCI1 gene (negative control plasmid).

Isomerization of a Δ^{3,5}-dienoyl-CoA to a Δ^{2,4}-dienoyl-CoA can be followed by monitoring changes of the UV absorption spectrum (Filppula et al., 1998; Zhang et al., 2001). The Δ^{3,5}-dienoyl-CoA has a maximum absorption peak at 240 nm, whereas the Δ^{2,4}-dienoyl-CoA has a maximum absorption peak at 260 nm and a shoulder peak at 300 nm. The difference of absorption at 300 nm is thus used to monitor the formation of the product of the Δ^{3,5},Δ^{2,4} isomerization reaction. A diagram showing the in vitro reaction cascade used is presented in Figure 3A. Synthesis of the substrate for the isomerization assay is achieved from the reaction of arachidonoyl-CoA (5,8,11,14-eicosatetraenoyl-CoA) with the yeast acyl-CoA oxidase, which also possesses an intrinsic Δ^{3,5},Δ^{2,4} isomerase activity, resulting in the formation of 3,5,8,11,14-eicosapentaenoyl-CoA. This reaction can be followed by an increase in the absorption at 240 nm (Filppula et al., 1998).

Enzyme assays with crude extracts from bacteria expressing the AtDCI1 protein were compared to extract from bacteria transformed with the negative control plasmid and grown under the same conditions. The conversion of 5,8,11,14-eicosatetraenoyl-CoA to 2,4,8,-11,14-eicosapentaenoyl-CoA was shown to be strictly dependent on both the expression of the AtDCI1 protein in E. coli (Fig. 3B, comparison of the black and white circles) and the prior incubation of the starting substrate 5,8,11,14-eicosatetraenoyl-CoA.
with the yeast acyl-CoA oxidase (Fig. 3B, white diamonds). No increase in $A_{300}$ was observed if extracts from bacteria transformed with the negative control plasmid were used or if the acyl-CoA oxidase was omitted from the reaction cascade. The reaction cascade from 5,8,11,14-eicosatetraenoyl-CoA to 2,4,8,11,14-eicosapentaenoyl-CoA was also monitored by the 220- to 340-nm absorbance spectra (Fig. 3C). Thus, the conversion of 5,8,11,14-eicosatetraenoyl-CoA to 3,5,8,11,14-eicosapentaenoyl-CoA by the yeast acyl-CoA oxidase and its intrinsic $\Delta^{3,5}$, $\Delta^{2,4}$ enoyl-CoA isomerase activity is shown by the increase in $A_{250}$ (Fig. 3C, white square and black diamond), while the isomerization of 3,5,8,11,14-eicosapentaenoyl-CoA to 2,4,8,11,14-eicosapentaenoyl-CoA was followed by the dramatic increase in $A_{300}$ (Fig. 3C, white triangle). The specific activity of the extracts measured in the linear range of activity using 100 $\mu$M arachidonoyl-CoA were 5.32 $\pm$ 0.26 $\mu$mol min$^{-1}$ mg$^{-1}$ ($n = 3$) for crude extracts and 16.63 $\pm$ 6.72 $\mu$mol min$^{-1}$ mg$^{-1}$ ($n = 5$) for nickel column affinity-purified fractions. The potential presence of a $\Delta^{3,5}$, $\Delta^{2,4}$ enoyl-CoA isomerase activity associated with the AtDCI1 protein was assayed as described by Palosaari et al. (1990) with crude and purified extracts. No such activities could be detected (data not shown).

Complementation of the dcil1A Mutant

The $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase activity of the AtDCI1 protein was tested in vivo through the expression of the plant protein in a yeast mutant having a deletion in the Dcil gene (YOR180c), encoding the $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase (Dci1p), and expressing a bacterial polyhydroxyalkanoate (PHA) synthase targeted to the peroxisome. Synthesis of PHA in the peroxisome of yeast and Arabidopsis has previously been shown to be a valuable tool to monitor the in

![Figure 2](image_url)

Figure 2. Subcellular localization of EYFP:AtDCI1 fusion protein in onion epidermal cells. Confocal microscopy projections of onion cells transiently coexpressing the fusion proteins EYFP:AtDCI1 and ECFP:MDH (glyoxisomal MDH). A, Fluorescence acquired in yellow channel (EYFP). B, Fluorescence acquired in the cyan channel (ECFP). C, Overlay of A and B. D, Light transmission image. Bar = 10 $\mu$m.

