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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.

To more precisely analyze *At*sPLA$_2$-α activity, the GST–sPLA$_2$ fusion protein and sPLA$_2$ (released from GST–sPLA$_2$ by thrombin digestion) were purified using affinity chromatography on glutathione-agarose as described [33,34]. The purified GST–sPLA$_2$ fusion protein and sPLA$_2$ were desalted on Sephadex G-25 columns (Amersham Pharmacia Biotech). To monitor protein expression levels, GST activity was measured using the 1-chloro-2,4-dinitrobenzene assay kit (Amersham Pharmacia Biotech). Aliquots of protein were obtained following each purification step to conduct PLA$_2$ activity analysis.

To compare the enzyme activities, the pre-processed and predicted mature forms of *At*sPLA$_2$-α were expressed fused to the *C*-terminus of DsbC in BL21(DE3) cells (Novagen). The fusion proteins were purified with the His·Bind purification kit (Novagen) and their expression levels were monitored with the S·Tag rapid assay kit (Novagen).

2.3. PLA$_2$ activity assays

The *sn*-specificity, acyl group preference and headgroup selectivity of the*At*sPLA$_2$-α enzyme were determined using the substrates 1,2-1-palmitoyl-2-[^{14}C]palmitoyl-PC, 1-palmitoyl-2-[^{14}C]linoleoyl-PC, 1,2-[^{14}C]dipalmitoyl-PC, and 1-palmitoyl-2-[^{14}C]linoleoyl-PE, mixed with unlabeled dipalmitoyl-PC, soybean PC, or soybean PE, as previously described [30,31]. PLA$_2$ activity assay for optimal pH and Ca^{2+} concentrations used as the substrate a mixture of 1-palmitoyl-2-[^{14}C]linoleoyl-PE with soybean PE in buffers with various levels of Ca^{2+} or pH [35]. Duration of the assay was 90 min.

2.4. Total RNA extraction and quantitative RT-PCR

Total RNA was isolated from *Arabidopsis* roots, stems, leaves, flowers, and siliques using RNA Isolator (Gibco) and treated with DNase RQI (Promega) to remove residual DNA. Relative quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed using an endogenous standard with the QuantumRNA™ 18 S kit (Ambion) as described previously [31]. The resulting, first strand cDNAs served as templates in PCR reactions with the gene-specific primers 5′ tctctgtctctgtctcggca′ and 5′ tcttggattggacacaagca′ chosen using the Primer Select Program (WICGR-MIT).

3. Results

Using the published *N*-terminal amino acid sequence of a purified Elm sPLA$_2$ [17] as query, we identified two related sequences in the *A. thaliana* database. Partial cDNAs were amplified by PCR from the Kieber and Ecker, *Arabidopsis*, cDNA library. Products were cloned into pGEM-T, sequenced, probable start and stop codons identified, and primers synthesized to obtain a cDNA encompassing the complete coding region of one sPLA$_2$. The cDNA of this gene, which we named *At*sPLA$_2$-α, was introduced into pGEX-4T. The *At*sPLA$_2$-α gene, located on the short arm of chromosome II, has been annotated as an expressed protein (At2g06925). The deduced amino acid sequence of *At*sPLA$_2$-α is given in Fig. 1A and its cDNA sequence has been reported to Genbank (accession #AY344842). The ATG at position 78–80 likely serves as the translation initiation codon because (1) it is the 5′-most ATG in the sequence, (2) there is an in-frame stop codon upstream, and (3) it designates the proper reading frame to align with the amino acid sequence of elm sPLA$_2$. The cDNA also contains an in-frame stop codon near its 3′ end designating the end of the coding region. A comparison of the cDNA with its corresponding genomic DNA (gene ID: 815261) indicated that *At*sPLA$_2$-α is composed of 4 exons. The open reading frame specifies a protein (accession #AAR04682) of 148 amino acids with a molecular weight of 16,310 and a pI of 7.61 (Fig. 1A).

Using the deduced amino acid sequence of *At*sPLA$_2$-α as query, BLAST searches of the NCBI database indicated that the protein contains the conserved Ca^{2+}-binding site (46–69% identity) and active site (62% identity) motifs, characteristic of the low molecular weight animal sPLA$_2$s (both motifs are designated in Fig. 1A). *At*sPLA$_2$-α does not share significant sequence homology to any of the animal sPLA$_2$s except within these two motifs.

