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Functional characterization of lysophosphatidic acid phosphatase from *Arabidopsis thaliana*

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ABSTRACT

Lysophosphatidic acid (LPA) acts as a signaling molecule that regulates diverse cellular processes and it can rapidly be metabolized by phosphatase and acyltransferase. LPA phosphatase gene has not been identified and characterized in plants so far. The BLAST search revealed that the At3g03520 is similar to phospholipase family, and distantly related to bacterial phosphatases. The conserved motif, (J)4XXXNXSFD, was identified in both At3g03520 like phospholipases and acid phosphatases. *In silico* expression analysis of At3g03520 revealed a high expression during phosphate starvation and abiotic stresses. This gene was overexpressed in *Escherichia coli* and shown to posses LPA specific phosphatase activity. These results suggest that this gene possibly plays a role in signal transduction and storage lipid synthesis.

1. Introduction

Lysophosphatidic acid (LPA), the smallest of all the glycerophospholipids, is one of the intermediates for *de novo* biosynthesis of phospholipids and neutral lipids. Apart from its role in lipid biosynthesis, it also acts as a signaling molecule [1]. Extracellular LPA mediates its action via G-protein coupled cell surface receptors, while intracellular LPA exerts its effect by binding to peroxisome proliferator-activated receptor γ, a transcription factor [2]. LPA acts as a mitogen [3] and involved in tissue remodeling [4]. The LPA is rapidly metabolized (Fig. 1) to either monoacylglycerol (MAG) or phosphatidic acid. LPA specific phospholipase was characterized from rat brain [5]. The excess of LPA is enzymatically hydrolyzed to a free form of phosphate and MAG, thereby maintaining the LPA levels. Recent reports have demonstrated that the extracellular LPA can be produced by the action of lysophospholipase D on lysophosphatidylcholine (LPC) [6]. It was shown that acylglycerol kinase can catalyze the formation of LPA from MAG [7]. The genes of soluble LPA phosphatase from mouse brain, prostatic acid phosphatase [8] and recently identified Phm8p of *Saccharomyces cerevisiae* have been shown to possess LPA phosphatase activity [9]. However, there is no report on LPA phosphatase gene from plant system.

In membrane bound triacylglycerol (TAG) biosynthetic pathway (Phosphatidic acid (PA) dependent), TAG is synthesized by the sequential acylation of glycerol-3-phosphate (G3P) in the microsomal membranes [10]. The LPA generated from G3P acylation is utilized for PA formation by LPA acyltransferase and the resulting PA is then converted to DAG by PA phosphatase. Eventually, DAG acts as a substrate for DAG acyltransferase to produce TAG. Apart from membrane bound pathway, an alternate TAG biosynthetic pathway (PA independent) has been proposed in the soluble fraction of immature peanut seeds [11]. Unlike membrane bound TAG biosynthetic pathway, the LPA, a product of G3P acylation, is dephosphorylated to MAG by LPA phosphatase. The corresponding enzyme was identified and purified from the soluble fraction of *Arachis hypogaea* [12]. The MAG formed through LPA phosphatase can be sequentially acylated to TAG by MAG and DAG acyltransferases [11,13]. This suggests that MAG is an intermediate in TAG biosynthetic pathway. However, recently it was shown that the DAG can also be synthesized from phosphatidylcholine (PC), indicating a direct role of PC in TAG biosynthesis [14,15]. It is possible that the intermediates, i.e., DAG, generated by membrane bound TAG biosynthetic pathway can also be channeled to soluble TAG biosynthesis.

The homology based search for At3g03520 gene product was performed and this protein was found to be a homologue to phospholipase C (PLC) from various organisms. It has DXDXT motif at the C-terminal region but none of its homologues contain DXDXT motif. This is the characteristic motif for phosphomonoesterase. We describe here that the comparative gene expression analysis of At3g03520 is utmost important for understanding expression profile in various conditions. In this article the *in vitro* characterization of

Abbreviations: LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; MAG, monoacylglycerol; PLA_2, phospholipase A_2; PLC, phospholipase C; TAG, triacylglycerol

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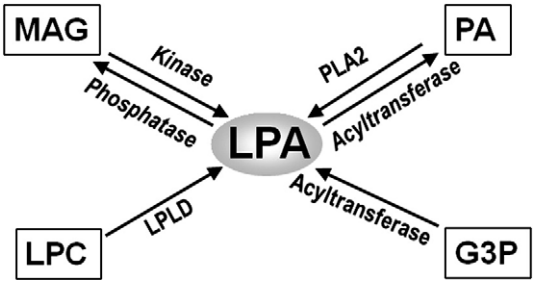

Fig. 1. A schematic representation of lysophosphatidic acid (LPA) metabolism. LPA is synthesized by the acylation of glycerol 3-phosphate (G3P) and the phosphorylation of monoacylglycerol (MAG). In addition, LPA can also be formed by the hydrolysis of lysophosphatidylcholine (LPC) and phosphatidic acid (PA) by the lysophospholipase D (LPLD) and phospholipase A2 (PLA2), respectively. LPA is acylated to PA by LPA acyltransferase and hydrolyzed to MAG by LPA phosphatase.

At3g03520 encoding a soluble LPA specific phosphatase is reported from *Arabidopsis thaliana*.

2. Materials and methods

2.1. Phylogenetic analysis of At3g03520

The At3g03520 related sequences were retrieved from the non-redundant database using BLAST program [16]. The similarity search parameters such as *E*-value and bit score were set to 10^{-5} and 80, respectively. The At3g03520 related sequences were multiply aligned using ClustalX 2.0 version program, and the phylogenetic dendrogram was constructed using bootstrap method with the help of MEGA software.

