

Cloning and Functional Characterization of a Phospholipid:Diacylglycerol Acyltransferase from *Arabidopsis*¹

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A new pathway for triacylglycerol biosynthesis involving a phospholipid:diacylglycerol acyltransferase (PDAT) was recently described (Dahlqvist A, Stahl U, Lenman M, Banas A, Lee M, Sandager L, Ronne H, Stymne S, [2000] Proc Natl Acad Sci USA 97: 6487-6492). The *LRO1* gene that encodes the PDAT was identified in yeast (*Saccharomyces cerevisiae*) and shown to have homology with animal lecithin:cholesterol acyltransferase. A search of the *Arabidopsis* genome database identified the protein encoded by the At5g13640 gene as the closest homolog to the yeast PDAT (28% amino acid identity). The cDNA of At5g13640 (AtPDAT gene) was overexpressed in *Arabidopsis* behind the cauliflower mosaic virus promoter. Microsomal preparations of roots and leaves from overexpressers had PDAT activities that correlated with expression levels of the gene, thus demonstrating that this gene encoded PDAT (AtPDAT). The AtPDAT utilized different phospholipids as acyl donor and accepted acyl groups ranging from C10 to C22. The rate of activity was highly dependent on acyl composition with highest activities for acyl groups containing several double bonds, epoxy, or hydroxy groups. The enzyme utilized both *sn*-positions of phosphatidylcholine but had a 3-fold preference for the *sn*-2 position. The fatty acid and lipid composition as well as the amounts of lipids per fresh weight in *Arabidopsis* plants overexpressing AtPDAT were not significantly different from the wild type. Microsomal preparations of roots from a T-DNA insertion mutant in the AtPDAT gene had barely detectable capacity to transfer acyl groups from phospholipids to added diacylglycerols. However, these microsomes were still able to carry out triacylglycerol synthesis by a diacylglycerol:diacylglycerol acyltransferase reaction at the same rate as microsomal preparations from wild type.

The acylation of diacylglycerols (DAGs) by acyl-CoAs catalyzed by diacylglycerol acyltransferase (DGAT) enzymes was until recently regarded as the only enzymatic step involved in triacylglycerol (TAG) biosynthesis. During the last 5 years three unrelated DGAT genes, the DGAT1, DGAT2, and a bifunctional DGAT/wax synthase, have been identified from different organisms (Cases et al., 1998; Lardizabal et al., 2001; Kalscheuer and Steinbuchel, 2003). Recently, a novel enzyme reaction for TAG biosynthesis involving the transacylation of acyl groups from phospholipids to DAGs was described in yeast (*Saccharomyces*

cerevisiae) and plants (Dahlqvist et al., 2000). The *LRO1* gene in yeast was shown to code for such an enzyme (phospholipid:diacylglycerol acyltransferase or PDAT; Dahlqvist et al., 2000; Oelkers et al., 2000). The *LRO1* has homology to the well-studied enzyme lecithin:cholesterol acyltransferase (LCAT), which catalyzes sterol ester synthesis in blood plasma.

PDAT activity with high specificity for ricinoleoyl groups was demonstrated in microsomal preparations from developing castor bean (*Ricinus communis*) seeds (Dahlqvist et al., 2000). These seeds accumulate TAG with about 85% of ricinoleate (12-hydroxy-octadeca-9-enoate). Ricinoleate and other unusual fatty acids produced by $\Delta 12$ desaturase-like enzymes are synthesized from acyl groups linked to phospholipids and then rapidly transferred from the phospholipids to TAG (Bafor et al., 1991). The mechanisms for the specific transfer of the unusual fatty acids from the phospholipids to TAG are not fully understood but PDAT was proposed to play an important role in some oilseeds (Dahlqvist et al., 2000).

In yeast, the DGAT and PDAT pathways appear to have different importance for TAG synthesis under different growth phases. The PDAT-pathway is the

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most important for TAG synthesis during active cell division (Oelkers et al., 2000, 2002). The DGAT-pathway, mainly catalyzed by the DGAT2 enzyme (DGA1), is dominant in the stationary growth phase when the yeast is storing significant amounts of TAG (Oelkers et al., 2002; Sandager et al., 2002). The PDAT pathway does not seem to contribute significantly to TAG synthesis in the stationary phase provided that the DGA1 enzyme is functional. However, the TAG content in the mutant *dga1* was reduced only to 50% of the wild type. A strain mutated in both DGAT and PDAT had only 1% of the TAG content of the wild type (Sandager et al., 2002), demonstrating that the PDAT can partly replace DGA1 in TAG synthesis.

In this paper, we show that there are six Arabidopsis gene sequences with homology to both the yeast PDAT (ScPDAT) and human LCAT (HsLCAT) sequences in the Arabidopsis genome database. The closest homolog to ScPDAT encodes the first identified plant PDAT enzyme (AtPDAT). The expression levels of the AtPDAT gene and its closest homolog in various tissues from wild-type Arabidopsis are discussed. Further, substrate specificities of the AtPDAT and the effect on lipid and fatty acid composition of over-expressing the gene in Arabidopsis are reported. It is also shown that an AtPDAT T-DNA insertion mutant has much lower (if any) PDAT activity in membrane fractions prepared from roots but still retains the same capacity as the wild type to synthesize TAG by transacylation between two DAG molecules.

RESULTS

Identification of the Arabidopsis PDAT Gene

We have previously identified the ScPDAT gene by the homology to HsLCAT (Dahlqvist et al., 2000). Six different genes with reasonable degrees of homologies were found in Arabidopsis when we used both the HsLCAT and ScPDAT to search the Arabidopsis genome database for homologous sequences. The sequences of the six putative Arabidopsis proteins together with the HsLCAT (GenBank accession no. AAR03499), human lysosomal phospholipase A₂ (HsPLA₂; GenBank accession no. AY072914; Hiraoka et al., 2002) and the ScPDAT (GenBank accession no. P40345) sequences were aligned using the CLUSTALX (1.81) program. The degree of homology is shown as a dendrogram in Figure 1. The sequences fall into three distinct subgroups with the sequence At1g04010 (GenBank accession no. NM_100282) falling outside these groups. The ScPDAT and the Arabidopsis sequences At5g13640 (AtPDAT, GenBank accession no. AAK96619) and At3g44830 (GenBank accession no. NP_190069) align together in one group. Another group consists of HsLCAT and HsPLA₂ together with the Arabidopsis At1g27480 sequence (GenBank accession no. NM_102512). The third subgroup, which is the most distantly related to the ScPDAT, is made up of

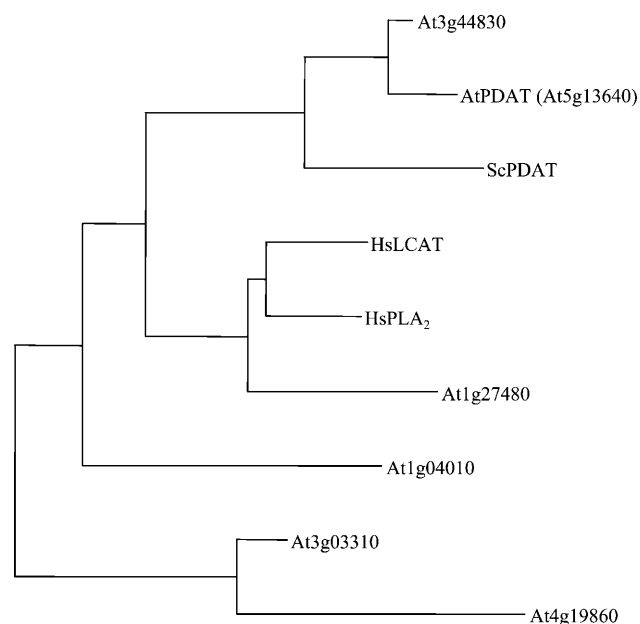


Figure 1. Evolutionary dendrogram showing the six Arabidopsis PDAT/LCAT homologs together with the HsLCAT, HsPLA₂, and the ScPDAT. The dendrogram was calculated from aligned protein sequences using the CLUSTALX multiple alignment program (Thompson et al., 1997) with default settings. Pair wise alignment scores were computed, and an unrooted tree was obtained from these scores by using the neighbor-joining method (Saitou and Nei, 1987) with correction for multiple substitutions and exclusion of gapped positions.