Three additional genes were identified in the genomic DNA sequence of *Arabidopsis thaliana* using as query the deduced amino acid sequence of *At*sPLA$_2$-α; the three predicted to encode sPLA$_2$s were designated *At*sPLA$_2$-β, -γ, and -δ (At2g19690, At4g29460, and At4g29470, respectively). The amino acid sequences of *At*sPLA$_2$-β, -γ, and-δ were derived from the cDNA sequences AF541915 [31], AY148346 [30], and AY148347, respectively. Interestingly, the positions of twelve cysteine residues are completely conserved in the four *Arabidopsis* sPLA$_2$ proteins (Fig. 1A). Some or all of these cysteines may be involved in intramolecular disulfide bridges that stabilize protein structure. Evidence that one or more disulfide bridges are important for *At*sPLA$_2$-α activity is presented below.

The deduced amino acid sequence of *At*sPLA$_2$-α was compared to the *N*-terminal amino acid sequence of purified elm sPLA$_2$ (17, Fig. 1B). Interestingly the homology between the two begins at amino acid 21 of *At*sPLA$_2$-α, which matches with amino acid 1 of elm sPLA$_2$. Because some animal sPLA$_2$s have *N*-terminal signal peptides [36–38] as do the closely related *At*sPLA$_2$-β and *At*sPLA$_2$-γ [30,31], it is presumed that the first 20 amino acids specified by the *At*sPLA$_2$-α cDNA serve as a signal peptide involved in protein sorting. The computer program pSORT (http://psort.nibb.ac.jp.ggoa/) concurs with this prediction and indicate that the protein will likely be shuttled to the vacuole or outside the cell.

The BLAST search also revealed that *At*sPLA$_2$-α shares sequence similarity to proteins encoded by expressed