![Figure 3](image_url)

Figure 3. In vitro $\Delta^{3,5}$, $\Delta^{2,4}$-dienoyl-CoA isomerase activity of AtDCI1. A, Diagram of the enzymatic cascade used for detection of $\Delta^{3,5}$, $\Delta^{2,4}$ dienoyl-CoA isomerase activity. B, Kinetics of 5,8,11,14-eicosatetraenoyl-CoA conversion to 2,4,8,11,14-eicosapentaenoyl-CoA were monitored at 300 nm. White and black circles, 50 $\mu$M 5,8,11,14-eicosatetraenoyl-CoA was preincubated for 10 min with 0.2 units of yeast acyl-CoA oxidase, and 12 $\mu$g of protein extract from control bacteria (black circles) or bacteria expressing AtDCI1 (white circles) was added at time point I (arrow I). White diamonds, addition to 50 $\mu$M 5,8,11,14-eicosatetraenoyl-CoA of 3.6 $\mu$g of crude extract from bacteria expressing AtDCI1 at time point I (arrow I) and 0.2 units of yeast acyl-CoA oxidase. C, UV absorption spectra of 50 $\mu$M 5,8,11,14-eicosatetraenoyl-CoA at 300 nm. White and black circles, 50 $\mu$M 5,8,11,14-eicosatetraenoyl-CoA was preincubated for 10 min with 0.2 units of yeast acyl-CoA oxidase and 12 $\mu$g of protein extract from bacteria expressing AtDCI1 (white circles) was added at time point I (arrow I). White diamonds, addition to 50 $\mu$M 5,8,11,14-eicosatetraenoyl-CoA of 3.6 $\mu$g of crude extract from bacteria expressing AtDCI1 at time point I (arrow I) and 0.2 units of yeast acyl-CoA oxidase. All baselines have been normalized to 0.
vivo carbon flux through the β-oxidation pathway (Mittendorf et al., 1998, 1999; Allenbach and Poirier, 2000; Poirier et al., 2001). In this system, PHA is synthesized from the polymerization of 3-hydroxyacyl-CoA intermediates generated by the β-oxidation of fatty acids via the activity of a PHA synthase from *Pseudomonas aeruginosa* modified for peroxisomal targeting (Poirier, 2002).

The Δ^3,5,Δ^2,4-dienoyl-CoA isomerase activity can be monitored in yeast synthesizing peroxisomal PHA from the β-oxidation of the fatty acid 10-cis,13-cis-nonadecadienoic acid (H.M. Bogdawa, Y. Poirier, and S. Marchesini, unpublished data). Based on current knowledge of the activity of yeast auxiliary enzymes involved in β-oxidation of unsaturated fatty acids, degradation of 10-cis,13-cis-nonadecadienoic acid gives the intermediate 2-trans-5-cis-undecadienoyl-CoA (Gurvitz et al., 1999; Hiltunen et al., 2003). This intermediate can be further metabolized via several pathways that are distinguishable by the nature of the 11-carbon 3-hydroxyacyl-CoA generated. Thus, in one pathway independent of the Dci1p, the intermediate 3-hydroxy,5-cis-undecenoyl-CoA is generated, while the intermediate 3-hydroxyundecanoyl-CoA and 3-hydroxy,4-trans-undecenoyl-CoA and 3-hydroxy,5-cis-undecenoyl-CoA is generated, while the intermediate 3-hydroxyundecanoyl-CoA and 3-hydroxy,4-trans-undecenoyl-CoA are generated by two other pathways requiring the participation of the Dci1p (Fig. 4). Whereas the intermediates 3-hydroxy, 5-cis-undecenoyl-CoA and 3-hydroxyundecanoyl-CoA are substrates for the bacterial PHA synthase, 3-hydroxyacyl-CoAs with an unsaturated bond on the fourth carbon, such as 3-hydroxy,4-trans-undecenoyl-CoA, are known to be very poor substrates for the PHA synthase (Mittendorf et al., 1998, 1999; Robert et al., 2005). Thus, the monomer 3-hydroxy,5-cis-undecenoic acid and 3-hydroxyundecanoic acid in PHA synthesized from cells degrading the fatty acid 10-cis,13-cis-nonadecadienoic acid are markers of the carbon flux through the pathways that are independent or dependent, respectively, of the Δ^3,5,Δ^2,4-dienoyl-CoA isomerase.