At3g03310 (GenBank accession no. NM_111202) and At4g19860 (GenBank accession no. NM_118106).

The two closest homologs to ScPDAT in Arabidopsis, At5g13640 (AtPDAT) and At3g44830, were further aligned to the ScPDAT and the HsLCAT sequences using the CLUSTALW algorithm (Fig. 2). In this alignment HsLCAT is shown with its signal peptide resulting in +24 in the numbering of the amino acids compared to what is given by Peelman et al. (1998). The two Arabidopsis sequences are 57% identical to each other, 28% and 26% to the ScPDAT, and 16% and 18% to the HsLCAT, respectively. As previously reported (McCartney et al., 2004) both Arabidopsis PDAT-like proteins have a C-terminal aromatic amino acid rich endoplasmic reticulum (ER) retrieval motif. All three PDAT and PDAT-like sequences are predicted to have a short N-terminal cytoplasmic tail, a transmembrane-spanning region (Krogh et al., 2001), and the rest of the protein localized on the lumen side of the ER.

There is a considerable amount of information regarding the sequence/function relationship of the animal LCAT. Residues S205, D369, and H401 in HsLCAT have, by site directed mutagenesis, shown to be the catalytic residues involved in the phospholipase A reaction of the enzyme (Peelman et al., 1998). These three residues are all conserved in ScPDAT, AtPDAT,

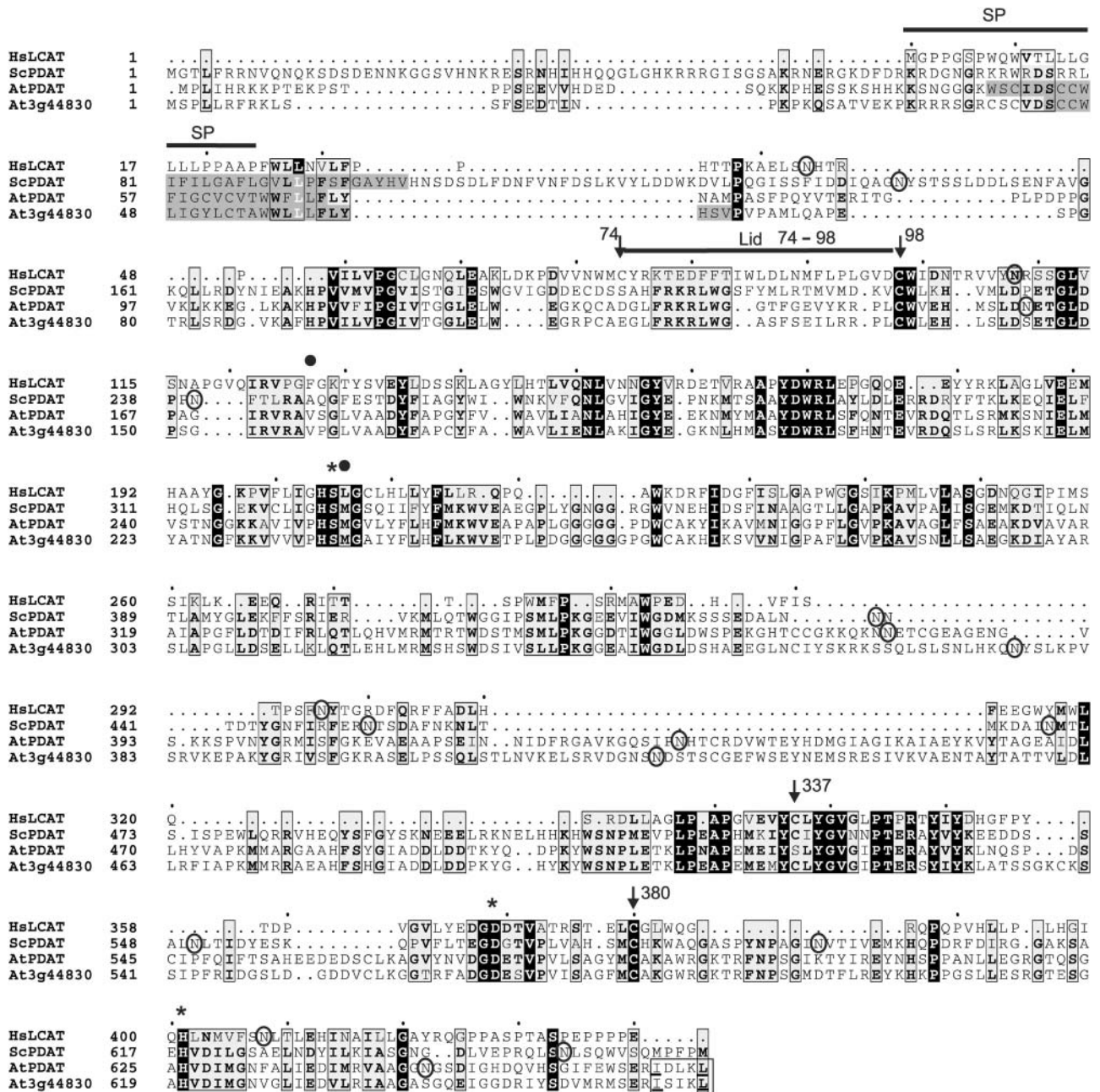


Figure 2. Alignment of the AtPDAT, At3g44830, HsLCAT, and ScPDAT sequences (accession nos. AAK96619, NP_190069, AAR03499, and P40345). Sequences were aligned by using the CLUSTALW algorithm at the PBIL web site (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) with default settings. Identical residues in all four sequences are highlighted with white letters on black background and similarities in at least three sequences are highlighted with black letters on light gray background. The signal peptide in the HsLCAT sequence is outlined as SP and the predicted membrane spanning regions in the three PDAT like sequences are marked with dark gray background. The three active site residues of HsLCAT, S205, D369, and H401, are indicated with a star above, and the two oxyanion hole residues of HsLCAT, F127 and L206, are indicated with a black circle above. The four Cys residues in HsLCAT which are involved in forming two disulfide bridges are indicated with arrows and numbers. N-glycosylations sites in HsLCAT and potential N-glycosylation sites (Asn-Gly-Ser) in the other proteins are encircled. The HsLCAT lid-structure (interfacial recognition site) spanning residue 74 to 98 is outlined as Lid. The C-terminal ER retrieval motif in AtPDAT and At3g44830 is outlined and the aromatic amino acids underlined. Note that HsLCAT is shown with its signal peptide resulting in +24 in the numbering of the amino acids compared to what is given by Peelman et al. (1998).

and At3g44830. The conserved Ser in HsLCAT as well as in ScPDAT is part of a conserved lipase motif with the consensus sequence GHSXG (Schrage and Cygler, 1997). The two Arabidopsis sequences differ from the lipase motif since the first Gly is replaced with a Pro. F127 in HsLCAT is one of the two suggested oxyanion hole residues that contributes to the stabilization of the intermediate oxyanion during the enzymatic reaction. F127 is in a region similar to F249 in ScPDAT but the Phe are more distantly positioned in the two Arabidopsis sequences (Fig. 2). While the second oxyanion hole residue is a Leu residue in HsLCAT as well as in most other animal LCATs, it is replaced by a Met in the aligned sequences from the two Arabidopsis proteins, ScPDAT (Fig. 2) and in the chicken LCAT (Peelman et al., 1998). Wang and co-workers (1997) have shown that residue E173 in HsLCAT influences the substrate specificity of the enzyme. A Glu to Ala mutation at residue 173 in the HsLCAT changes the *sn*-2 acyl specificity from linoleate (18:2) to arachidonate (20:4). The mutation is suggested to influence the size of the cavity of the enzyme and facilitate the binding of the more bulky 20:4. None of the PDAT-like sequences has a Glu aligning with this position but instead has Ala or Ser, which have smaller side chains.

