At4g24160, a Soluble Acyl-Coenzyme A-Dependent Lysophosphatidic Acid Acyltransferase

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Human CGI-58 (for comparative gene identification-58) and YLR099c, encoding Ict1p in Saccharomyces cerevisiae, have recently been identified as acyl-CoA-dependent lysophosphatidic acid acyltransferases. Sequence database searches for CGI-58 like proteins in Arabidopsis (Arabidopsis thaliana) revealed 24 proteins with At4g24160, a member of the α/β-hydrolase family of proteins being the closest homolog. At4g24160 contains three motifs that are conserved across the plant species: a CX5XG lipase motif, a HX4D acyltransferase motif, and V(X)3HGF, a probable lipid binding motif. Dendrogram analysis of yeast proteins being the closest homolog. At4g24160 placed these three polypeptides in the same group. Here, we describe and characterize At4g24160 as, to our knowledge, the first soluble lysophosphatidic acid acyltransferase in plants. A lipodomics approach revealed that At4g24160 has additional triacylglycerol lipase and phosphatidylcholine hydrolyzing enzymatic activities. These data establish At4g24160, a protein with a previously unknown function, as an enzyme that might play a pivotal role in maintaining the lipid homeostasis in plants by regulating both phospholipid and neutral lipid levels.

Acylation of glycerol-3-phosphate (G3P) is the first step in the biosynthesis of glycerolipids in plants. Most of the enzymes involved in this pathway were shown to be membrane bound (Somerville and Browse, 1991). However, a soluble G3P acyltransferase has been reported in plants, which acylates G3P to lysophosphatidic acid (LPA) in an acyl-(acyl carrier protein)-dependent manner (Murata and Tasaka, 1997). The role of other soluble enzymes in the glycerolipid biosynthesis pathway is well documented. Cytosolic monoacylglycerol acyltransferase (Tumaney et al., 2001), diacylglycerol acyltransferase (Saha et al., 2006), and LPA phosphatase (Shekar et al., 2002) were shown to be present in the immature seeds of Arachis hypogaea. Recently, cytosolic LPA phosphatase (Reddy et al., 2008) and phosphatidic acid (PA) phosphatase have also been reported in Saccharomyces cerevisiae (Han et al., 2006). In addition, we demonstrated earlier the presence of a soluble LPA acyltransferase (LPAAT) as a part of the cytosolic multienzyme complex for the synthesis of triacylglycerol (TG) in Rhodotorula glutinis (Gangar et al., 2008). In S. cerevisiae, Ict1p catalyzes the acylation of LPA to PA, thereby enhancing phospholipid biosynthesis under cellular stress. A Δict1 deletion strain was shown to be calcofluor white sensitive and exhibited a defective phospholipid biosynthesis, suggesting a role of Ict1p in the maintenance of the cell membranes (Ghosh et al., 2008a).

BLAST analysis of the human genome with the Ict1p sequence resulted in the identification of a gene named CGI-58. Mutations in human CGI-58 are responsible for a rare autosomal recessive genetic disorder known as Chanarin Dorfman syndrome (Zechner et al., 2009). CGI-58 is a member of the α/β-hydrolase family of proteins and has a conserved lipase motif GXNXG, where the Ser is replaced by an Asn. Biochemical characterization of human CGI-58 revealed that it acylates LPA to PA. Heterologous overexpression in yeast showed that expression of CGI-58 enhanced the biosynthesis of total phospholipids, especially PA, phosphatidylethanolamine, and phosphatidylcholine (PC). CGI-58 was found to localize to the lipid bodies isolated from the mice white adipose tissues, but the LPAAT activity in the soluble fraction from adipose tissue was also attributed to CGI-58 (Ghosh et al., 2008b).

So far, a soluble LPAAT from plants has not been identified, although the importance of such enzymes in other experimental systems has been envisaged (Tumaney et al., 2001; Ghosh et al., 2008a). Being aware of the important role of Ict1p and CGI-58 in...
phospholipid metabolism and in combating stress, we started a systematic search for CGI-58-like proteins in plants. The availability of the complete genome sequence of Arabidopsis (Arabidopsis thaliana) allowed us to perform a comprehensive genome-wide survey of CGI-58 like proteins in Arabidopsis. As will be described in this study, a BLAST analysis of CGI-58 in Arabidopsis revealed At4g24160 as its closest homolog. Biochemical characterization of At4g24160 showed its ability to acylate LPA to PA in an acyl-CoA-dependent manner. The recombinant protein has the capability to hydrolyze TG and PC to a lesser extent. Expression analysis of At4g24160 and its homologs suggests the significance of these genes under various stress conditions. In summary, At4g24160 is a soluble acyltransferase with lipase and phospholipase functions from Arabidopsis belonging to the α/β-hydrolase superfamily of proteins.