The gas chromatography-mass spectrometry profiles of the 11-carbon monomers present in PHA synthesized in yeast cells grown in media containing 10-cis,13-cis-nonadecadienoic acid are shown in Figure 5. Hydroxyacid monomers present in PHA are identified with the prefix H, followed by the number of carbons and the number of unsaturated bonds. While both the H11:1 and H11:0 monomers are present in wild-type cells, only the H11:1 monomer is detectable in the dci1Δ mutant, thus confirming that the generation of the intermediate 3-hydroxyundecanoyl-CoA is dependent on the presence of the Δ^3,5,Δ^2,4-dienoyl-CoA isomerase encoded by Dci1 (Fig. 5, A and B). Expression of the Arabidopsis *AtDCII* gene in the dci1Δ strain resulted in a PHA containing both the H11:1 and H11:0 monomers (Fig. 5C), thus revealing that AtDCII possesses Δ^3,5,Δ^2,4-dienoyl-CoA isomerase activity in vivo.

**Expression Pattern of AtDCII**

The temporal and spatial expression profile of *AtDCII* was determined by northern-blot analysis. Transcript level of *AtDCII* in whole seedlings strongly increases upon germination, decreasing slightly from day 2 to day 4, and increasing again at day 5 and day 7 (Fig. 6A). A similar pattern of expression was also observed for the *AtAIM1* gene encoding the peroxisomal multifunctional enzyme, with the exception of the high expression level of *AtAIM1* present in hydrated seeds at 4°C (0 d after imbibition [DAI];

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**Figure 4.** Degradation pathway of 10-cis,13-cis-nonadecadienoic acid in yeast. The alternative pathways involved in the metabolism of the intermediate 2-trans,5-cis-undecenoyl-CoA are highlighted. I, Conversion of 10-cis,13-cis-nonadecadienoic acid to the intermediate 2-trans,5-cis-undecenoyl-CoA requires three turns through the core β-oxidation cycle to generate 2-trans,4-cis,7-cis-tridecatrienoyl-CoA isomerase; Dci1p, 2,4-dienoyl-CoA isomerase activity in vivo. 2,4-dienoyl-CoA reductase leading to the intermediate 2-trans,7-cis-tridecadienoic acid, which then undergoes one more cycle of β-oxidation. II, Action of the enoyl-CoA hydratase activity of the Fox2p multifunctional enzyme on enoyl-CoAs to generate 2-trans,5-cis-undecadienoyl-CoA requires three turns through the core β-oxidation cycle to generate 2-trans,4-cis,7-cis-tridecatrienoyl-CoA isomerase; Dci1p, 2,4-dienoyl-CoA isomerase activity in vivo. 2,4-dienoyl-CoA reductase leading to the intermediate 2-trans,7-cis-tridecadienoic acid, which then undergoes one more cycle of β-oxidation. III, Conversion of 10-cis,13-cis-nonadecadienoic acid to the intermediate 2-trans,5-cis-undecenoyl-CoA requires three turns through the core β-oxidation cycle to generate 2-trans,4-cis,7-cis-tridecatrienoyl-CoA isomerase; Dci1p, 2,4-dienoyl-CoA isomerase activity in vivo. 2,4-dienoyl-CoA reductase leading to the intermediate 2-trans,7-cis-tridecadienoic acid, which then undergoes one more cycle of β-oxidation. IV, Conversion of 10-cis,13-cis-nonadecadienoic acid to the intermediate 2-trans,5-cis-undecenoyl-CoA requires three turns through the core β-oxidation cycle to generate 2-trans,4-cis,7-cis-tridecatrienoyl-CoA isomerase; Dci1p, 2,4-dienoyl-CoA isomerase activity in vivo. 2,4-dienoyl-CoA reductase leading to the intermediate 2-trans,7-cis-tridecadienoic acid, which then undergoes one more cycle of β-oxidation.
Richmond and Bleecker, 1999). The AtDCI1 gene appears to be expressed at a similar level in green leaves, senescing leaves, flowers, and floral stems, but at lower level in roots.