Lipases, as well as HsLCAT, contain a so-called lid domain which is closed by a disulfide bridge. The lid structure occurs between the two Cys, C74 and C98, at the N- and C- terminal parts of the segment in HsLCAT (Fig. 2). This 20 to 25 amino acid long, highly mobile element covers the hydrophobic active site of the enzymes. These lids have highly variable sequences in lipases and are able to form an amphipathic helix interacting with the lipid interface. It has been suggested that they destabilize the bilayer and facilitate both the binding of the hydrophobic substrate and its diffusion into the active site cavity of the enzyme (Peelman et al., 1999). A Trp residue in the lid is thought to bind the cleaved fatty acid in the active site of the enzyme (Martinelle et al., 1996). The yeast and the two Arabidopsis sequences have one Cys residue aligning with the LCAT C98 and another in close proximity to the C74 residue. However, there is no homology to the LCAT in the lid sequence between the yeast and the two Arabidopsis sequences although the three sequences have a conserved block of several basic amino acids. The C337 and C380, which form the second disulfide bridge in LCAT, are conserved in two of the PDAT-like sequences but not in the AtPDAT, which has a Ser aligning with LCAT C337. The AtPDAT sequence has, however, two Cys residues (C545 and C562) further down the sequence. AtPDAT has four potential *N*-glycosylation sites (Asn-Gly-Ser) at N161, N381, N434, and N647 (Fig. 2). None of these are conserved in the other sequences but N161 is in close proximity to one (N108) of the four *N*-glycosylation sites in HsLCAT, N381 is close to one potential glycosylation sites in ScPDAT (N439), and N434 is close to one (N425) in At3g44830.

Functional Expression of AtPDAT

In order to investigate whether the AtPDAT gene encodes an enzyme with PDAT activity and to further characterize the potential PDAT activity, the cDNA coding for AtPDAT was overexpressed in Arabidopsis using the constitutive cauliflower mosaic virus promoter (35S). The AtPDAT coding sequence was obtained from a cDNA clone, p5D6T7P, of the AIMS database. The clone was sequenced and found, by comparison to the GenBank genomic sequence (AB006704), to contain an insertion of one base that introduces a frame shift in the open reading frame. This extra base was deleted through site directed mutagenesis.

To test whether the transformed Arabidopsis plants expressed the AtPDAT gene, RNA was extracted from T₂ plants transformed with empty vector (control) or AtPDAT. Since the T₂ seedlings were segregating for the inserted gene, seeds were germinated on agar plates containing kanamycin to eliminate nontransgenic segregating individuals. T₂ seedlings from Arabidopsis transformed with empty vector and T₂ seedlings from three independent transgenic lines containing AtPDAT were then grown in liquid culture and RNA was prepared from leaves and roots for northern blotting. Expression of AtPDAT gene was not detectable by northern blots in either leaves or roots of Arabidopsis transformed with empty vector but expression was clearly detectable in both roots (Fig. 3A) and leaves (data not shown) in all three 35S-AtPDAT transgenic lines. AtPDAT was most highly expressed in the transgenic line 1-1-6, while lines 1-3b-44 and 1-2-13 gave bands with approximately 70% and 25%, respectively, of the radioactivity of the 1-1-6 hybridizing band when measured by electronic autoradiography (Fig. 3A).

PDAT Activities in Microsomal Preparations

PDAT activity was determined in microsomal preparations of leaves and roots of T₂ plants of Arabidopsis transformed with empty vector (control) and from the three independent 35S-AtPDAT lines. Plants used for microsomal preparation were grown under the same conditions as plants used for detection of AtPDAT mRNA by northern blotting. PDAT assays were performed with microsomal preparations from roots with phosphatidylcholine (PC) containing [¹⁴C]labeled ricinoleate in the *sn*-2 position and di-oleoyl-DAG as substrates. The amount of synthesized [¹⁴C]labeled 1-ricinoleoyl-TAG correlated positively with the expression levels of the AtPDAT gene in the plant material with membrane preparations from the highest expresser yielding about 10 times more radioactive TAG than the control (Fig. 3A). It was further shown that addition of DAG stimulated the production, indicating limiting amounts of endogenous DAG in the microsomes (Fig. 3B).

Since some radioactivity also was found in DAG after the incubation, we performed an experiment to

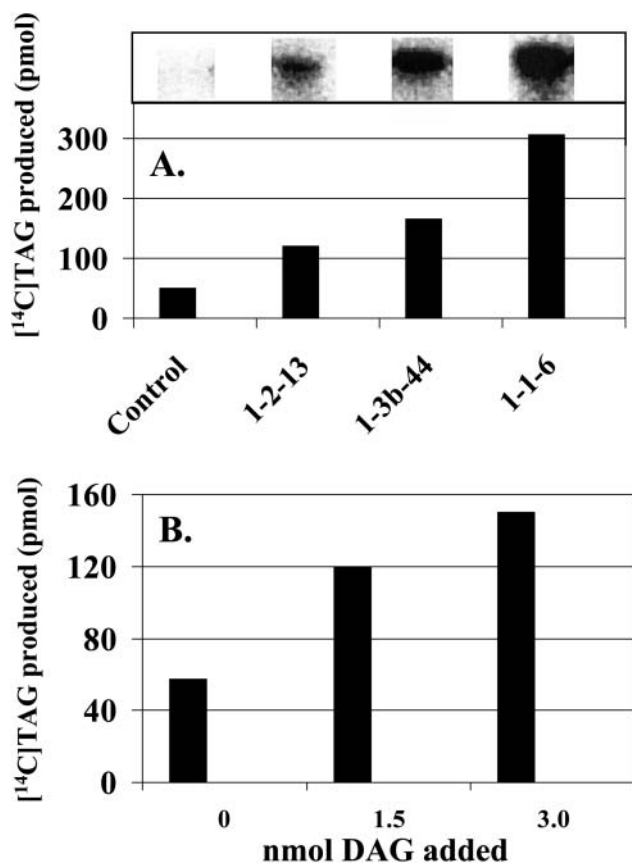


Figure 3. PDAT activity in microsomal preparations of Arabidopsis transformants expressing the AtPDAT gene under the control of the 35S promoter. A, PDAT activity in microsomal preparations of roots from control plants (transformed with empty vector) and three different transgenic lines expressing the AtPDAT under the control of the 35S promoter and (Top) northern-blot analysis of total root RNA from corresponding plants. PDAT activity was measured with *sn*-1-oleoyl(18:1)-*sn*-2-[¹⁴C]ricinoleoyl-PC and unlabeled di-18:1-DAG as substrates. B, PDAT activity as a function of DAG concentrations in microsomal preparations of roots from AtPDAT overexpresser (line 1-3b-44). The PDAT activity was measured with *sn*-1-18:1-*sn*-2-[¹⁴C]18:3-PE and with di-18:1-DAG added in amounts indicated in the figure.