### RESULTS

#### Evolutionary Relationship of CGI-58-Like Proteins from Arabidopsis

To search for the homologs of CGI-58 in plants the Arabidopsis genome was screened as described in “Materials and Methods.” After identifying the individual protein sequences, we eliminated the repeated sequences by comparisons with theoretical cDNA and genomic DNA sequences in The Arabidopsis Information Resource database. This strategy led to the identification of 24 proteins that were found to be homologous to CGI-58 (Table I). All 24 of these proteins belong to the α/β-hydrolase or esterase/lipase superfamily.

Multiple sequence alignment of all the 24 proteins revealed three conserved motifs (Fig. 1A) and several conserved Ser, Asp, and His residues that may serve as part of the catalytic triad, the active site in most of the

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Figure 1. (Legend appears on following page.)
known hydrolases/lipases. Motif I is V(X)3HGF, where X represents hydrophobic residues such as Val, Ile, Phe, Met, and Leu. This is supposedly the lipid binding motif of these polypeptides. The motif II is GXSXG, the signature motif of known lipases, phospholipases, and lysophospholipases. The motif III is H(X)4D/E, previously identified as a signature motif of most acyltransferases. Interestingly, among all the 24 proteins identified, the H(X)4D motif was found only in At4g24160, whereas the other polypeptides contained an H(X)4E motif. In order to gain an insight into CGI-58-like proteins in other plants, a global BLAST search analysis was performed using the plant database at the National Center for Biotechnology Information (NCBI). Multiple sequence alignment revealed the presence of all the three motifs that were seen in Arabidopsis to be conserved in other plant species (data not shown).

To examine the relationship among CGI-58-like proteins, a topographic dendrogram was constructed (Fig. 1B). ICT1, CGI-58, and At4g24160 were clustered in the same group. However, CGI-58 and its homologs in nonchordates, amphibians, nematode, and higher mammals diverged to form a different subgroup, indicating a final attainment of function in the evolutionary timescale. Interestingly, none of the other 23 CGI-58 homologs from Arabidopsis was present in the same group, suggesting that At4g24160 diverged from other members of this polypeptide family in the evolutionary history to perform specific catalytic functions that may be important in the physiology of the plant. The dendrogram also suggests that the remaining 23 hydrolases have markedly diverged during evolution, attaining specific functions, a feature common among members of the \( \alpha/\beta \)-hydrolase family of proteins. As an example, BODYGUARD 1 domain-containing proteins, At4g24140, At5g41900, and At1g64670, form a distinct group of polypeptides. Multiple sequence alignment of the proteins present in the groups I and II showed that most of the residues

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**Figure 1.** Phylogenetic analysis of CGI-58 and its homologs in yeast, plants, and animals. A. BLAST search was performed using amino acid sequence of CGI-58 followed by ClustalW alignment of the close homologs. B. The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in <50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap tests (500 replicates) is shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were a total of 183 positions in the final data set. Phylogenetic analyses were conducted in MEGA4.
were conserved throughout the family (Fig. 1B). Motifs I, II, and III were distinctly present in all the members of the group, suggesting their probable catalytic role per se. An interesting observation made in the sequence alignment was that the Ser of GXSXG motif is replaced by Asn in all the vertebrates starting from amphibians. The effect of such a conversion needs to be examined. The conversion also suggests the divergence of these polypeptides from the other members of Group I.

Domain Analysis and Subcellular Distribution of CGI-58-Like Proteins in Arabidopsis

Using only the information of the amino acid sequence, it has been difficult to assign a function to proteins of the $\alpha/\beta$-hydrolase superfamily, especially because of low sequence similarities among the members investigated. Moreover, members of this superfamily may catalyze a wide variety of reactions acting as lipases, esterases, epoxide hydrolases, acyltransferases, and/or Ser proteases (Nardini and Dijkstra, 1999). To obtain additional information about these proteins, we analyzed the different functional domains and the subcellular localization of CGI-58-like proteins in Arabidopsis. The eFP cell browser was used (Winter et al., 2007; http://www.bar.utoronto.ca/) to retrieve information on subcellular localization, and domain structure was analyzed based on the Pfam database (http://www.sanger.ac.uk/Software/Pfam/).

A detailed domain analysis suggested possible biological roles for these hypothetical proteins. At1g52750 possesses a putative redox active protein domain with a very high expression in the mitochondria and a moderate expression in the nucleus. At3g10840 was found to have a SARS lipid binding domain, suggesting a possible association of the protein with long-chain fatty acids (Meier et al., 2006). At1g13820 localized mainly in the chloroplast and harbors a putative l-type