**Identification of Plant Homologs of AtDCI1**

The sequences available in public databases were used to identify homologs to AtDCI1 in a large spectrum of plants, including both a gymnosperm (*Pinus taeda*) and several angiosperms belonging to the Monocotyledon (*Oryza sativa, Zea mays*) or Dicotyledon (*Nicotiana benthamiana, Lycopersicon esculentum, Citrus sinensis, Medicago truncatula*) classes. An alignment of the sequences revealed a high level of amino acid conservation distributed throughout the protein with the exception of a region of low homology corresponding to amino acids 74 to 93 in AtDCI1 (Fig. 7). Furthermore, DCI1 from monocots have an additional 12- to 14-amino acid extension at the N terminus compared to dicots. This N-terminal extension does not appear to contain any targeting information to a subcellular compartment (data not shown).

**DISCUSSION**

Searches of the Arabidopsis genome for proteins with homology to the mammalian $\Delta^{3,5}, \Delta^{2,4}$-dienoyl-CoA isomerase gave two potential candidate genes coding...
for previously uncharacterized proteins, namely At5g43280 and At4g16210. These proteins had two characteristics expected to be present in a plant Δ^3,Δ^2,4-dienoyl-CoA isomerase, namely the presence of an enoyl-CoA hydratase/isomerase fingerprint and the existence of a peroxisomal targeting peptide sequence. Alignment of these proteic sequences with the rat Δ^3,Δ^2,4-dienoyl-CoA isomerase revealed that only At5g43280 contained the conserved aspartic and glutamic residues shown to be important for catalytic activity of the rat enzyme (Modis et al., 1998; Zhang et al., 2001). Analysis of the enzymatic activity of the protein encoded by At5g43280 was first performed by an in vitro assay via the expression of the plant protein in *E. coli*, a host that does not contain an endogenous Δ^3,Δ^2,4-dienoyl-CoA isomerase. Δ^3,Δ^2,4-Dienoyl-CoA isomerase activity was clearly detected in both the crude extracts of bacteria expressing At5g43280 as well as with purified protein from the same extracts, while extracts from bacteria transformed with negative control vector were devoid of the same activity.