elucidate if the synthesis of radioactive TAG could have occurred by a DAG/DAG transacylase that was previously postulated to occur in plants (Stobart et al., 1997). Incubation of microsomes from the overexpresser and control with *sn*-2-[¹⁴C]18:2-PE and vernoloyl-DAG was compared with incubation with *sn*-2-[¹⁴C]18:2-DAG and vernoloyl-DAG in equimolar amounts (Fig. 4A). Since TAGs with vernoloyl groups are separated from TAGs without any epoxidized acyl groups in the thin-layer chromatography (TLC) system used, acylation of exogenous added nonradioactive DAG could easily be distinguished from acylation of endogenous DAG. When radioactive PE was used as substrate, 84% of the radioactive TAG had a vernoloyl group (6.8% of

total radioactivity), demonstrating that the added vernoloyl-DAG efficiently out competed endogenous DAG in the production of radioactive TAG. When radioactive nonepoxidized DAG was used, 90% of the radioactive TAG was found in molecules with no vernoloyl groups, indicating that acyl groups from endogenous phospholipids rather than DAG was used as acyl donor. Thus, it could be concluded that regardless if radioactive DAG or PE was used as substrate, phospholipids were the main acyl donor for [¹⁴C]TAG synthesis in the overexpresser. The small amount of radioactivity found in vernoloyl-TAG in incubations with radioactive DAG (0.7%, Fig. 4) could either come from vernoloyl groups entering phospholipids from DAG or from a minor DAG/DAG transacylase activity. We therefore separated the polar lipids by TLC after an incubation with radioactive DAG and measured radioactivity in the different lipid classes. We could not find any detectable activity in polar lipids other than in a very polar compound not cochromatographing with any known phospholipids and this amounted to less than 0.2% of total activity (data not shown). Although no detectable radioactivity from DAG entered phospholipids, significant radioactivity from PE entered DAG during the incubation (see Fig. 4A). In order to further discriminate between the possible transacylation reactions and enzymes carrying out TAG synthesis, we did similar enzymatic assays with microsomal preparations from an AtPDAT T-DNA insertion mutant (SALK_032261) and a segregant of this line, not having the insert in the AtPDAT gene (null-segregant). The microsomal preparations from the null-segregant produced four times more radioactive vernoloyl-TAG (0.4% of added activity) than nonepoxidized [¹⁴C]TAG when [¹⁴C]PE was used as substrate (Fig. 4B). In a corresponding incubation with membranes from the T-DNA mutant, the amount of [¹⁴C]vernoloyl-TAG was 25% (0.1% of total radio activity) of that formed by the null-segregant but the mutant produced the same amount of nonepoxidized [¹⁴C]TAG as the null segregant. In incubations with the T-DNA mutant, the ratio of radioactive vernoloyl-TAG to nonvernoloyl-TAG was 1:1 regardless if radioactive DAG or PE were used as substrate. Since some radioactive DAG was produced from [¹⁴C]PE by the membranes (see Fig. 4A and B) it is likely that all radioactive TAG produced from [¹⁴C]PE in the assays with the membranes from the T-DNA mutant was a result of DAG:DAG transacylation.

Since LCATs have been shown to have phospholipase activity (Aron et al., 1978) we investigated if AtPDAT also had this activity. We could not observe any consistent difference in the amount of radioactive free fatty acids between assays with microsomal fractions from wild type and overexpresser, whether or not exogenous DAG was added (data not shown). However, the phospholipase activities varied much more than PDAT activities from one microsomal preparation to another.

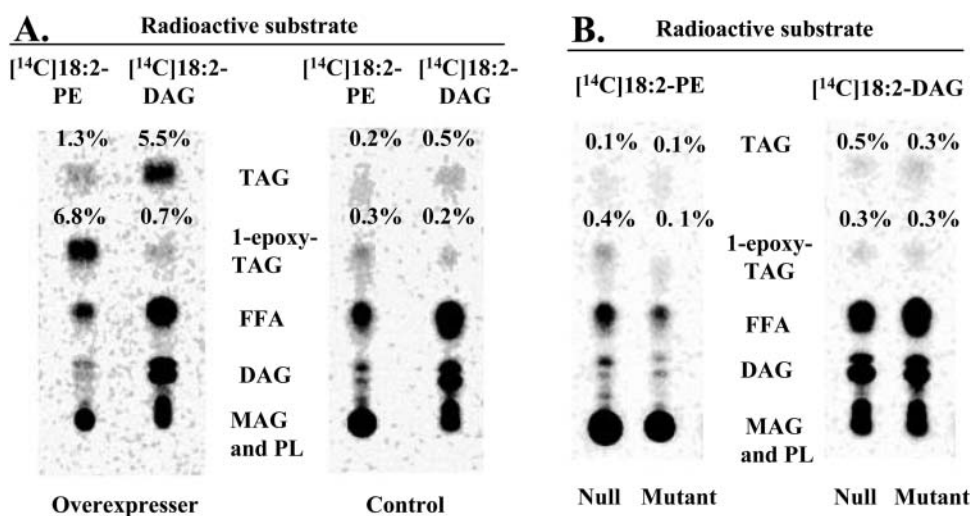


Figure 4. Autoradiogram of neutral lipid fraction separated on TLC after incubation of microsomal preparations of roots from different *Arabidopsis* lines with *sn*-1-18:1-*sn*-2-[¹⁴C]18:2-PE or *sn*-1-18:1-*sn*-2-[¹⁴C]18:2-DAG in the presence of vernoloyl-DAG. A, Incubations with microsomal preparations from AtPDAT overexpresser (line 1-1-6) and control plants (transformed with empty vector). B, Incubation with microsomal preparations from AtPDAT T-DNA insertion mutant (mutant) and the null segregant (null) to this mutant. The figures given correspond to percentage radioactivity in the TAG areas of total radioactivity in the lane. Amount of substrates used in each assay: *sn*-1-18:1-*sn*-2-[¹⁴C]18:2-PE: Figure A, 2.5 nmol; Figure B, 5 nmol; *sn*-1-18:1-*sn*-2-[¹⁴C]18:2-DAG: Figure A, 1.5 nmol; Figure B, 6 nmol; vernoloyl-DAG: Figure A, 1.5 nmol; Figure B, 6 nmol. TAG, triacylglycerol; 1-epoxy-TAG, TAG with one vernoloyl moiety; FFA, free fatty acids; DAG, diacylglycerol; MAG, monoacylglycerol; PL, polar lipids.

Positional Specificity of AtPDAT

In order to investigate the positional specificity of the AtPDAT, microsomal preparations from leaves of overexpresser and control plants were incubated with either the *sn*-1-[¹⁴C]labeled or *sn*-2-[¹⁴C]labeled di-18:1-PC. The activity of the *sn*-1 substrate was one-third of the activity of the *sn*-2 substrate, demonstrating a clear preference of the enzyme for the *sn*-2 position (Fig. 5). That the activity with the *sn*-1 labeled substrate was catalyzed by the AtPDAT is evident by comparing the assays with those from control microsomes, where

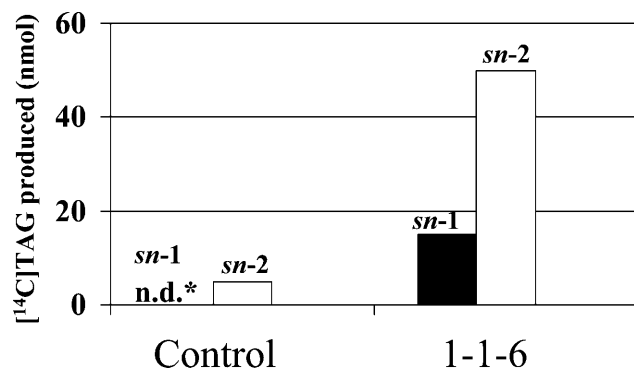


Figure 5. Positional specificity of AtPDAT. PDAT activity was measured in microsomal preparations from leaves of *Arabidopsis* control plants (transformed with empty vector) and an AtPDAT overexpresser (line 1-1-6) with 5 nmol of either *sn*-1-[¹⁴C]18:1-*sn*-2-18:1-PC or *sn*-1-18:1-*sn*-2-[¹⁴C]18:1-PC.

activity was below detection limit. When checked with phospholipase A₂, the *sn*-1 labeled substrate had, at the most, 10% of the label in the *sn*-2 position (see "Materials and Methods"). Consequently the major part of the radioactive TAG must have been derived from *sn*-1 position of PC in assays with the *sn*-1 labeled substrate.