Figure 3. Purification of the recombinant At4g24160. A, At4g24160 from E. coli BL21 (DE3) was purified using Ni$^{2+}$-NTA affinity column chromatography. Proteins were resolved on a 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, supernatant of induced lysate; lane 2, pellet of induced lysate; lane 3, the purified At4g24160. M, Molecular mass standard. B, Specificity for cosubstrate (lyso)phospholipids on the acyltransferase activity of purified At4g24160. Values are average of two independent experiments. C, Two-dimensional TLC showing PA as the product formed after the enzymatic acylation of LPA by the purified recombinant At4g24160. First-dimensional solvent system is chloroform:methanol:ammonia (65:35:5, v/v), and the second-dimensional solvent system is chloroform:methanol:acetic acid:water (40:20:5:0.5, v/v). D, Acyl-CoA-dependent formation of PA catalyzed by the purified recombinant At4g24160. Assay was performed with 50 $\mu$M [3H]LPA (0.25 $\mu$Ci/tube), 10 $\mu$M acyl-CoA donors, and 5 $\mu$g enzyme in a final volume of 100 $\mu$L. The reaction was carried out for 20 min. Values are means ($\pm$SD) for four independent determinations.
calcium channel-like domain. At5g17720, a member of cupin superfamily is probably involved in the plant secondary metabolite production (Dunwell et al., 2000) and localizes to the cell wall, cytosol, mitochondria, and chloroplast. At5g41900 has a moderate expression in the plasma membrane and localizes to the mitochondria. It is related to zona pellucida-like-domain-containing proteins, which are known to play structural roles in the plasma membranes (Zazwinska and Affolter, 2004). At4g24140, At1g64670, and At5g41900 were found to contain a domain commonly known as BODYGUARD and may probably be involved in the biosyntheses of cell wall-associated lipids (Kurdyukov et al., 2006). Another member of this group of proteins belongs to the DNA polymerase family of proteins with a putative helix hairpin helix motif, though the subcellular localization suggested a cytosolic association. At4g25290 is related to DNA photolyases, and it is highly expressed in mitochondria followed by the chloroplast. The other proteins in the family do not possess any specialized domain distinction and were grouped under the broad α/β-hydrolase superfamily. Among them, At4g24160 was found to be localized in the mitochondria and the chloroplast. At3g24420 and At5g17780 were found to be intriguing because of their nuclear localization.

Expression Analysis of CGI-58-Like Proteins

The Genevestigator online search tool Meta-Analyzer was used to analyze Arabidopsis Affymetrix microarray data. Relative gene expression was studied in different plant organs, at various growth stages, and under different stress conditions. Many of these genes were found to be up-regulated during biotic stress. The expression profiles of these 24 proteins under different stress conditions are summarized in Table 1. At4g24160 was present from the early seedling to the later stages of development but was found to be specifically expressed in the roots. It was up-regulated during Pseudomonas syringae infection and salicylic acid treatment, while abscisic acid (ABA) and zeatin treatment resulted in its down-regulation. The expression pattern of At4g24160 under abiotic stresses like cold, heat, salt, hypoxia, osmotic, genotoxicity, wound, and drought was found to be interesting. At4g24160 is up-regulated under high salt stress with the maximum expression in the roots (eFP database), which is a particularly important observation because salt stress has been shown to be associated with high phospholipid biosynthesis. The other stress conditions that seem to have an effect on the expression of At4g24160 are cold, osmotic stress, and hypoxia.

At4g24160 Is the Closest Homolog of ICT1 and CGI-58

BLAST analysis of Ict1p in the nonredundant database of NCBI revealed plant At4g24160 and human CGI-58 as its closest homologs (Fig. 2). At4g24160, a 418-amino acid protein, is a member of the α/β-hydrolase family of proteins. In addition to the hydrolase domain, At4g24160 possesses an esterase (pfam 00756), hydrolase/acyltransferase (COG 0596), and lysophospholipase domain (COG 2267). A distinct functional motif H(X)₄D, a characteristic of most acyltransferases (Heath and Rock, 1998) is also present at the C-terminal region of At4g24160. At the N-terminal region, At4g24160 possesses the highly conserved GXSXG motif found in the known lipases, phospholipases, lysophospholipases, esterases, and Ser proteases.

Biochemical Characterization of At4g24160

As a prerequisite for biochemical investigations, At4g24160 was cloned in pRSET A and overexpressed in BL21 (DE3) cells. Immunoblot analysis using anti-(His)₆ monoclonal antibody was performed to confirm the expression. The recombinant protein was purified by Ni²⁺-nitrilotriacetic acid (Ni²⁺-NTA) column chromatography (Fig. 3A).

Lysophospholipid Acyltransferase Activity. As one of the most important enzymatic assays, we analyzed lysophospholipid acyltransferase activity of the purified At4g24160 recombinant protein. As can be seen from Figure 3B, At4g24160 was found to be highly specific for LPA as a substrate. Two-dimensional thin-layer chromatography (TLC) analysis confirmed that PA was the product of this enzymatic conversion (Fig. 3C). At4g24160 was found to be capable of using different acyl-CoA donors for the formation of PA; however, the specificity toward oleoyl-CoA was maximum followed by stearoyl-CoA (Fig. 3D). The enzyme

![Figure 4](image-url)
Figure 5. (Legend appears on following page.)
showed a protein- and time-dependent incorporation of oleoyl-CoA into PA (data not shown). The LPAAT activity was also analyzed by electron spray ionization-mass spectrometry (ESI-MS; Supplemental Fig. S1, A–C). At this point, it is important to mention that At4g24160 homologs of yeast and human were also shown to exhibit a similar LPAAT activity (Ghosh et al., 2008a, 2008b). ESI-MS analysis also confirmed the LPAAT activity of this recombinant purified enzyme.