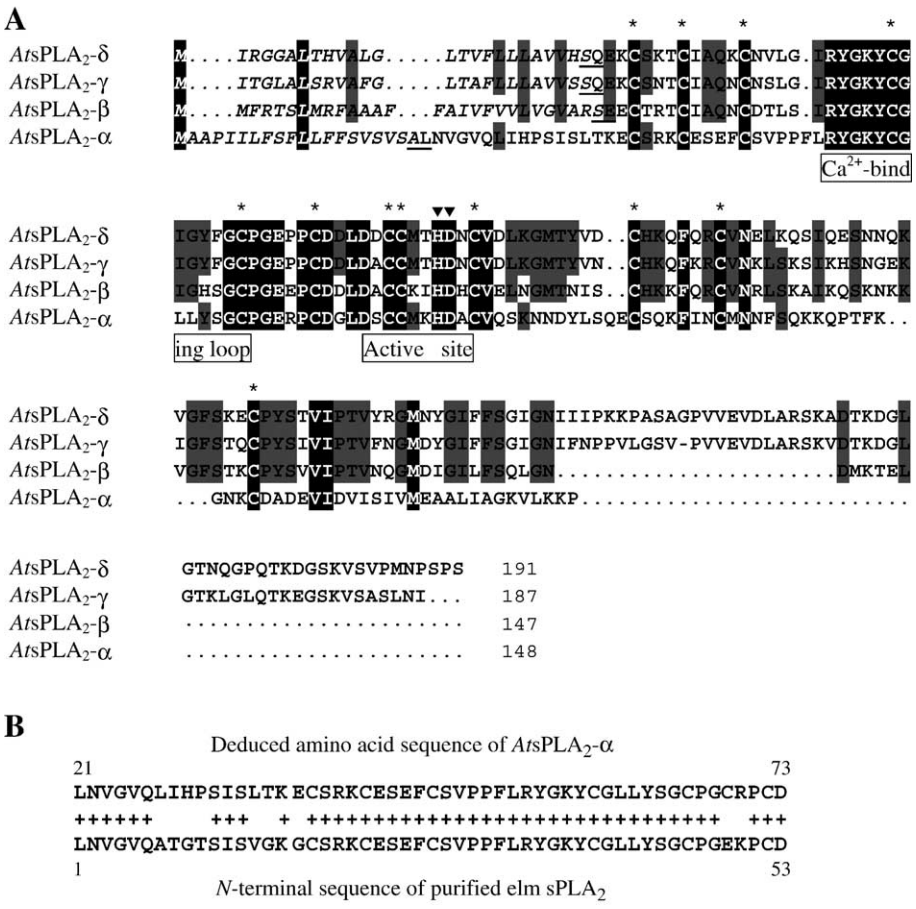

Fig. 1. Amino acid sequence comparison between *At*sPLA$_2$-α (At2g06925), and three related *Arabidopsis* sPLA$_2$s (*At*sPLA$_2$-β, -γ, and-δ). (A) Alignment of the deduced amino acid sequences of four *At*sPLA$_2$ genes. The putative *N*-terminal signal peptides of *At*sPLA$_2$s are italicized and the predicted cleavage sites for removal of the signal peptides are underlined. The domains having homology to the Ca^{2+}-binding loop (YGKYCGxxxxGC) and active site (DACCxxHDxC) motifs of animal sPLA$_2$s are indicated in open squares. Each of the 12 conserved cysteine residues among *At*sPLA$_2$s is marked with an asterisk. The conserved histidine/aspartate dyad in the active site domain is marked with triangles. (B) The deduced amino acid sequence of *At*sPLA$_2$-α compared to the *N*-terminal amino acid sequence of purified elm sPLA$_2$. Only the part of *At*sPLA$_2$-α which aligns with the elm sPLA$_2$ sequence obtained by Ståhl et al. (1998) is given. The respective amino acid sequences are numbered beginning with the first methionine encoded by the *Arabidopsis* cDNA and the first sequenced amino acid for the elm enzyme. +s indicate positions with an identical amino acid.

sequence tags from carnation (AF064732), populus (AI16-4389), tomato (AI487873, BG127 198), cotton (BF275891), soybean (AI522932, AW318251, BE802011, BG045671), pine (AW289670), and rice (AJ238116, AJ238117, AAK-50122). The overall amino acid sequence identity scores were between 40 and 60%, indicating a moderate relationship to *At*sPLA$_2$-α. The regions with the greatest sequence conservation are the Ca^{2+}-binding loop motif, and the short sequence that separates the Ca^{2+}-binding loop from the active-site domain.

The pGEX-4T *PLA$_2$* construct was introduced into BL21(DE3)PlysS cells to over-express *At*sPLA$_2$-α fused to the *C*-terminus of GST. The GST–*At*sPLA$_2$-α fusion protein was found to have an approximate molecular weight of 46 kDa, while GST produced by pGEX-4T has an apparent molecular weight of 30 kDa [data not shown]. This suggested that the molecular weight of *At*sPLA$_2$-α is about 16 kDa, in good agreement with the calculated molecular weight of 16,310.

To determine if the cloned cDNA encodes a functional PLA$_2$ enzyme, GST–*At*sPLA$_2$-α and *At*sPLA$_2$-α (released from GST–*At*sPLA$_2$-α by thrombin digestion) were purified from cell-free extracts using glutathione-agarose beads and tested for enzyme activity. The *Arabidopsis* cDNA was indeed found to encode an enzyme that, like snake venom sPLA$_2$, digests PC to yield LPC and FFA as determined by thin-layer chromatography (TLC) (data not shown).

To compare the enzymatic activities of the pre-processed (with putative signal peptide) and predicted mature (without signal peptide) forms of *At*sPLA$_2$-α, we expressed the respective proteins in *E. coli* strain BL21(DE3) cells fused to the *C*-terminus of DsbC to enhance disulfide bond formation. For unknown reasons, the expression level of the pre-processed form was low, but that of the mature form was much higher [data not shown]. By equalizing the molar concentrations of enzymes added, the specific activity of PC hydrolysis by the pre-processed form of *At*sPLA$_2$-α was determined to be ~80% lower than that of the mature form

(Fig. 2A, lanes 1 and 2). It was also determined that the mature form retained 76% of its activity after a 5-min boiling treatment but lost 82% of its activity when assayed in the presence of the reducing agent DTT (Fig. 2A, lanes 3 and 4). Similar results were found on the influence of DTT on pre-processed enzyme (data not shown).