Acyl and Lipid Specificity of the AtPDAT

The acyl specificity of the AtPDAT for different acyl groups at position *sn*-2 of PC was investigated in microsomal preparations from the overexpresser (Fig. 6A). The enzyme activity was highly dependent on the nature of the acyl group with stearic acid (18:0) and erucic acid (22:1) giving the lowest activities and with ricinoleoyl group yielding 23-fold higher activity. Increased number of double bonds greatly increased activity as did the introduction of functional groups such as epoxy or hydroxy groups. However, replacement of a double bond with an acetylenic bond decreased the activity. Caproyl(10:0)-PC was utilized much better than 18:0-PC and somewhat better than 18:1-PC. When phosphatidic acid (PA), PC, and PE with [¹⁴C]labeled 18:1 in the *sn*-2 position were compared as substrates, the enzyme showed a 5.6-fold and 1.7-fold higher activity with PE than with PA and PC, respectively (Fig. 6B). The relative activities of the enzyme for the different phospholipids appeared to be similar if phospholipids with 10:0 or ricinoleoyl groups (ricinoleoyl-PE was not tested) were used as substrates (Fig. 6B).

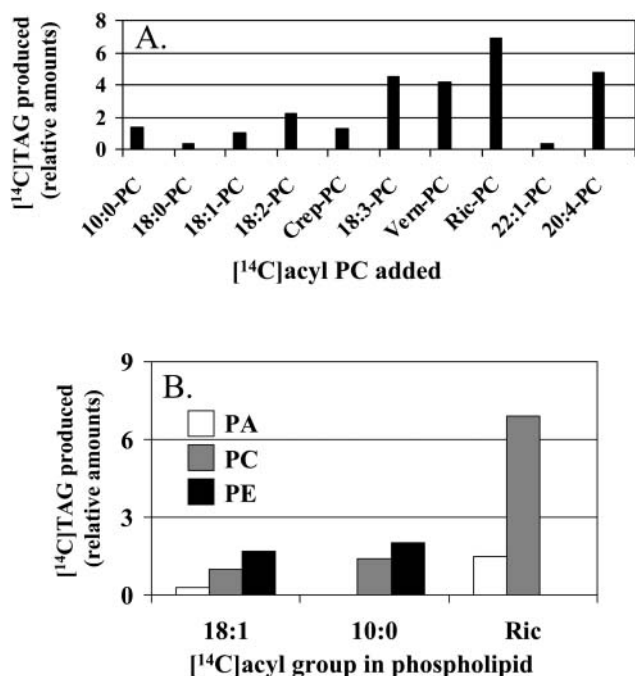


Figure 6. Acyl (A) and lipid (B) specificity of the AtPDAT. The PDAT activity was measured in microsomal preparation of leaves from AtPDAT overexpressor (line 1-1-6) with *sn*-2 labeled substrates. Labeled 18:0-PC, 20:4-PC, 22:1-PC, 10:0-PC, and 10:0-PE had 16:0 at position *sn*-1, whereas all other substrates had 18:1 in the *sn*-1 position. The results are presented as relative amounts of radioactive TAG synthesized compared to assays with *sn*-2-[¹⁴C]18:1-PC. Crep-PC, crepenynoyl-PC; Vern-PC, vernoloyl-PC; Ric-PC, ricinoleoyl-PC; Ric, ricinoleoyl.

Fatty Acid and Lipid Composition and Content in AtPDAT Overexpressor

Fatty acid composition and lipid content of 17-d-old seedlings of the highest AtPDAT overexpressor were compared with control (wild-type) seedlings (Table I). No significant differences were found in total acyl composition or in the amount of polar lipids, TAG or other neutral lipids. It should be noted that the amount of TAG was very low (0.6%–0.8% of all acyl groups) in these seedlings. The amount of acyl groups per fresh weight (FW) was also identical between the overexpressor and the wild type. In order to investigate if there were any differences between individual polar lipids in the overexpressor compared to wild type, we analyzed the relative distribution and acyl composition of the major polar lipids in leaves and roots from plants grown for 14 d in liquid culture. We found no significant differences between overexpressor and wild type in any major polar lipid classes or in the lyso-PC (data not shown). The amount of lyso-PC was very low in roots and leaf tissues of both wild type and overexpressor (0.3%–0.4% of total acyl groups).

We also analyzed the fatty acid composition and oil content per FW and per seed of the T₃ seeds from all three overexpressors and did not find any significant differences compared to the wild type (data not

shown). It should be noted that northern blots showed that the developing T₃ seeds of the highest overexpressor had an expression level of the AtPDAT that was about 30% of that of leaves from the T₂ plants as judged on northern blots by electronic autoradiography (data not shown). The expression level of AtPDAT in developing seeds from wild type was below detection limit on our northern blots (data not shown).

Expression of AtPDAT and At3g44830 Genes in Different Tissues

Since the expression of the AtPDAT gene was below detection limit in wild-type *Arabidopsis* in our northern blots, we examined the expression of AtPDAT and its closest homolog At3g44830 by semi-quantitative RT-PCR. We detected expression of AtPDAT at similar levels in leaves, roots, flowers and developing seeds (Fig. 7). At3g44830 was clearly expressed in developing seeds but only at a very low levels in leaves and flowers while no transcript was detected in roots.

DISCUSSION

Catalytic Activities of PDAT and LCAT-Like Proteins

The gene encoding ScPDAT from yeast was previously cloned based on the assumption that the catalytic similarities between LCAT and PDAT would also be reflected in sequence similarities (Dahlqvist et al., 2000). The only sequence found in the human genome that is homologous to LCAT/ScPDAT, apart from LCAT itself, is the LCAT-like lyso-phospholipase sequence (Taniyama et al., 1999) which recently have been shown by Hiraoka and co-workers to have phospholipase A₂ and phospholipid:ceramide transacylase activities (Hiraoka et al., 2002). There are six sequences with reasonable homology to either LCAT or ScPDAT in *Arabidopsis*. Of these six proteins, one (At1g04010)

Table I. Fatty acid and lipid composition and amounts in wild-type and PDAT-overexpressing (1-1-6) *Arabidopsis* 17-d-old seedlings (*n* = 4)

Mean values (\pm se) are shown.		
Acyl Group	Wild Type	1-1-6
%		
16:0	13.7 \pm 0.5	13.4 \pm 0.3
16:1	1.8 \pm 0.9	1.2 \pm 0.6
16:3	15.4 \pm 1.9	15.1 \pm 0.4
18:0	1.6 \pm 0.1	1.6 \pm 0.3
18:1	2.8 \pm 0.4	4.2 \pm 1.4
18:2	18.0 \pm 0.6	18.0 \pm 0.6
18:3	46.6 \pm 0.8	46.5 \pm 1.1
Percent acyl groups in TAG	0.8 \pm 0.1	0.6 \pm 0.1
Percent acyl groups in other neutral lipids	3.3 \pm 0.4	3.5 \pm 0.5
Total acyl group/mg FW (nmol)	12.8 \pm 1.0	12.9 \pm 2.3

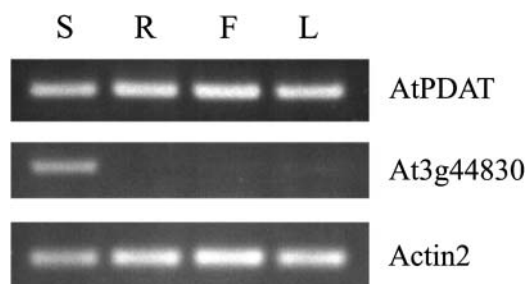


Figure 7. RT-PCR analysis of the expression of the genes encoding AtPDAT and At3g44830. One microgram of total RNA from developing seeds (S), roots (R), flower (F), and leaves (L) were used for cDNA synthesis. Transcripts shown are obtained after 35 cycles using cDNA as template. ACTIN2 was used as a control.

has been reported orally in a conference to encode a phospholipid:sterol acyltransferase (in P14, Stymne et al., 2003) and another (At3g03310) a phospholipase A₁ enzyme (in Meta-22, Bouvier-Navé et al., 2003). In this paper we show, unequivocally, that a third protein, (At5g13640 or AtPDAT), which is the closest Arabidopsis homolog to the ScPDAT gene, is a PDAT enzyme. The second closest ScPDAT homolog in Arabidopsis, At3g44830, has high homology (57% identical) to AtPDAT (Fig. 2) and it is tempting to conclude that this is a second AtPDAT. However, the At1g04010 has been reported to be a sterol acyltransferase but does not group together with either the LCATs or the PDATs and the HsPLA₂ clearly groups together with the LCATs (Fig. 1) but does not have LCAT activity (Hiraoka et al., 2002). Therefore no presumption can be made regarding catalytic activity that is only based on overall sequence similarities. We here show that the gene encoding At3g44830 protein was clearly expressed in developing seeds but only at extremely low levels in leaves and flowers. Since the AtPDAT gene, on the other hand, was constitutively expressed, this indicates that the two proteins might have different physiological functions with At3g44830 perhaps having a specific role in TAG deposition.