Lipase and Phospholipase Activity. Since the GXSXG motif was found in At4g24160 and in its yeast homolog Ict1p, the purified recombinant proteins of At4g24160 and ICT1 were analyzed for esterase, lipase, and phospholipase A₂ activities using para-nitrophenyl stearate, triolein, and PC (dipalmitate), respectively, as substrates. Both the bacterially expressed purified proteins (At4g24160 and Ict1p) were able to hydrolyze triolein and PC in a time-dependent manner. At4g24160 has a higher PLA₂ activity (Supplemental Fig. S3, A and B) as compared to the lipase activity (Supplemental Fig. S2, A and B). On the contrary, Ict1p has higher lipase activity (Supplemental Fig. S4, A–C) than the PLA₂ activity (Supplemental Fig. S5, A and B). Lipase and phospholipase activities in yeast and plant enzyme were also confirmed by ESI-MS analysis (Fig. 4). Zero minute and no substrate reactions were kept as controls. These analyses revealed that both enzymes hydrolyze triolein to diacylglycerol, monoaoylglycerol, and fatty acids and PC in a phospholipase A₂-type reaction to lysophosphatidylcholine (LPC) and free fatty acid. On the other hand, both At4g24160 and Ict1p did not show esterase activity (data not shown).

A cocktail assay comprising of all the three substrates, i.e. LPA, TG, and PC, was also performed to determine the substrate preference of both At4g24160 and Ict1p. It was observed that the LPAAT activity for proteins was maximum, and they were capable of hydrolyzing TG and PC to a lesser extent. The PLA₂ activity of At4g24160 was higher than its TG lipase activity; however, the case was vice versa for Ict1p. Zero minute and no substrate reactions were kept as controls (Fig. 5, A–F).

Lysophospholipase Activity. Since At4g24160 was found to possess a putative lysophospholipase domain, we analyzed the LPA lysophospholipase activity of the purified enzyme using [³H]LPA; however, no lysophospholipase activity was detected. The same experiment was also done by ESI-MS using unlabeled LPA (1-oleoyl), and the entire substrate provided was recovered at the end of the reaction without any product formation, clearly indicating the absence of lysophospholipase activity (Supplemental Fig. S6, A and B).

At4g24160 Overexpression Alters Phospholipids in S. cerevisiae

To study the effect of At4g24160 and Ict1p overexpression on the levels of cellular phospholipids and neutral lipids, S. cerevisiae was transformed with pYES2-At4g24160 and pPS189-ICT1. Immunoblotting with anti-Ict1p antibodies confirmed the expression of the At4g24160 protein (Fig. 6A). Overexpression of At4g24160 in yeast led to an approximately 2-fold increase in PA as analyzed by [³²P]labeling of phospholipids in vivo (Fig. 6, B and C). In addition, total phospholipids were quantified as described by Broekhuysen (1968). An increase of 1.5-fold in total