Proof that At5g43280 encodes a protein with a Δ^3,Δ^2,4-dienoyl-CoA isomerase activity in vivo was provided by the complementation of the yeast *dci1Δ* mutant. Previous studies showed that the *dci1Δ* mutant, although deficient in Δ^5,Δ^2,4-dienoyl-CoA isomerase activity, could grow as well as wild type in media containing unsaturated fatty acid and was essentially symptomless (Gurvitz et al., 1999). Complementation of the *dci1Δ* mutant was thus assayed by a method relying on the synthesis of PHA from the polymerization of the β-oxidation intermediates 3-hydroxyacyl-CoAs by a bacterial peroxisome-targeted PHA synthase. Although synthesis of PHA in plant and yeast peroxisomes can be employed for the production of biodegradable polymers with plastics properties, this system has also been successfully exploited to learn fundamental aspects of fatty acid metabolism in the peroxisome, including the pathways involved in the degradation of unsaturated fatty acids (Allenbach and Poirier, 2000). In a recent study aimed at understanding the contribution of the yeast Dci1p protein to the degradation of 10-cis,13-cis-nonadecadienoic acid, it was demonstrated that the pathway requiring the participation of Dci1p for the conversion of 3-trans,5-cis-undecadienoyl-CoA to 2-trans,4-trans-undecadienoyl-CoA could be distinguished by the generation of the β-oxidation intermediate 3-hydroxyundecanoyl-CoA, which is itself a substrate for PHA synthase (H.M. Bogdawa, S. Marchesini, and Y. Poirier, unpublished data). Thus, while wild-type yeast cells expressing a peroxisomal PHA synthase and grown on 10-cis,13-cis-nonadecadienoic acid produce a PHA containing both the monomers H11:1 and H11:0, the isogenic *dci1Δ* strain synthesized a PHA containing the H11:1 monomer, but not the H11:0 monomer (Fig. 4). Functional complementation of the *dci1Δ* mutant with the At5g43280 gene was shown by the presence of the H11:0 monomer in PHA. Based on the demonstration of Δ^5,Δ^2,4-dienoyl-CoA isomerase activity by in vitro and in vivo assays, At5g43280 was thus named *AtDCI1*.

*AtDCI1* terminates at the carboxy terminus with the tripeptide Ala-Lys-Leu, which has been defined as major PTS1 for plants (Reumann, 2004). Since the presence of a PTS1-like C-terminal tripeptide can also be

![Figure 7. Sequence alignment of AtDCI1 with homologs in other plants. At, Arabidopsis; Pt, Pinus taeda; Nb, Nicotiana benthamiana; Le, Lycopersicon esculentum; Cs, Citrus sinensis; Mt, Medicago truncatula; Os, Oryza sativa cv japonica; Zm, Zea mays. Stars, Putative important residues for catalytic activity by similarity to rat dienoyl-CoA isomerase. Shading threshold for identity/similarity is 75%.

found in nonperoxisomal proteins, we confirmed the peroxisomal localization of AtDC1 in Arabidopsis thaliana. This was achieved by showing that a fusion protein between the EYFP and AtDC1 was colocalized with the peroxisomal marker protein Dci1p. Furthermore, complementation of the yeast dci1Δ mutant with AtDC1 showed that the plant protein was adequately targeted to the peroxisomes in yeast, since β-oxidation in this host occurs exclusively in the peroxisomes. Such cross-species targeting is not unexpected, as peroxisomal targeting sequences are known to be generally well conserved between insect, mammal, fungi, and plants (Gould et al., 1990; Subramani, 1993). The mammalian Δ^3,5,Δ^2,4-dienoyl-CoA isomerase contains both a C-terminal peroxisomal targeting signal and an N-terminal mitochondrial targeting signal, and the protein was shown to be present in both organelles (Filippula et al., 1998). In contrast, no evidence of an N-terminal mitochondrial targeting sequence was found on AtDC1. These results are in agreement with the plant β-oxidation occurring primarily, if not exclusively, in peroxisomes, while in animal cells the cycle occurs in both mitochondria and peroxisomes.

AtDC1 shows greater homology to the mammalian Δ^3,5,Δ^2,4-dienoyl-CoA isomerases than to the yeast counterpart, both at the whole protein level, as well as for the aspartic and glutamic residues found to be important for catalysis in the rat Δ^3,5,Δ^2,4-dienoyl-CoA isomerase (Modis et al., 1998; Zhang et al., 2001). The divergence of the yeast Δ^3,5,Δ^2,4-dienoyl-CoA isomerase to the mammalian homologs has been noted in previous studies (Gurvitz et al., 1999). Another distinctive feature of the fungi Δ^3,5,Δ^2,4-dienoyl-CoA isomerase is the presence of a Δ^3,Δ^5-enoyl-CoA isomerase activity within the protein, while no such activity has been found with the mammalian enzymes (Gurvitz et al., 1999; Zhang et al., 2001). No Δ^3,Δ^5-enoyl-CoA isomerase activity could be detected associated to AtDC1 either with in vitro enzyme assays with purified protein or with in vivo assays via complementation of the yeast ecc1Δ mutants (data not shown).