Sequence Comparison Studies of PDATs and LCAT

Reasonable amino acid sequence similarities exist between the two Arabidopsis PDAT-like sequences, the ScPDAT and the HsLCAT sequences throughout the proteins. Putative active site residues, Cys involved in disulfide bridges and putative glycosylation sites are all relatively conserved in the PDAT-like sequences. The HsLCAT lid domain is suggested to interact and bind to the lipid substrates, but there is no homology to this domain in the three PDAT-like sequences (Fig. 2). The PDAT-like proteins might not need an interfacial recognition sequence since they are predicted to have an N-terminal located membrane-spanning region (Fig. 2) and would by this be anchored to the membrane and thus be in close con-

tact with the substrates. The three PDAT-like proteins are predicted to have small cytosolic N-terminal tails before the predicted membrane spanning regions and with the rest of the proteins facing the lumen side of the ER. The Arabidopsis proteins have an aromatic amino acid rich stretch in the C-terminal end that recently have been shown to act as a ER retrieval motif (McCartney et al., 2004). The putative N-glycosylation sites found in the PDAT-like protein are more indicative of their noncytosolic localization. This predicted lumen orientation, which has important implications for understanding the function of these proteins, suggests that the LCAT, which is a secreted protein, has evolved from a membrane bound ancestor. However, before cell biology data on the actual subcellular localization of AtPDAT are available, discussions regarding the link between the physiological role of the enzyme and the subcellular targeting should be made with caution.

Characterization of Catalytic Activity

Assays with microsomal preparations of leaves and roots from AtPDAT overexpressers and control plants (empty plasmid) showed that the PDAT activity correlated positively with expression levels. The activity was 10-fold higher in the membranes from the highest overexpresser than in the control. The evolutionarily related LCAT, that normally catalyzes the esterification of cholesterol, can also esterify alcohols, DAGs, lyso-phospholipids as well as catalyze hydrolysis of phospholipids (Czarnecka and Yokoyama, 1993). (¹⁴C)Acyl labeled phospholipids were converted to a small extent to DAG in our microsomal assays and therefore we performed experiments that showed that the majority of the radioactive TAG produced in the overexpresser utilized phospholipids as donor and DAG as acceptor and was not a result of DAG/DAG transacylation activities (Stobart et al., 1997). However a minor DAG/DAG transacylase activity appeared to be present in the microsomal membranes since radioactive vernoloyl-TAG was produced without any detectable activity entering phospholipids when [¹⁴C]18:2-DAG and vernoloyl-DAG was added. The level of this activity was similar in microsomal preparations from roots of an AtPDAT T-DNA insertion mutant and a control (the null-segregant of this mutant) even though the formation of radioactive TAG from radioactive phospholipids was barely detected in the mutant. It could be concluded on the basis of these experiments that a diacylglycerol:diacylglycerol acyltransferase which is distinct from AtPDAT and has similar rates of activity as the AtPDAT is present in microsomal membranes from Arabidopsis roots. It is therefore possible that the transacylation reaction studied by Stobart et al. (1997) in microsomal preparations of safflower was a result of a mixture of DAG/DAG transacylation and PDAT activities. It is doubtful if the root microsomal membranes from the

mutant possessed any PDAT activity at all since a small amount of the added [^{14}C]PE was converted to radioactive DAG, which could then be converted into radioactive TAG by the DAG/DAG transacylase. Membranes from the overexpresser produced two to three times more radioactive vernoloyl-TAG from [^{14}C]DAG than membranes from control and mutant plants, whereas PDAT activity was 10 times higher in membranes of the overexpresser. Thus it appears that the PDAT enzyme itself has a DAG/DAG transacylation activity which is about one-tenth of the PDAT activity.

The positional specificity of the AtPDAT was tested using radioactive di-18:1-PC labeled with [^{14}C]18:1 in either *sn*-1 or *sn*-2 position. The enzyme was shown to have a preference for *sn*-2 position but utilized the *sn*-1 position at about one-third of the rate of the *sn*-2 labeled substrate. When corresponding assays were performed with yeast PDAT it showed a 7-fold preference for the *sn*-2 position (Dahlqvist et al., 2000). The animal LCAT was shown to have activity toward the *sn*-1 group and this activity was totally dependent on the acyl moiety at the *sn*-2 position. When palmitate (16:0) was present at position *sn*-1, the relative activity of the enzyme toward this position could vary between 1% and 72%, depending on the acyl group at the other position (Subbaiah et al., 1994).

The AtPDAT enzyme showed a broad acceptance for acyl groups of different chain lengths but had a strong preference for acyl groups with multiple double bonds or a functional group such as a hydroxy or epoxy group. In general, the AtPDAT accepted acyl groups ranging from C_{10} to C_{22} and with the activity of the enzyme negatively correlating to the melting point of the acyl group except for the hydroxy and epoxy fatty acids. PDAT activity was originally identified in microsomal preparations from developing seeds of castor bean (*Ricinus communis*) and *Crepis palaestina* plants, which accumulate high levels of hydroxy (ricinoleoyl) and epoxy (vernoloyl) acyl groups, respectively, in their seed TAGs (Dahlqvist et al., 2000). It was suggested that PDAT was involved in the transfer of these oxygenated acyl groups from the phospholipids into TAG. Our results show that the AtPDAT has similar acyl specificity for vernoloyl and ricinoleoyl groups as the PDAT activity assayed in the microsomal preparations from developing castor bean endosperm, indicating that the plant PDAT acyl specificities are general and not anything that has evolved only in plants producing unusual fatty acids. An interesting difference in acyl specificity of the PDAT activity in the yeast and *C. palaestina* compared to AtPDAT and castor bean can be noted. Whereas the two latter enzymes showed strong preference for ricinoleoyl as well as vernoloyl groups, ricinoleate was a poor acyl donor for *Crepis* PDAT(s) and vernoloyl groups were poorly utilized by the yeast PDAT (Dahlqvist et al., 2000). It is pertinent to note that linolenate acid (18:3) was utilized four and one-half times faster than 18:1 and 2-fold faster than 18:2 by the AtPDAT and yet the transformants having 10-fold higher PDAT activity than the wild type did not have

any alteration in fatty acid composition in either vegetative parts or in the oil accumulating seeds. The relative proportion of major lipid classes and the lysophospholipid classes also were not altered in the overexpresser. Thus, the results obtained in this study do not give any evidence for a significant role of AtPDAT in controlling the lipid composition or the redistribution of particular acyl groups between lipids. However, it should be pointed out that other PDAT enzymes, like the seed specific expressed putative PDAT protein At3g44830, might display more obvious properties of channelling enzymes. The AtPDAT showed, like the ScPDAT, higher activity with PE than PC but this preference was less pronounced than for the yeast enzyme (1.7-fold and 3.4-fold preference, respectively). PA was a much less preferred acyl donor. However, the acyl group had much greater influence on the activity than the type of phospholipid. For example, ricinoleoyl-PA was nearly as good acyl donor as 18:1-PE, whereas 18:1-PA was only utilized with one-fifth of the rate of corresponding PE substrate.