Figure 5. ESI-MS analysis of LPAAT, lipase, and phospholipase activities (cocktail assay). A, Control reaction (without substrate) was performed with assay buffer and 10 μg At4g24160 enzyme in a final reaction volume of 100 μL. The reaction was carried out for 40 min at 30°C. The lipids were extracted with butanol, dried, and analyzed by ESI-MS. The ESI analysis was done in a positive mode. The control reaction clearly reveals that the products formed are not contaminants from the enzyme source. B, Control reaction (without enzyme) was performed with assay buffer, 1 mM LPA (1-oleoyl), 10 μM oleoyl-CoA, 1 mM PC (dipalmitate), and 1 mM triolein in a final reaction volume of 100 μL. The reaction was carried out for 40 min at 30°C. The lipids were extracted with butanol, dried, and analyzed by ESI-MS. The ESI analysis was done in a positive mode. The neutral lipid molecules were sodiated. The control reaction clearly reveals that the products formed are not contaminants from the substrate. C, A cocktail assay was performed with assay buffer and 10 μg purified recombinant At4g24160 enzyme and three substrates, i.e. 1 mM LPA (1-oleoyl), 10 μM oleoyl-CoA, 1 mM PC (dipalmitate), and 1 mM triolein, in a final reaction volume of 100 μL. The reaction was carried out for 40 min at 30°C. The lipids were extracted with butanol, dried, and analyzed by ESI-MS. The ESI analysis was done in a positive mode. The neutral lipid molecules were sodiated. The control reaction clearly reveals that the products formed are not contaminants from the enzyme source. D, Control reaction (without enzyme) was performed with assay buffer and 10 μg Ict1p enzyme in a final reaction volume of 100 μL. The reaction was carried out for 40 min at 30°C. The lipids were extracted with butanol, dried, and analyzed by ESI-MS. The ESI analysis was done in a positive mode. The neutral lipid molecules were sodiated. The control reaction clearly reveals that the products formed are not contaminants from the substrate. F, A cocktail assay was performed with assay buffer and 10 μg Ict1p enzyme and three substrates, i.e. 10 μM oleoyl-CoA, 1 mM LPA (1-oleoyl), 1 mM PC (dipalmitate), and 1 mM triolein, in a final reaction volume of 100 μL. The reaction was carried out for 40 min at 30°C. The ESI analysis was done in a positive mode, and the neutral lipids were sodiated. The LPA provided was acylated to PA in the presence of oleoyl-CoA. TG was hydrolyzed to diacylglycerol and monoaoylglycerol, whereas as LPC was formed from the hydrolysis of PC. The LPAAT activity of the purified Ict1p was the most pronounced followed by its TG lipase activity. The PLA₂ activity was found to be the least.
phospholipid was observed when At4g24160 was overexpressed in S. cerevisiae. The phospholipids in the wild type were found to be 23.8 mg PL/(A_{600} = 5) of cells, whereas on At4g24160 overexpression the total phospholipids were estimated to be 36.8 mg PL/(A_{600} = 5) of cells. Besides the increase in total phospholipids, At4g24160 overexpression showed an approximately 47% increase in PA. The overexpression of ICT1 increased the total cellular phospholipid levels by 1.9-fold and a decrease of nearly 3-fold in the PA formation was observed in ICT1 deleted strain (Ghosh et al., 2008a). Thus, the overexpression of At4g24160 and Ict1p resulted in an increase in PA formation.

Moreover, yeast cells overexpressing At4g24160 and Ict1p were labeled with [14C]acetate in an induction medium to address the question of their effect on neutral lipid metabolism, which suggested that At4g24160 and Ict1p may also be involved in TG turnover in yeast cells (data not shown).

**DISCUSSION**

LPAAT is a crucial enzyme controlling the metabolic flow of LPA into the pool of PA, which plays a key role in many physiological aspects, such as cell signaling, cell polarity, and apoptotic signaling cascades in higher eukaryotes (Park et al., 2004). It was also shown that PA specifically regulates the genes involved in modulating cell shape and organization. PA has also been found to target proteins to the membranes, thereby modulating their catalytic activity (Fang et al., 2001; Anthony et al., 2004; Zhang et al., 2004; Huang et al., 2006). A recent study also demonstrated that PA is involved in the regulation of stomatal movements by influencing the ABA level. It binds to the ABA-interacting protein phosphatase to signal ABA-promoted stomatal closure, whereas phospholipase D and PA interact with G protein to mediate ABA inhibition of stomatal opening. This signaling is essential for maintaining the hydration status of plants (Mishra et al., 2006).

In Arabidopsis, five AtLPAAT1 to AtLPAAT5 genes encoding LPAATs have been reported based on their amino acid sequence similarity (Kim and Huang, 2004). Arabidopsis has one plastidial LPAAT, AtLPAAT1, and the deletion of this gene is known to be embryonic lethal (Kunst et al., 1988). AtLPAAT2 encodes another LPAAT, which is ubiquitous and endoplasmic reticulum located. A related gene, AtLPAAT3, is highly expressed in pollen presumably for a rapid membrane turnover in the pollen tube (Kim et al., 2005). All AtLPAATs identified so far are membrane bound through transmembrane domains. In contrast, several soluble acyltransferases have been identified from various plant sources (Murata and Tasaka, 1997; Tumaney et al., 2001; Saha et al., 2006). This study provides an additional example of a soluble acyltransferase in plants whose enzymatic properties deserve our attention as biochemists and cell biologists.
Based on the BLAST analysis, 24 proteins were identified in Arabidopsis that are homologs of CGI-58 and Ict1p (Fig. 1A). Among these polypeptides At4g24160 is the closest homolog to the two templates, and in the dendrogram analysis it is grouped (Fig. 1B) in close vicinity to CGI-58 and ICT1. All the three proteins possess no transmembrane domains. Most notably, none of the known AtLPAATs fulfill the latter criterion. Earlier studies have shown that Ict1p in S. cerevisiae is highly expressed during organic solvent exposure (Miura et al., 2000; Matsui et al., 2006) and acylates LPA to PA, thereby bringing about an overall increase in the biosyntheses of membrane phospholipids (Ghosh et al., 2008a). A Δict1 mutant was found to have reduced phospholipid biosynthesis and showed sensitivity to calcifluor, suggesting a defect in membrane formation (Miura et al., 2000). Our recent analysis also revealed that the overexpression of Ict1p not only increased the level of phospholipid but also facilitated the degradation of TG, resulting in an enhanced supply of fatty acyl-CoA for PA synthesis and membrane biogenesis during solvent stress. Similarly, the human homolog of Ict1p, CGI-58, was found to be capable of acylating LPA to PA in an acyl-CoA-dependent manner. Mutations in CGI-58 are responsible for a rare hereditary disorder known as Chanarin Dorfman syndrome. Fibroblasts from such patients showed an abnormal biosynthesis of phospholipids (Ghosh et al., 2008b). Ict1p and human CGI-58 both encode a soluble acyl-CoA-dependent LPAAT responsible for enhanced phospholipid synthesis. Here, we demonstrate that the purified recombinant At4g24160 is capable of acylating LPA to PA (Fig. 3B), and it is, to our knowledge, the first soluble LPAAT to be reported in plants.