BLAST searches identified another gene, namely At4g16210, encoding a protein with relatively weak homology to Dci1p of yeast. The same Arabidopsis protein also showed some weak homology to the rat Δ^3,5,Δ^2,4-dienoyl-CoA isomerase. This homology is largely based on the presence of an enoyl-CoA hydratase/isomerase fingerprint in all three proteins. The function of the protein encoded by At4g16210 is at present unknown. A recent analysis of putative peroxisomal proteins in Arabidopsis based on bioinformatic tools had tentatively grouped At5g43280 with the Δ^3,5,Δ^2,4-dienoyl-CoA isomerase clade, while At4g16210 was grouped in a different clade with other peroxisomal proteins of undefined function (Reumann et al., 2004). No functional complementation of the yeast dci1Δ or ecc1Δ mutants was achieved through expression of the At4g16210 gene, indicating that, at least in yeast, the protein encoded by At4g16210 does not have a Δ^3,5,Δ^2,4-dienoyl-CoA isomerase or a Δ^3,Δ^2-enoyl-CoA isomerase activity (data not shown).

The AtDC1 gene is expressed in a broad range of tissues, including various photosynthetic tissues (leaves, stems) and to a lower, but detectable, extent in roots, but is particularly enhanced during seed germination. Germination is a period of high β-oxidation requirement in oleaginous plants. The similarity between the expression pattern of AtDC1 and that of other genes coding for peroxisomal proteins involved in β-oxidation is in accord with the involvement of AtDC1 in fatty acid catabolism (Hooks et al., 1999; Eastmond and Graham, 2000).

The physiological contributions of the Δ^3,5,Δ^2,4-dienoyl-CoA isomerase to the degradation of unsaturated fatty acids in eukaryotes remains unclear in both yeast and mammals. Deletion of Dci1 in yeast was shown to be associated with no deficiencies in growth or utilization of oleic acid (Gurvitz et al., 1999). Furthermore, degradation of the conjugated fatty acid 9-cis-11-trans-octadecadienoic acid in yeast was recently shown to be largely mediated by a pathway that was independent of Dci1p (Bogdawa et al., 2005). Studies with mitochondrial extracts from rat heart revealed that the degradation of oleic acid was also largely mediated by a pathway not requiring the participation of the Δ^3,5,Δ^2,4-dienoyl-CoA isomerase (Ren and Schulz, 2003). Similar conclusions were also reached for the degradation of 10-cis,13-cis-nonadecadienoic acid in yeast (H.M. Bogdawa, S. Marchesini, and Y. Poirier, unpublished data).

The apparent dispensable role of Δ^3,5,Δ^2,4-dienoyl-CoA isomerase in the degradation of unsaturated fatty acids in yeast and mammals appears paradoxical considering that the conservation of this enzyme across the microbial, plant, and animal kingdoms suggests a positive selective pressure. Proteins with high homology to AtDC1 are found in a broad range of plants, including gymnosperms and angiosperms, suggesting an important physiological role for the Δ^3,5,Δ^2,4-dienoyl-CoA isomerase in plants. Several recent studies have revealed the involvement of β-oxidation enzymes into developmental processes and stress responses in plants (Eastmond and Graham, 2000; Rylott et al., 2003; Castillo et al., 2004). Thus, the study of Δ^3,5,Δ^2,4-dienoyl-CoA isomerase in Arabidopsis may provide novel valuable information on the physiological role of this enzyme.