At this stage we can only speculate about the physiological relevance of the AtPDAT. The PDAT reaction catalyzes a breakdown of the major membrane lipids (PC and PE), thus forming lysophospholipids, which indicate that PDAT could be involved in various signal transduction pathways. LPC and LPE are products from the PDAT as well as of phospholipase A_2 activities but, unlike the lipase reaction, no free fatty acids are formed. Lysophospholipids have been reported to stimulate a number of different activities in the cell (Palmgren and Sommarin, 1989; Ryu et al., 1997; Munnik et al., 1998; Paul et al., 1998; Viehweger et al., 2002; Lee et al., 2003). However, when analyzing the lipid composition from AtPDAT overexpresser we could not see any increase in lyso-PC content. It should be pointed out that lysophospholipid content was on the limit of detection in both control plants and the overexpresser. Another possible physiological role for AtPDAT could be as a scavenger for the membranes. The preference for oxygenated acyl groups by the PDAT might be a membrane repair mechanism in response to situations where oxygenated fatty acids are formed. LCAT has been suggested to play an important role in hydrolysis of oxygenated PC molecules during lipoprotein oxidation in plasma (Goyal et al., 1997).

The identification of the PDAT gene in plants and its functional expression as well as the identification of a plant PDAT T-DNA insertion mutant, as described in this work, open the possibility to experimentally address PDAT's potential involvement in storage lipid synthesis and other metabolic processes in plants. Our experiments with the AtPDAT mutant have also revealed the presence of a third type of enzyme reaction, a DAG:DAG transacylation, involved in TAG biosynthesis in plant tissues. This reaction is carried out by an enzyme(s) other than AtPDAT and thus, adds another number to the growing list of transacylases found in plants.

MATERIALS AND METHODS

Plant Material

Arabidopsis (ecotype Columbia-0) plants were grown in peat-based soil media in a growth chamber with 70% humidity and 16 h light (200 $\mu\text{mol radiation m}^{-2} \text{s}^{-1}$) at 20°C and 8 h dark at 18°C regime. Six-week-old plants, with inflorescence cut back once, were used for transformation. Plant material for gene expression and enzymatic studies were obtained by germinating Arabidopsis seeds in petri dishes containing one-third (1.4 g/L) Murashige and Skoog medium, 1% Suc in absence (nontransgenic) or presence of kanamycin (50 $\mu\text{g}/\text{mL}$) for 10 d. The seedlings were then transferred into beakers with liquid medium containing one-half Murashige and Skoog medium and 1% (w/w) Suc and grown for additional 27 d at 23°C under constant light (80 $\mu\text{mol radiation m}^{-2} \text{s}^{-1}$) and gentle shaking (80 rpm).

Identification of an AtPDAT T-DNA Insertion Mutant

A putative AtPDAT insertion mutation line, SALK_032261 (Alonso et al., 2003), was identified after a BLAST search of the Salk Institute T-DNA insertion library database (<http://signal.salk.edu/cgi-bin/tdnaexpress>) and seeds were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, UK). Detailed analysis of this line revealed a T-DNA insertion in the middle of the 6th (last) exon. The insertion site is upstream of the proposed active sites, D573 and H626, suggesting that AtPDAT's activity is eliminated in this line. Individual plants homozygous for a T-DNA insertion in the AtPDAT gene were identified by PCR screening using primers specific for the AtPDAT gene and the T-DNA left border. Individual plants lacking a T-DNA insertion in the AtPDAT gene (null-segregants) were also identified in the same PCR screening. It should be noted that the SALK_032261 line contained other T-DNA inserts in addition to the one in the AtPDAT gene.

Construction of Plant Expression Vector

The cDNA clone, p5D6T7P, coding for a putative Arabidopsis PDAT (AtPDAT) was obtained from the AIMS database. The clone was sequenced and found, by comparison to the GenBank genomic sequence, to contain an insertion of one base at position 302 counting from the A in the start codon, which caused a frame shift in the open reading frame. This extra base was deleted through site directed mutagenesis and in the same mutagenesis a *SmaI* site was created by a single and silent base change of a thymine to a guanine at nucleotide number 326. The mutagenesis was performed in two consecutive PCR reactions run with the AIMS clone, pD6T7P, as DNA template and with Taq DNA polymerase (Sigma, St. Louis). In the first PCR reaction two 45-bp overlapping DNA fragments, 326 and 1,751 bp long, were amplified using the primers (5'-GGAATTCCATGCCCTTATTCATCGG-3' and 5'-CGCC-TTAAGACCTTCTTTTTGAGCTTAACCCCGGGCGGTCAGG-3') for the N-terminal short fragment and the primers (5'-CCTGACCCGCCGGGT-TAAGCTCAAAAAAGAAGTCTTAAGGCG-3' and 5'-GCTCTAGATCA-CAGCTCAGGTCAATA-3') for the C-terminal long fragment. The primers introduced an *EcoRI* site just before the start codon and an *XbaI* site right after the stop codon of the cDNA. The two PCR fragments were gel purified and equal amounts of each band, together with the end primers and Taq DNA polymerase, were used for the second PCR reaction. The amplified PCR fragment from the second reaction was gel purified and cloned into the plasmid pUni/V5-HIS-TOPO (Invitrogen, Carlsbad, CA) thus generating the plasmid pUS56. The insert in pUS56 was verified by sequencing.

The pUS56 insert, harboring the AtPDAT cDNA, was cut out by an *EcoRI*/*XbaI* digestion and ligated into a *EcoRI*/*XbaI* digested cloning vector. The cloning vector used was pART7-35S (Gleave, 1992) containing the 35S promoter in front of the multiple cloning site and the octopine synthase terminator (MacDonald et al., 1991). Restriction enzyme digestions verified the orientation of the insert and the cloning borders were sequenced. The expression cassette from pART7-35S containing the AtPDAT cDNA were cut out by *NotI* digestion and ligated into a *NotI* digested binary vector, pART27 (Gleave, 1992), generating the binary transformation vector pAtPDAT-27-35S. The binary vector was transferred into *Agrobacterium tumefaciens* GV3101 by the freeze thaw method (Holsters et al., 1978).

Transformation of Arabidopsis

Arabidopsis plants were transformed with *A. tumefaciens* GV3101 harboring the binary plasmid pAtPDAT-27 to 35 s (overexpressers) or the pART27

equipped with an empty expression cassette from pART7-35S (control plants) using the floral dip method (Bent and Clough, 1998). T₁-seeds were harvested and seeds from each plant were kept as separate pools. Seeds from each seed pool were screened on plates containing one-third \times Murashige and Skoog medium, 0.8% agar (Sigma), and kanamycin monosulfate (50 $\mu\text{g}/\text{mL}$). A total of 10 resistant T₁ seedlings were chosen from each of the seed pools and transferred to soil. These primary transformants were grown to maturity and their T₂ seeds harvested. T₂ kanamycin resistant seeds, representing different transgenic lines, were used for further studies. Developing and mature T₃ seeds from T₂ kanamycin resistant plants were collected for northern blotting and lipid analysis.

RNA Isolation and RT-PCR

Total RNA was isolated from root and leaf tissue of wild-type plants grown on agar plates and from open flowers and developing seeds (mid stage) from soil-grown wild-type plants using Concert Plant RNA reagent (Invitrogen, Paisley, UK) as described by the manufacturer. One microgram of RNA was reverse transcribed at 43°C using Moloney murine leukemia reverse transcriptase and the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas GmbH, St. Leon-Rot, Germany) with oligo(dT) as primer according to the protocol provided by the supplier. The resulting cDNA was diluted 10-fold and 1 μL used as template for 35 cycles of PCR amplification using Taq DNA Polymerase (Sigma-Aldrich) with the specific oligo-nucleotides for AtPDAT (forward 5'-TCAAAGCTATCGCTGAGTATAAGG-3'; reverse 5'-CGGTAATTTTGTCTCTAACGGATT-3'), AT3g44830 (forward 5'-CTAACGTCGAAGAACTGTCAAG-3'; reverse 5'-GTGCTCCGGTAATTTGG-3'). Amplification of the ACTIN2 control transcript was done using the primers Actin2-1 (5'-TCCCTCAATCTCATCTTCTCC-3') and Actin2-1 (5'-GACCTGCCTCATCAATCTTCTCC-3'). In order to avoid amplification of genomic products, one primer in each primer pair was designed to span an exon border. The PCR products were analyzed on ethidium bromide stained agarose gels.