Interestingly, At4g24160 was also found to possess lipase and phospholipase activities similar to Ict1p (Figs. 4 and 5). Another single polypeptide exhibiting esterase, lipase, and phospholipase activity was reported earlier in the plant system. Thermally stable alkaline lipase from rice bran was shown to hydrolyze para-nitrophenyl palmitate, TG, and phospholipids (Bhardwaj et al., 2001). In addition, adipocyte triglyceride lipase, a single polypeptide, was shown to have phospholipase A2, TG hydrolase, and acylglycerol transacylase activities (Jenkins et al., 2004; Zechner et al., 2009). Here, we report for the first time, to our knowledge, a soluble protein in the plant system, At4g24160, which exhibits acyl-CoA-dependent LPAAT, TG lipase, and phospholipase activities with the LPAAT activity being most profound. The TG lipase and PLA2-specific activities of At4g24160 and Ict1p were found to be low in comparison to the previously demonstrated Arabidopsis lipases. The low activities could be attributed to the heterologous expression of the enzyme in Escherichia coli. The TG lipase-specific activity of sugar-dependent 1 was found to be 40 μmol/mg protein/min and was determined by using the protein purified from yeast (Eastmond, 2006). The baculovirus-expressed AtLip1 (At2g15230) showed a TG hydrolysis with the specific activity of 45 μmol/mg protein/min. However, the E. coli-expressed protein did not show TG hydrolysis (El-Kouhen et al., 2005). On the other hand, our data clearly establish a detectable TG lipase and PLA2 activity for the E. coli-expressed recombinant protein. In the ESI-MS cocktail assay where the recombinant enzyme was presented with a mixture of substrates at the end of reaction period, the products were analyzed by MS (Fig. 5) that revealed the presence of all the three enzyme activities.

In plants, PA plays a pivotal role as a multifunctional stress signal. PA is generated either by the action of phospholipase D or by the sequential action of phospholipase C and diacylglycerol kinase (van Leeuwen et al., 2004; Wang, 2004; Testerink and Munnik, 2005). At4g24160 can generate PA by acylating LPA to PA, or the diacylglycerol produced by the hydrolysis of TG can be channeled toward PA synthesis. Hence, At4g24160 might be involved in generating an independent cytosolic PA pool. The PLA2 activity of this enzyme might keep a check on membrane biogenesis in the presence of excess PA formed. The gene expression analysis also revealed that At4g24160 was highly expressed during salt stress, emphasizing the role of this gene in plant stress signaling. In conclusion, data presented here provide important insights into the probable physiological role of At4g24160 in maintaining lipid homeostasis, thereby regulating the cellular physiology of plants.

**MATERIALS AND METHODS**

**Materials**

At4g24160 clone was obtained from the Arabidopsis Biological Resource Center. \[1^{14}C\]Oleoyl-CoA (54 mCi/mmol), \[3^{14}C\]acetate (51 mCi/mmol), \[9,10-3H\]Hiriolein (53 Ci/mmol), [2-palmitoyl-9,10-3H]phosphatidylcholine (92.3 Ci/mmol), and \[9,10-3H\]LP (47 Ci/mmol) were purchased from Perkin-Elmer Biosciences. \[13^{13}C\]Phosphotriphosphate (5000 Ci/mmol) was obtained from the Board of Radiation and Isotope Technology, Bhabha Atomic Research Centre (Mumbai, India). Silica gel 60 F254 TLC plates were from Merck. Oligonucleotide primers, TG, diacylglycerol, phospholipids, lysophospholipids, and solvents were purchased from Sigma-Aldrich. Acyl-CoA donors were obtained from Avanti Polar Lipids. Polyclonal antibodies were raised against Ni2+-NTA affinity-purified recombinant Ict1p as described (Ghosh et al., 2008a).

**Bioinformatics Analysis**

**Sequence Retrieval, Alignment, and Comparison**

cDNA, EST, and protein sequences were identified by searching public databases available at NCBI (http://www.ncbi.nlm.nih.gov) and The Arabidopsis Information Resource (http://www.arabidopsis.org/) with the BLAST algorithms (Altschul et al., 1990, 1997). Sequences were aligned using the ClustalX program (Thompson et al., 1994). The nonredundant protein sequence database was searched using default parameters, and the sequences with E value of \(10^{-5}\) and score of 80 were retrieved and subjected to sequence alignment.

