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Characterization of a cDNA encoding *Arabidopsis* secretory phospholipase A_2-α, an enzyme that generates bioactive lysophospholipids and free fatty acids

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Abstract

Phospholipase A_2s (PLA_2s) are enzymes that liberate lysophospholipids and free fatty acids (FFAs) from membrane phospholipids in response to hormones and other external stimuli. This report describes the cloning and functional characterization of a PLA_2 cDNA from *Arabidopsis thaliana*, *At*sPLA_2-α, which represents one of four secretory PLA_2 (sPLA_2) genes in *Arabidopsis*. The encoded protein is 148-amino acid polypeptide and is predicted to contain a 20-amino acid signal peptide at its amino terminus. The predicted mature form (M_r=14,169) of *At*sPLA_2-α exhibited approximately 5 times the specific activity of its pre-processed form. Different from animal sPLA_2s, *At*sPLA_2-α showed a significant preference for the acyl group linoleic acid over palmitic acid in phospholipid hydrolysis. Like some animal sPLA_2s, however, it has a slight preference for phosphatidylethanolamine over phosphatidylcholine as the substrate. The specific activity of *At*sPLA_2-α continuously increased as the Ca^{2+} concentration was increased to 10 mM, and the optimal pH range was very broad and biphasic between 6 and 11. *At*sPLA_2-α transcript was detected at low levels in roots, stems, leaves, and flowers but not in siliques.

Keywords: Phospholipase A_2; Phospholipid; Bioactive lipid

1. Introduction

Phospholipase A_2s (PLA_2s) hydrolyze glycerophospholipids specifically at the *sn*-2 position to yield free fatty acids (FFAs) and lysophospholipids. Animal PLA_2s are well studied and have been found to play key roles in diverse cellular responses, including phospholipid digestion and metabolism, host defense and signal transduction [1–3], and generation of precursors for the synthesis of eicosanoids, platelet-activating factor and some bioactive lysophospholipids [4]. Studies in plants suggest that PLA_2s may be involved in auxin-induced cell elongation [5]. Auxin treatment resulted in the rapid elevation of FFA, lysophosphatidylcholine (LPC), and lysophosphatidylethanolamine (LPE) levels in microsomes, implying an increase in PLA_2 activity [6–10]. Furthermore, auxin-dependent elongation of hypocotyl segments was inhibited by the addition of PLA_2 inhibitors [11]. Our group found that the lysophospholipid LPE could retard senescence in leaves, flowers, and fruits [12,13] and accelerate fruit ripening [14] when exogenously sprayed onto plants. LPE suppressed the production of ethylene, a plant hormone involved in stimulating senescence. LPE and lysophosphatidylinositol also inhibit phospholipase D, a key enzyme promoting membrane deterioration that leads to plant senescence [15].

Abbreviations: FFA, free fatty acid; GST, glutathione S-transferase; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, Phospholipase A_2; RT-PCR, reverse transcription-polymerase chain reaction; sPLA_2, secretory low-molecular weight PLA_2; TLC, thin-layer chromatography

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During embryo maturation, a PLA_2-like activity has been implicated in the selective removal of unusual fatty acids from phospholipids in the ER to allow for their reintroduction into triacylglycerols [16–18]. PLA_2s are involved in plant responses to external stimuli such as wound stress and pathogen elicitors. Products of reactions catalyzed by PLA_2s rapidly accumulated following the subjection of plants to localized wounding [19]. Linolenic acid, a FFA, is the precursor of the plant hormone jasmonic acid [20], which activates the expression of defense-related genes. PLA_2 activity is also elevated in response to pathogen elicitors, and may play a role in initiating/triggering the oxidative burst [5,21].

Plant PLA_2s can be classified into 2 groups based on sequence data and biological properties [5]: the secretory low-molecular-weight PLA_2s ($sPLA_2$s), and the patatin-like PLAs (PAT-PLAs), which are homologous to the intracellular animal Ca^{2+}-independent PLA_2 but show combined activities of PLA_2 and phospholipase A_1. Patatin-like PLAs have been reported in plants and are thought to be involved in insecticidal and anti-oxidant activities as well as in auxin-induced hypocotyl elongation [22–28]. $sPLA_2$ activity in plants has been reported in partially purified preparations [17,18] and several cDNAs encoding putative $sPLA_2$s have been reported [18,29]. Two *Arabidopsis* $sPLA_2$ isoforms were found to encode functional $sPLA_2$ enzymes [30,31]. In this study, we cloned and functionally characterized a third *Arabidopsis* $sPLA_2$ gene which we named *At*$sPLA_2$-α. We compared the specific activities of the pre-processed and mature forms of *At*$sPLA_2$-α, determined the pH and Ca^{2+} concentrations for optimal enzymatic activity, and demonstrated its *sn*-2 acyl specificity and headgroup preference. Also, we showed the spatial expression pattern of the gene in plant tissues.