The *sn*-position specificity of *At*sPLA$_2$-α activity with or without signal peptide was determined using 3 substrates as described in Materials and methods. Purified *At*sPLA$_2$-α was incubated separately with each substrate, the reaction products were separated on TLC plates, and the location of the ^{14}C was used to determine the site of hydrolysis. Radioactive LPC was not released when the protein was incubated with 1-palmitoyl-2-[^{14}C]palmitoyl-PC or 1-palmitoyl-2-[^{14}C]linoleoyl-PC, indicating that both the pre-processed and mature forms of the enzyme are not active at the *sn*-1 position of PC (Table 1). When 1,2-[^{14}C]dipalmitoyl-PC was used as a substrate for the mature form of the enzyme, a similar level of radioactive carbon was measured in the LPC and FFA spots, indicating that the protein indeed hydrolyzes specifically the *sn*-2 position and is therefore a PLA$_2$ rather than a PLA$_1$. For unknown reasons, the pre-processed form of the enzyme appeared to release more radioactive carbon in the FFA spot than in the LPC spot. *At*sPLA$_2$-α hydrolyzed the linoleoyl acyl chain from 1-palmitoyl-2-[^{14}C]linoleoyl-PC at a higher rate than the palmitoyl acyl chain from 1-palmitoyl-2-[^{14}C]palmitoyl-PC, indicating that it exhibits an acyl preference (Table 1). In contrast, *At*sPLA$_2$-β showed the opposite acyl group preference, while *At*sPLA$_2$-γ and-δ did not exhibit any significant acyl group preference [30,31,39].

Headgroup preference of *At*sPLA$_2$-α was determined using the substrates 1-palmitoyl-2-[^{14}C]linoleoyl-PC and 1-palmitoyl-2-[^{14}C]linoleoyl-PE. This and all subsequent enzyme activity assays were conducted only with the mature form of *At*sPLA$_2$-α. In simultaneous, 1 h experiments, *At*sPLA$_2$-α hydrolyzed about 70% of the PE into FFA and LPE, and about 50% of the PC into FFA and LPC

Table 1
sn-Specificity and acyl group preference of the pre-processed (P) and mature (M) forms of recombinant *At*sPLA$_2$-α enzyme

Enzyme	% of input ^{14}C recovered as	
	LPC	FFA
Substrate= 1-Palmitoyl-2-[^{14}C]palmitoyl-PC		
P	0.4±0.1	34.8±0.7
M	0.9±0.1	68.0±8.0
Substrate= 1-Palmitoyl-2-[^{14}C]linoleoyl-PC		
P	2.6±0.1	60.5±0.2
M	0.4±0.1	92.4±5.8
Substrate= 1,2-[^{14}C]Dipalmitoyl-PC		
P	21.7±0.1	31.9±0.3
M	46.6±1.3	43.6±6.1

Values shown are means±S.E. obtained three independent activity assays (n=3). Duration of the assay was 90 min.

(Fig. 2B). This indicates a slight preference for PE over PC, the same as observed for *At*sPLA$_2$-β [39]. In contrast, *At*sPLA$_2$-γ and-δ both showed a very strong preference for PE over PC [30,39].

The catalytic properties of *At*sPLA$_2$-α were examined with respect to pH and Ca^{2+} levels to characterize the optimal conditions for its activity using 1-palmitoyl-2-[^{14}C]linoleoyl-PE mixed with soybean PE as the substrate. Interestingly, *At*sPLA$_2$-α exhibited a very broad and biphasic optimal pH range between pH 6 and 11, although specific activity was somewhat greater in the pH 9–11 range than in the pH 6–8 range (Fig. 3A). In contrast, the other isoforms of *At*sPLA$_2$ showed narrower optimal pH ranges: pH 6–7 for *At*sPLA$_2$-β, pH 7–9 for *At*sPLA$_2$-γ, and pH 8–9 for *At*sPLA$_2$-δ [30,39]. Similar to animal sPLA$_2$s but different from the other *At*sPLA$_2$s, the specific activity of *At*sPLA$_2$-α continuously increased as the Ca^{2+} concentration was increased [40] to 10 mM (Fig. 3B). A similar phenomenon was observed for *At*sPLA$_2$-γ when PC served as the substrate [30]. However, when re-examined with PE,