**Phylogenetic Tree Construction**

The obtained multiple sequence alignment was subjected to bootstrap resampling. These bootstrap replicate alignments were then used to construct phylogenetic trees by the neighbor-joining method (Saitou and Nei, 1987).
Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 software (Tamura et al., 2007). The results were analyzed using the bootstrap method (1,000 replicates) to provide confidence level for tree topology (Felsenstein, 1996). Consensus trees summarizing the topologies found among the bootstrap replicate trees are presented. The tree topology was generated by neighbor joining, with bootstrap support at critical nodes indicated as percentage.

**Examination of Conserved Protein Domains**
Conserved protein domains were examined using the conserved domain database at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and pdam database (http://pdam.sanger.ac.uk/; Bateman et al., 2000).

**Gene Expression Analysis**
The Genevestigator online search tool Meta-Analyzer (http://www.genevestigator.ethz.ch) was used to retrieve microarray expression data.

**Cloning and Expression of At4g24160**

pUNI vector containing At4g24160 open reading frame was used as a template for amplification of the gene. Forward primer (5’-ATGATCCCAT- GAACCTGCGCTTGTGCTT-3’) and reverse primer (5’-ATGATTCC- TAACCACATTCGTAGACCATGGC-3’) were used. PCR (1 min denaturation at 94°C; 1 min annealing at 55°C; and 1 min elongation at 72°C) was performed using Pfu polymerase for 30 cycles with 10 pmol concentration of each primer. The purified PCR product and pSET A vector (N-terminal His tag) were digested with BamHI and Xhol and ligated directionally. The construct was transformed into Escherichia coli BL21 (DE3) cells and induced with 1 mM isopropylthio-galactoside for 4 h at 37°C. The cell pellet was resuspended in lysis buffer containing 50 mM Tris-HCl (pH 8.0) and 300 mM NaCl. Cells were disrupted by sonication. The inclusion bodies were separated and solubilized in lysis buffer containing 6 M urea and 25 mM imidazole. The 10,000 g supernatant of solubilized inclusion bodies was allowed to bind to the Ni2+-NTA matrix. The column was washed with lysis buffer containing 25 mM imidazole. The bound elution was eluted with 250 mM imidazole in lysis buffer. Fractions (1 mL each) were collected and analyzed by 12% SDS-PAGE followed by Coomassie Brilliant Blue staining.

For overexpression of At4g24160 in Saccharomyces cerevisiae, full-length At4g24160 cDNA was cloned from pUNI vector containing At4g24160 to the BamHI-EcoRI site and transformed into yeast cells by lithium chloride method (Schiestl and Gietz, 1989). Transformants were selected on synthetic minimal medium devoid of uracil (SM-U) containing 2% (w/v) Glc and were grown to the late log phase. The cells were harvested by centrifugation and inoculated in an Amplex of 0.4 in SM-U medium containing 2% (w/v) Gal and grown for 24 h. To confirm the expression, cells (A600 = 5) were resuspended in 50 mM Tris-HCl (pH 7.5) and 2% (w/v) SDS and then were disrupted by glass beads. The proteins were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. The overexpression of At4g24160 was confirmed using anti-ct1p antibodies at a dilution of 1:1,000 (1/ν/v).

**LPAAT Assay**
The reaction mixture contained 10 μM [1-14C]oleoyl-CoA (110,000 dpm/assay), 1 to 5 μg enzyme, and 50 μM LPA (1-oleoyl) in assay buffer with a total volume of 100 μL. The reaction was carried out at 30°C for 10 min and terminated by extracting the lipids (Ghosh et al., 2008a, 2008b). Lipids were also analyzed by two-dimensional TLC using chloroform:methanol:ammonia:acetic acid:water (40:20:5:0:5, v/v) as the second-dimensional solvent system. The TLC plates were subjected to autoradiography, and the PA spots were scraped and counted with toluene-based scintillation cocktail. Control incubations were carried out for zero time and in the absence of enzyme. The control value was subtracted from the actual assay value, and the specific activity was calculated after the correction. For ESi-MS analysis, unlabeled substrates 10 μM oleoyl-CoA and 1 mM LPA (1-oleoyl) were used. The reactions were stopped by addition of 100 μL of butanol. The samples were dried and subjected to ESi-MS analysis. The mass by charge peaks obtained were analyzed using the lipid metabolites and pathways strategy (http://www.lipidmaps.org).

**Soluble Lysophosphatidic Acid Aclyltransferase**

**Lipase Assays**
The purified recombinant At4g24160 and Ict1p (10 μg protein) were incubated in a reaction buffer containing 50 mM Tris-HCl, pH 8.0, for 40 min at 30°C in the presence of a sonicated suspension of 1 μM triolein. Reactions were stopped by addition of 100 μL of butanol. The butanol layer containing the lipids was washed with 50 mM sodium acetate. The samples were dried and subjected to ESi-MS analysis. The mass by charge peaks obtained were analyzed using the lipid metabolites and pathways strategy.