2. Materials and methods

1-Palmitoyl-2-[^{14}C]palmitoyl-phosphatidylcholine (PC), 1-palmitoyl-2-[^{14}C]linoleoyl-PC, 1,2-[^{14}C]dipalmitoyl-PC, and 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylethanolamine (PE) were purchased from Amersham Pharmacia Biotech. Unless otherwise stated, all other chemicals were purchased from Sigma. The *Arabidopsis* cDNA library was donated by J. Kieber and J. Ecker to the *Arabidopsis* Biological Resource Center in Columbus, Ohio [32].

2.1. Identification and cloning of the *AtsPLA*$_2$-α gene

The two degenerate oligonucleotides 5′tgtcctggtgaga/gaa/gccttgtgat and 5′caaga/catcaagac/tcatcacaa (degenerate positions are underlined; one or the other indicated nucleotide was introduced with equal probability) were designed based on two putative *Arabidopsis* PLA_2 genes identified by a BLAST Search (tblastn) of the *Arabidopsis* genomic DNA database (http://genome.www.stanford.edu/Arabidopsis/) using the *N*-terminal amino acid sequence of Elm PLA_2 as query. These two oligonucleotides, in combination with either the T3 or T7 promoter primer, were used to amplify PLA_2 cDNAs from the Kieber and Ecker, *Arabidopsis*, size-selected, cDNA libraries. The PCR reactions, containing 6 pmol of each primer and 1 unit ExTaq polymerase (Takara Mirus Bio), were subjected to 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min. Several amplification products were isolated from a 0.8% agarose gel, cloned into pGEM-T (Promega) and sequenced using the Dideoxy–Sanger method. Sequence comparisons against the GenBank sequence databases were performed using BLAST programs.

To obtain a clone containing the complete coding region of *At*$sPLA_2$-α and to express the encoded protein in *E. coli*, its cDNA was amplified from the 0.5 to 1 kb size-selected cDNA library using the oligonucleotides: 5′gcaggatccatggcatggcggctccgatcatac and 5′catggatccttagggtttcttgaggactttg (translational start and stop codons underlined) using the same cycling program as above. The amplified cDNA was digested with *Bam*H1 and ligated into the *Bam*H1 site of expression vector pGEX-4T (Amersham Pharmacia Biotech), which generates a GST fusion protein, to construct pGEX-4T *PLA*$_2$-α.

To express *At*$sPLA_2$-α without its predicted signal peptide (predicted mature form), its cDNA was amplified with the oligonucleotides 5′cggatcccccttaacgtcggtgttcagctc and 5′cctcgaggggtttcttgaggactttgcc and ligated between the *Bam*HI and *Xho*I sites of pET40b(+) (Novagen). This construct was designed to express the predicted mature form of *At*$sPLA_2$-α as a *C*-terminal fusion to DsbC that catalyzes disulfide bond formation and induces protein export into the periplasm, a more favorable environment for folding and disulfide bond formation. To synthesize the pre-processed form that includes the signal peptide, the cDNA was amplified with 5′cggatcccatggcggctccgatca and 5′cctcgagggtttcttgaggactttgcc prior to *Bam*HI and *Xho*I digestion and ligation into pET40b(+).

2.2. Expression and purification of recombinant *AtsPLA*$_2$-α

pGEX-4T *PLA*$_2$-α was introduced into BL21(DE3)PlysS cells (Novagen) to overexpress *At*$sPLA_2$-α fused to the *C*-terminus of glutathione S-transferase (GST). A single colony from the transformation was used to inoculate 2 ml of LB medium containing 150 μg/ml ampicillin and the culture was grown at 37 °C overnight. Fifty μl of the culture were diluted into 25 ml of fresh medium and grown for an additional 3 h prior to inducing GST-PLA_2 expression by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM final concentration). The cells were harvested by centrifugation (5 min at 3000×*g*) after 2 h further incubation at 27 °C and resuspended in 1 ml of STE buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM EDTA) containing 0.5 mM PMSF. The resuspended cells were briefly sonicated and centrifuged 5 min at 10,000×*g* to obtain a cell-free extract to measure PLA_2 and GST activities.