MATERIALS AND METHODS

Sequence Analysis

Searches against the Arabidopsis (Arabidopsis thaliana) sequence database were done using the BLASTP algorithm available from the TAIR database (http://www.arabidopsis.org/Blast). The characterized Δ^3,5,Δ^2-dienoyl-CoA isomerases cited in this article are from yeast (Saccharomyces cerevisiae; ScDci1p; YOR180c; Geisbrecht et al., 1999; Gurvitz et al., 1999), rat (Rattus norvegicus; RnECH1; NP_072116; FitzPatrick et al., 1995), and humans (Homo sapiens; HsECH1; AAH17408; FitzPatrick et al., 1995). Protein sequences of the Δ^3,5,Δ^2-dienoyl-CoA isomerase from Arabidopsis, rat, human, and yeast were

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aligned using ClustalW (http://www.ebi.ac.uk/clustalw). Genes homolo-
gous to the Arabidopsis Δ13,Δ5-dienoyl-CoA isomerase in other plants were
searched by TBLASTN using the AtDCI1 sequence and performed against
Orca sativa and Nicotiana benthamiana expressed sequence tag (EST) collec-
tions available on GenBank. The DCII in O. sativa and N. benthamiana are
encoded by the EST XM_463670 and EST CK294507, respectively. For other
plants, genes homologous to AtDCI1 were found by TBLASTN using se-
quenences available on the plant genome database (http://www.plantgdb.org;
Dong et al., 2004). Accession references for the corresponding EST or Genome
Survey Sequence (GSS) contigs are Zea mays ZmGSSuc04-27-04-27328; Lyc-
opersicon esculentum LeTUC02-10-21.1245; Citrus sinensis CsTUC04-08-17.4127;
and Medicago truncatula MHTUC04-08-17.5308. For Pinus taeda, the deduced
PdDCI1 gene was derived from the assembly of ESTs CF470462 and CF40018.

Bacterial Growth, Protein Purification, and
Enzymatic Assays

Escherichia coli DH5α was used to maintain and propagate all plasmids,
according to Sambrook and Russell (2001), with the exception of Gateway
native plasmids (pDEST17, pDONR207), which were maintained in E. coli
DB31 (Invitrogen, Breda, The Netherlands). For experiments aimed at
protein expression in E. coli, the strain BL21 (DE3) pLySs was used. The bac-
terial expression vector pDEST17-AtDCI was constructed by Gateway re-
combination. High-fidelity (Roche, Rotkreuz, Switzerland) PCR was performed
on pYE352-AtDCI (see yeast section) using the primers AtB1-DCI
5′-ATATCCAGTTATGACCGAC-3′ and AtB2-DCI 5′-ATATCCAGTTA-
CCAGCC-3′. The product was recombined in a BP reaction to the
pDONR207 plasmid (Invitrogen), resulting in the plasmid pDONR207-AtDCI.
This entry clone was finally LR recombined with pDEST17 (Invitrogen)
to result in the pDEST17-AtDCI. Bacteria carrying this plasmid were grown to
saturation in selective Luria-Bertani medium at 37°C. This starter culture was used to initiate a selective NCYZM medium culture. Cells were grown at
29°C to an OD560 of approximately 0.6, induced with 1 mM isopropylthio-
galactoside, and grown for an additional 3 to 4 d before harvesting them for PHA analysis as previously described (Poirier et al., 2001). 10-cis,13-cis-Nonadecadienoic acid
was purchased from Nu-Check-Prep (Elysian, MN).

Microscopy

The plasmids pCAT-ECFP-MDH and pCAT-EYPF-Not have previously
been described (Fulda et al., 2002). In-frame fusion of AtDCI with the EYPF
was achieved by amplifying AtDCI from pYE352-AtDCI with the prim-
ers dcI-YPF-5′ 5′-TTAAAGGCGCCCTGAAATTGCTA-3′ and dcI-YPF-3′ 5′-
TAATCAGATGATCAGAAATTGTA-3′, restricting the fragment with
Bsp120I and XhoI and inserting the fragment into pCAT-ECFP-Not digested
with NotI and XhoI. Plasmids were precipitated on gold beads and trans-
formed into the epidermis of fresh onions (Allium cepa) as previously des-
cribed (Mueller et al., 1997). Tissues were left 12 h in the dark on solid
medium containing half-strength Murashige and Skoog salts. Images were
acquired with a Leica TCS SP2 confocal scanning microscope (Leica, Heidel-
berg). Wavelengths were set on appropriate parameters for YFP and CFP.