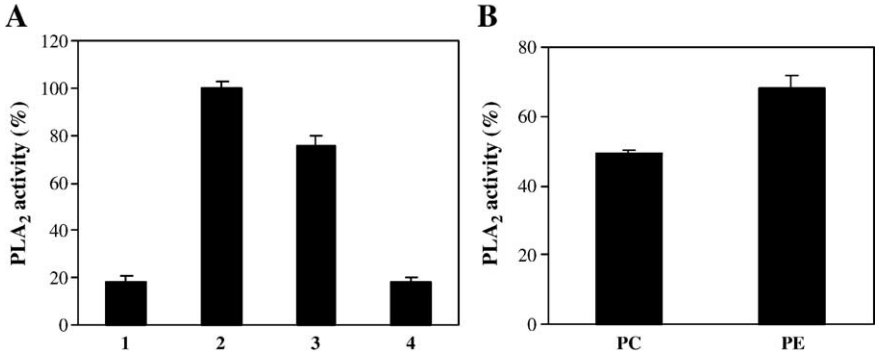

Fig. 2. Enzymatic activities of the mature and pre-processed forms of recombinant *At*sPLA$_2$-α expressed in *E. coli*. (A) Relative enzyme activities of the pre-processed (lane 1) and mature (lane 2) forms of the recombinant *At*sPLA$_2$-α. The effects of a 5 min boiling treatment (lane 3) or the reducing agent DTT at 5 mM (lane 4) on the activity of the mature form of the enzyme were also analyzed. (B) Headgroup preference of the mature form of the recombinant *At*sPLA$_2$-α. Data (mean with standard deviations) in (A) and (B) were obtained from three independent experiments and are presented as the percents of recovered ^{14}C radioactivity in the enzymatic products, lysophospholipids and free fatty acids, from the radio-labeled substrates.

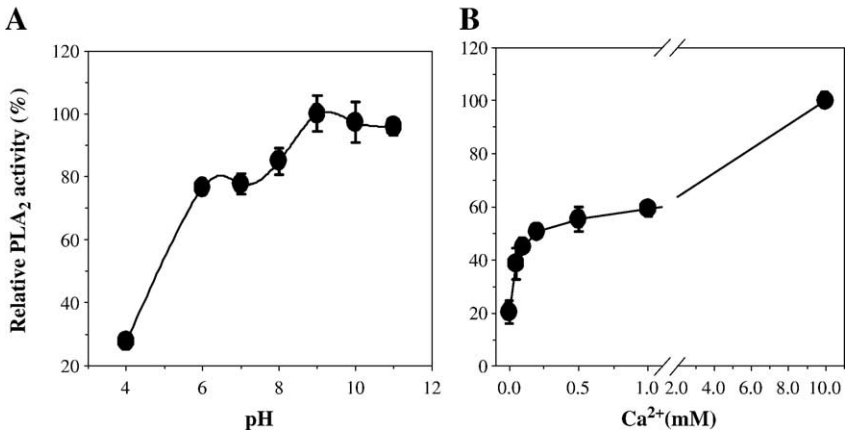

Fig. 3. Enzymatic properties of the mature form of recombinant *At*sPLA$_2$-α on the substrate 1-palmitoyl-2-[^{14}C]linoleoyl-PE. (A) The effect of pH on *At*sPLA$_2$-α activity. (B) The effect of Ca^{2+} concentration on *At*sPLA$_2$-α activity. Mean values and standard deviations were calculated from data collected in 3 independent experiments. Enzyme activities are presented relative to the maximum obtained for each treatment type.

its preferred substrate, the activity of *At*sPLA$_2$-γ was found to plateau at micromolar levels of Ca^{2+}, the same as observed for *At*sPLA$_2$-β and-δ [39]. Thus, only *At*sPLA$_2$-α appears to require millimolar levels of Ca^{2+} for optimal activity.

To investigate the expression of the *At*sPLA$_2$-α gene in different plant organs, we isolated total RNA from roots, stems, leaves, flowers, and siliques. We were unable to detect *At*sPLA$_2$-α transcript in any of these tissues by Northern blot analysis [data not shown]; therefore, we used relative quantitative RT-PCR. Concomitant amplification of an 18S rRNA fragment as an internal standard using the ratio 1 18S rRNA primer: 9 18S competimers resulted in near equal amplification of *At*sPLA$_2$-α cDNA and rRNA cDNA (Fig. 4) indicating that *At*sPLA$_2$-α transcript is extremely rare. *At*sPLA$_2$-α transcript is detected at similar abundance in roots, stems, and leaves and at a slightly higher level in flowers; they were barely discernable in siliques (Fig. 4).