RNA-Blot Analysis (Functional Expression of AtPDAT)

T₂ plants and T₃ developing seeds, either transformed with empty vector or with pAtPDAT-27-35s, were used for RNA extraction. Total RNA was extracted by a modified phenol extraction method (Sambrook et al., 1989). Ten micrograms of total RNA was separated on 1.5% agarose gels with 5% formaldehyde and transferred to nylon (Hybond-N+; Amersham Biosciences Europe GmbH, Freiburg, Germany) membranes. Blots were hybridized with the full-length 2-kb AtPDAT cDNA in 0.5 M NaHPO₄ (pH 7.2), 7% SDS, and 1 mM EDTA at 64°C. After hybridization overnight, blots were washed once in 1 \times SSC 0.1% SDS, once in 0.5 \times SSC 0.1% SDS, and twice in 0.1 \times SSC 0.1% SDS at room temperature for 5 min each time, followed by one 5 min wash with 0.1 \times SSC 0.1% SDS at 64°C. Hybridization was visualized and radioactivity quantified by electronic autoradiography (Instant Imager, Packard Instruments, Meriden, CT).

Lipid Substrates

Radiolabeled ricinoleic (12-hydroxy-9-octadecenoic) and vernolic (12,13-epoxy-9-octadecenoic) acids were synthesized enzymatically from [1-¹⁴C]18:1 and [1-¹⁴C]18:2, by incubation with microsomal preparations from seeds of *R. communis* and *Euphorbia lagascae*, respectively (Bafar et al., 1991; Stahl et al., 1995). Radiolabeled crepenynic acid (9-octadecen-12-ynoic acid) was synthesized enzymatically from [1-¹⁴C]linoleic acid by incubation with microsomal preparations from developing seeds of *Crepis alpina* (Lee et al., 1998). [1-¹⁴C]labeled 10:0, 18:0, 18:1, 18:2, 18:3, 22:1, 20:4, and di-[1-¹⁴C]18:1-PC, *sn*-1-16:0-*sn*-2-[¹⁴C]18:2-PE were obtained from Amersham Biosciences (Buckinghamshire, UK). [1-¹⁴C]22:1 acid was purchased from American Radiolabeled Chemicals (St. Louis). *sn*-1-18:1-lyso-PC, *sn*-1-16:0-lyso-PC, *sn*-1-16:0-lyso-PE, and unlabeled fatty acids were purchased from Sigma. *sn*-1-18:1-lyso-PE, and *sn*-1-[¹⁴C]18:1-lyso-PC were obtained by phospholipase (*Naja mossaibica mossaibica*; Sigma) treatment of di-18:1-PE (Avanti Polar Lipids, Birmingham, AL) and di-[1-¹⁴C]18:1-PC, respectively, followed by purification by TLC. The synthesis of PC, PE and PA with [¹⁴C]labeled acyl groups in the *sn*-2 position was performed using either enzymatic (Banas et al., 1992), or chemical acylation of the corresponding [¹⁴C]acyl tri-flour anhydride

(Kanda and Wells 1986) to 18:1-lyso-PC, 16:0-lyso-PC or 18:1-LPE. *sn*-1-[¹⁴C]18:1-*sn*-2-18:1-PC was synthesized by chemical acylation of *sn*-1-[¹⁴C]18:1-lyso-PC with 18:1. All radioactive substrates (specific activities of 5,000–10,000 dpm/nmol) were checked on positional distribution of radioactivity by phospholipase A₂ (from *Naja mossaibica mossaibica* or bee venom; Sigma) treatment, subsequent separation of the lipids by TLC and measuring the relative distribution of radioactivity between lyso-phospholipids and free fatty acids. All *sn*-2-[¹⁴C]substrates were found to have more than 95% of the radioactivity in the free fatty acids, whereas the *sn*-1-[¹⁴C]18:1-*sn*-2-18:1-PC had more than 90% of the radioactivity in lyso-PC. However, it was found that both phospholipase preparations used contained some PLA₁ or lyso-phospholipase activities, yielding an underestimation of the positional specificity of the *sn*-1-[¹⁴C]18:1 substrate. Thus, it is likely that the *sn*-1-[¹⁴C]18:1 substrate also was more than 95% positional specific, since the method used for the synthesis was identical to synthesis of the *sn*-2-[¹⁴C]substrates. *sn*-2-[¹⁴C]18:2-*sn*-1-*rac*-18:1-DAG was synthesized by acylation of *sn*-*rac*-1-oleoyl-glycerol with [¹⁴C]18:2-trifluor anhydride (method modified from Kanda and Wells, 1986). *sn*-2-[¹⁴C]acyl-PA was obtained by phospholipase D (from *Streptomyces chromofuscus*; Sigma) treatment of corresponding [¹⁴C]PC substrate and subsequent separation by TLC. Acyl-vernoloyl-DAG was prepared from *C. palaestina* TAGs by partial lipase treatment (*Rizhopus arrhizus*; Sigma) and subsequent separation by TLC with hexane/diethyl ether/acetic acid (60:40:1.5 by volume) and isolation of the DAGs with one vernoloyl group.

Microsomal Membrane Preparations and Enzyme Assays

Microsomes from leaves and roots from plants grown in liquid culture were prepared using the procedure described by Stobart and Stymne (1985). Aliquots of crude microsomal fractions (corresponding to 12 nmol of microsomal PC) were lyophilized overnight. Substrates (2.5 nmol of radioactive PC, PE, or PA with [¹⁴C]labeled acyl groups in the *sn*-2 position and 1.5 nmol of di-18:1-DAG or at concentrations indicated in the figures) dissolved in 14 μ L benzene were then added to the dried microsomes. The benzene was immediately evaporated under a stream of N₂ at 35°C, leaving the lipids in direct contact with the membranes, after which 0.1 mL of 50 mM potassium phosphate (pH 7.2) was added. The suspension was thoroughly mixed and incubated at 30°C for 60 min. It should be noted that prolonged exposure of the microsomes to benzene severely affected enzymatic activity. Lipids were extracted from the reaction mixture into chloroform (Bligh and Dyer, 1959) and separated by TLC with hexane/diethyl ether/acetic acid (30:70:1.5 by volume) using silica gel 60 plates (Merck, Darmstadt, Germany). The radioactive lipids were visualized and quantified on the plates using electronic autoradiography (Instant Imager, Packard Instruments). Assays were done at least in duplicates and the amounts of synthesized radioactive TAG never deviated more than 10% between duplicate samples. Data shown are standard means of replicates.

Fatty Acid and Lipid Analysis

Plant material was homogenized in chloroform:ethanol:0.15 M acetic acid (1:2:0.9) using a Potter Elvehjem homogenizer and extracted into chloroform according to Bligh and Dyer (1959). Individual lipids in the chloroform phase was separated by TLC as described above for neutral lipids or in chloroform:methanol:acetic acid:water (85:15:10:3.5) for separation of polar lipids. Gel from areas corresponding to lipid standards were removed and methylated in situ on the gel with 2% H₂SO₄ in dry methanol (60 min at 90°C). The methyl esters were extracted with hexane and analyzed by gas liquid chromatography equipped on a WCOT fused-silica 50 m \times 0.32 mm ID coating CP-Wax 58-CB DF = 0.2 capillary column (Chrompack International, Middleburg, The Netherlands) with methyl-heptadecanoic acid added as an internal standard.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permission will be the responsibility of the requestor.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AAR03499, AY072914, P40345, NM_100282, NM_102512, NM_111202, NM_118106, AAK96619, NP_190069, AAR03499, and AB006704.

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