Yeast Strains, PHA Production, and Analysis

Wild-type yeast strain BY4742 (matα his3Δ1 leu2Δ0 trp1Δ0 ura3Δ0) and the isogenic dcIΔ mutant (YOR180c::kanMX4) were obtained from EUROSCARF
(www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). Plasmids were
transformed into yeast strains by the lithium acetate procedure (Gietz et al.,
1992). Yeast strains were propagated on selective media composed of 0.67%
yeast nitrogen base without amino acids (DIFCO Laboratories, Detroit), 0.5%
ammonium sulfate, 2% Glc, and appropriate dropout supplement (CLON-
TECH, Palo Alto, CA). The yeast shuttle plasmid Yplac111-PHA containing the
PHA1/2 synthase from Pseudomonas aeruginosa modified for peroxisomal
targeting by the addition, at the carboxyl end of the protein, of the last 34
amino acid residues of the B. subtilis α-amylase isocitrate lyase, has previously been
described (Marchesini and Poier, 2003). The yeast shuttle plasmid pYE352-AtDCI
was constructed by replacing the catalase A gene from pYE352-CTA (Filippula
et al., 1995) by AtDCI. This was achieved by amplification of AtDCI from the
cDNA clone A096999 (Genome Systems, St. Louis) with high-fidelity poly-
ermerase (Roche) using the primers 5′-CACGCCAGTCTCATACCAGTCT-3′
and 5′-CACACCTGAGAGTATCAGAAAG-3′, followed by restriction with
SacI and Xhol restriction enzymes. The resulting plasmid was verified by
sequencing. For experiments analyzing PHA synthesis in cells growing in
media containing 10-cis,13-cis-nonadecadienoic acid, a stationary phase cul-
ture of cells grown in selective media containing 2% Glc was harvested by
centrifugation, cells were washed once in water, and resuspended at a 1:10
dilution in fresh selective medium containing 0.1% (w/v) Glc, 2% Pluronic-
dec-127 (w/v; Sigma), and 0.05% (v/v) 10-cis,13-cis-nonadecadienoic acid. Cells
were grown for an additional 3 to 4 d before harvesting them for PHA analysis as
previously described (Poirier et al., 2001). 10-cis,13-cis-Nonadecadienoic acid
was purchased from Nu-Check-Prep (Elysian, MN).

Plant Culture, RNA Extraction, and
Northern-Blot Analysis

Seeds of Arabidopsis, accession Columbia (Col-0), were sterilized and
plated on media containing half-strength Murashige and Skoog salts, 1% Suc,
and 0.8% agar. Plates were first placed at 4°C for 48 h before placing them
under constant illumination (70 μE m−2 s−1) at 21°C (defined as 0 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 7 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 on
plants grown in liquid half-strength Murashige and Skoog medium supple-
mented with 2% Suc. Total RNA was extracted with hot borate protocol (Van
and Wilkins, 1994) for seedlings from 0 to 5 DAI and with the LiCl protocol
(http://www2.unil.ch/bpv/WWWPR/Docs/rna_preparation.htm) for all
other samples. Northern blots were made with 20 μg of total RNA, according
to Sambrook and Russell (2001). [35P]PldCTP-labeled probes were made by
random priming using the prime-a-gene labeling system (Promega, Madison,
WI) and purified on the MegaBind kit (Amersham BioSciences). Prehybrid-
ization and hybridization were carried out in sodium phosphate buffer,
25 mM, 7% SDS, 1% bovine serum albumin, 0.372 g L−1 EDTA at 65°C. Washes
were done at 65°C, twice for 10 min with 2x SSC, 0.1% SDS, then twice for
10 min in 1x SSC, 0.1% SDS. Autoradiographic signals were quantified using
ImageJ software (http://rsb.info.nih.gov/ij). For ratio calculations, the
autoradiographic signals were divided by the negative intensity of the ethi-
dium bromide signal of the corresponding sample.

Upon request, all novel materials described in this publication will be
made available in a timely manner for noncommercial research purposes.

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