4. Discussion

Our isolation of the *Arabidopsis* gene encoding *At*s-PLA$_2$-α is an important first step to define the role of sPLA$_2$s in the physiology of higher plants. The sequences of other isoforms of plant sPLA$_2$s have been reported in the GenBank database. *At*sPLA$_2$-α shares little amino acid sequence similarity to any of the animal sPLA$_2$s except in their catalytically important Ca^{2+}-binding loops and active-site motifs. It, like the animal sPLA$_2$s of type V [38], contains 12 cysteine residues (6 potential intramolecular disulfide bridges); however, it shares higher sequence similarity in its Ca^{2+}-binding loop and active site motif to animal sPLA$_2$s of type I (67%) than of type V (57%).

*At*sPLA$_2$-α displays a *sn*-2 acyl group preference for linoleoyl acyl chains over palmitoyl acyl chains (Table 1). In contrast, none of the animal sPLA$_2$s show any acyl group preference. Several other plant sPLA$_2$s also exhibit acyl preference at the *sn*-2 position of the phospholipid substrate. For instance, the sPLA$_2$ purified from developing elm seeds showed a preference for oleoyl acyl chains over palmitoyl acyl chains [17], and *At*sPLA$_2$-β for palmitoyl acyl chains over linoleoyl acyl chains [31]. Like the animal sPLA$_2$s, some plant sPLA$_2$s, such as *At*sPLA$_2$-γ and -δ, do not exhibit significant acyl group preference [39].

The cellular functions of the *Arabidopsis* secretory low molecular weight sPLA$_2$s remain to be elucidated. In animals, sPLA$_2$s have been suggested to play an important role in signaling in the processes of inflammation, cell injury, and tumor promotion [2,3,41]. In plants, PLA$_2$s have also been implicated in diverse cellular responses, including cell elongation, shoot gravitropism, and defense responses [5,9,31]. Since PAT-PLAs are non-specific acyl transferases that have a combined activity of PLA$_1$ and PLA$_2$, sPLA$_2$s are the only true PLA$_2$s that have been identified so far in plants. Four different isoforms of sPLA$_2$ have been cloned in *Arabidopsis* [5,39]. Interestingly, *At*sPLA$_2$-α shows enzymatic properties that are distinguishable from other three isoforms. The optimal pH range of *At*sPLA$_2$-α was very broad and biphasic between pH 6 and 11, compared to the narrow optimal pH ranges of the other isoforms of *At*sPLA$_2$. Moreover, it requires millimolar levels of Ca^{2+} for optimal activity, whereas the other isoforms require only

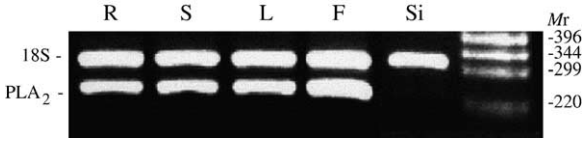

Fig. 4. Tissue-specific expression of the *At*sPLA$_2$-α as detected by relative RT-PCR analysis. Total RNA isolated from roots (R), stems (S), leaves (L), flowers (F) and siliques (Si) was reverse-transcribed and used as template in PCR reactions with primers that amplify a 228-bp fragment from *At*sPLA$_2$-α cDNA and a 315-bp fragment from 18S rRNA cDNA. The sizes of the RNA marker bands (lane M_r) are indicated on the right.

micromolar levels of Ca^{2+}. It also showed weaker head-group preference but stronger acyl group preference than the other *At*sPLA$_2$s. While *At*sPLA$_2$-γ and-δ isoforms are expressed predominantly in flower tissues and *At*sPLA$_2$-β in young seedlings, actively growing tissues and open flower tissues [31,39], *At*sPLA$_2$-α is expressed in most plant tissues except siliques. These results suggest that *At*sPLA$_2$-α may play a distinct role that differ from those of *At*sPLA$_2$s in the plant. *At*sPLA$_2$-α was sorted into a separate cluster (cluster I) since its amino acid sequence is notably different in from other three *Arabidopsis* sPLA$_2$s (cluster II) [39]. It is noteworthy, however, that most plant sPLA$_2$s that have been identified in a variety of plant species such as elm, carnation, rice, and tomato are grouped into cluster I with *At*sPLA$_2$-α [39]. This study opens a door to study the cellular roles of *At*sPLA$_2$-α in plants during growth and development, as well as in response to external stimuli.

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