2.2. Microarray expression analysis of At3g03520

The GENEVESTIGATOR online search tool meta-analyzer method was used to retrieve the levels of gene expression during various conditions such as growth, stimuli and mutations [17]. The expression levels are given as ratio of expression and microarray signal intensity.

2.3. Heterologous expression of At3g03520

The At3g03520 gene was obtained in pUni51 vector from Arabidopsis Resource Centre, Ohio State University, Ohio, USA. The cDNA of At3g03520 was amplified using 5′-TATACTGCAGATGGTGGAGGA-AACGAGC-3′ and 5′-TAGGTACCTCAATTATCACAAATCAAACACGAG-3′ as sense and antisense primers, respectively. PCR reaction mixture consisted of 100 ng template, 10 pmol sense and antisense primers, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 U of XT-5 DNA polymerase (Bangalore Genei, India) and 1× reaction buffer. The gene amplification was carried out under the following conditions: initial denaturation of the template at 94 °C for 4 min followed by 30 cycles at 94 °C for 1 min (denaturation), 55 °C for 1 min (annealing) and 72 °C for 1 min (extension). The final extension was done at 72 °C for 20 min. The amplified product was cloned into pRSET-C vector (Invitrogen) at *Kpn*I and *Pst*I restriction sites. The resultant plasmids containing the gene were confirmed by sequencing. The expression of recombinant protein in *E. coli* Origami (DE3) pLysS cells (Novagen) was induced by adding 0.5 mM IPTG, and the induction was continued for 4 h at 37 °C. The over expressed protein was purified by Ni-NTA chromatography. Overexpression of At3g03520 was confirmed by immunoblot with anti-His_6 antibody. The protein was estimated by Bio-Rad DC protein assay kit.

2.4. LPA phosphatase assay

LPA phosphatase assay was performed as described earlier [12]. Briefly, [1-oleoyl-9,10-^{3}H]LPA (47 Ci/mmol) was suspended in 0.1% Triton X-100 and sonicated for 5 min in a sonic bath. The substrate stock solution was diluted 100-fold in the assay. LPA phosphatase assay mixture consisted of 50 mM MES (pH 6.5), 50 μM [^{3}H]LPA, 2 mM $MgCl_2$ and 1 μg of the purified recombinant enzyme in a total volume of 100 μl. The incubation was carried out at 37 °C for indicated time points and stopped by the addition of 500 μl of 2% phosphoric acid followed by 600 μl of chloroform:methanol (1:2, v/v). Following lipid extraction, the lower chloroform-soluble fraction was separated by TLC on 250 μm silica gel G plates using chloroform:methanol:water (98:2:0.5, v/v) as the solvent system. The lipids were visualized by staining with iodine vapor, and spots of MAG were scraped off for determination of radioactivity by liquid scintillation counting. The substrate alone and substrate with boiled enzyme were used as controls, and the control values were subtracted from the values measured in the presence of an active enzyme source.

2.5. LPA phosphatase assay with different LPAs

The different LPAs (lauroyl(C12:0), myristoyl(C14:0), palmitoyl (C16:0), stearoyl(18:0) and oleoyl (18:1)) were obtained from Avanti Polar Lipids. The LPA phosphatase assay with different LPA species was performed as described above and the phosphate, the hydrolyzed product of LPA phosphatase, was estimated using malachite green method [18].

2.6. Other enzyme assays

Fifty micromoles of diacylglycerol pyrophosphate or sphingosine-1-phosphate were incubated with the purified recombinant At3g03520 and the release of phosphate was monitored in the aqueous phase by using malachite green method [18]. Assay component and conditions are same as described for LPA phosphatase assay. [glycerol-^{14}C(U)] Dipalmitoyl-PA (50 μM; 165, 000 dpm/reaction) was incubated with the purified recombinant At3g03520 for 30 min and the reaction was stopped by adding 2% phosphoric acid followed by lipid extraction using chloroform-methanol in the ration of 1:2 (v/v). The lower chloroform phase was spotted on TLC and estimated the formation of DAG in chloroform:methanol:water (98:2:0.5, v/v) as the solvent system.

The lysophospholipids were prepared by treating radiolabeled [^{3}H] PC and PE with Pseudomonas lipase (Sigma-Aldrich). After the reaction, the products were purified from silica-TLC (110,000 dpm/reaction) and incubated with the purified At3g03520 for 30 min at 30 °C. Reaction was stopped by the addition of 2% phosphoric acid followed by lipid extraction using chloroform and methanol in the ratio of 2:1 (v/v). The lower chloroform soluble fraction was spotted on TLC and looked for the formation of MAG using chloroform: methanol:water (98:2:0.5, v/v) as the solvent system. Assay components and conditions are same as described for LPA phosphatase assay.

Glycerol-3-phosphate hydrolysis was carried out with the purified recombinant At3g03520 by incubating 100 μM of [^{14}C]glycerol-3-phosphate (220, 000 dpm/reaction) for 30 min and the reaction was stopped by the addition of 2% phosphoric acid. To this equal volume of chloroform was added to precipitate the protein, vortexed, centrifuged for 2 min and an aliquot of the supernantent was spotted on a TLC. The TLC was developed in 0.1 M ammoium acetate in 67% ethanol [19] and exposed to phosphorimaging screen and quantified using liquid scintillation counter.

3. Results and discussion

3.1. Domain, hydrophathy and phylogenetic analysis of At3g03520

Based on sequence similarity and conserved domain search against NCBI non-redundant protein database, the At3g03520 gene product with unknown function was found to possess phosphoesterase domain