Lipase assay was also performed in a reaction buffer containing 50 mM Tris-HCl, pH 7.5, and 100 μM sodium taurocholate for 40 min at 30°C in the presence of sonicated suspension of 100 μM [9,10-3H]tri olein (0.25 μCi/tube). Reactions were stopped by addition of 100 μL of butanol, and the samples were resolved on a silica-TLC plate along with oleic acid standard using chloroform:methanol:ammonia (65:35:5, v/v). The region corresponding to the oleic acid standard was scraped and quantified by liquid scintillation counting. The control value was subtracted from the actual assay value, and the specific activity was calculated after the correction.

For esterase assay, pnu-nitrophenyl esterate (2.5 μM) was used as a substrate, and the hydrolytic product pnu-nitrophenol was monitored at 410 nm. The absorbance was measured against the reference cell to which buffer had been added instead of dialyzed enzyme solution.

**Phospholipase Assays**
The reaction mixture contained 1 μM sonicated vesicles of dipalmitate and 10 μM enzyme in a total volume of 100 μL assay buffer (0.05 μTris-HCl, pH 7.5, and 2 mM dithiothreitol). The reaction was carried out at 30°C for 40 min and terminated by extracting the lipids with butanol. The lipids were dried and analyzed by ESi-MS. The mass-to-charge ratio (m/z) peaks of the substrate and the product were identified using lipid maps.

Radiometric assay consisted of 100 μM sonicated vesicles of [2-palmitoyl-9,10-3H]phosphatidylcholine (1 μCi/assay) and 10 μg enzyme in total volume of 100 μL assay buffer (0.05 μTris-HCl, pH 7.5, and 2 mM dithiothreitol). The reaction was carried out at 30°C for 40 min and terminated by extracting the lipids by the method of Bligh and Dyer (1959). Lipids were analyzed by silica-TLC (Bhardwaj et al., 2001). The region of the plate corresponding to free fatty acid standard was scraped and counted with toluene-based scintillation cocktail.

**Cocktail Assays**
The purified recombinant At4g24160 and Ict1p (10 μg protein) were incubated in a reaction buffer containing 50 mM Tris-HCl, pH 8.0, for 40 min at 30°C in the presence of a sonicated suspension of 10 μM oleoyl-CoA, 1 mM LPA (1-oleoyl), 1 μM triolein, and 1 mM PC (dipalmitate). The reaction was terminated by extracting the lipids with butanol. A sodium acetate wash was given to the butanol layer. The lipids were dried and analyzed by ESi-MS. The m/z peaks of the substrate and the product were identified using lipid maps.

**ESi-MS Analysis**
The butanol soluble fraction was dried and suspended in HPLC-grade methanol and subjected to ESi-MS analysis (Bruker Esquire 3000 plus electrospray ion trap instrument). The sample was applied directly into the ESi source through a polytetrafluoroethylene line at the rate of 4 μL/min. The ESi-MS settings used were as follows: turbo electrospray ionization source was maintained at 260°C, and the data were collected in the positive ion mode. The experiments were repeated three times, and a similar spectrum was obtained in all the cases. The m/z peaks obtained were analyzed by lipid maps (http://www.lipidmaps.org).

[32P]Orthophosphate Incorporation into Phospholipids

pYES2-At4g24160 and pYES2 transformants were grown to the late log phase in 5 mL SM-U containing 2% (w/v) Glc and then transferred to 50 mL of the same media, such that the absorbance was 0.1. The cells were grown till the absorbance reached 3. A600 = 0.4 cells were inoculated in a fresh medium containing 2% (w/v) Gal and 200 μM [32P]orthophosphate and grown for 24 h. Cells (A600 ≥ 25) were harvested by centrifugation, and lipids were extracted and analyzed by two-dimensional TLC. The solvents for the first dimension

Planta Vol. 151, 2009 879

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were chloroform:methanol:ammonia (65:35:5, v/v), and solvents for the second dimension were chloroform:methanol:acetic acid:water (40:20:5:0.5, v/v; Ghosh et al., 2008a).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. LPAAT activity analysis of A4tg24160 by ESI-MS.

Supplemental Figure S2. TG lipase assay for recombinant purified A4tg24160 by ESI-MS.

Supplemental Figure S3. Phospholipase A2 assay for recombinant purified A4tg24160 ESI-MS.

Supplemental Figure S4. TG lipase assay for recombinant purified Ict1p by ESI-MS.

Supplemental Figure S5. Phospholipase A2 assay for recombinant purified Ict1p by ESI-MS.

Supplemental Figure S6. Lyso phospholipase activity of A4tg24160 by ESI-MS.

Received July 3, 2009; accepted August 17, 2009; published August 21, 2009.

LITERATURE CITED


