sPLA$_2$ and PLA$_1$: Secretory Phospholipase A$_2$ and Phospholipase A$_1$ in Plants

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Abstract Plant phospholipase As (PLAs) are classified into two major types, PLA$_1$ and PLA$_2$, according to the hydrolysis sites of their membrane lipids. The lipid products released by PLAs have been suggested to act as bioactive molecules that mediate cellular signaling pathways functioning in plant growth and development, as well as responses to abiotic and biotic stimuli. The past few years have witnessed a wealth of new information regarding the function of these phospholipases in various biological processes. In this chapter, we discuss recent insights into lipid-based signaling mediated by PLAs and their lipid products, with particular emphasis on their emerging role as lipid mediators.

Keywords PLA$_2$ • PLA$_1$ • Signaling • Cellular roles Lipid mediators

1 Introduction

Phospholipid-derived products generated by phospholipase A (PLA), such as free fatty acids (FFAs) and lysophospholipids, play critical roles in plants, as these products are the precursors of second messengers in signal transduction pathways. In addition, these signaling molecules function in important physiological processes such as cell elongation, gravitropism, anther dehiscence, biosynthesis of jasmonic acid (JA), and defense signaling (Chen et al. 2011; Ryu 2004; Wang et al. 2012). To understand the highly regulated production of these lipid mediators in response to diverse extracellular stimuli, it is important to study the functions and regulatory mechanisms of the diverse PLA enzymes. Recent developments in

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genetic and biochemical analysis have facilitated our understanding of the cellular functions of plant PLAs. The PLA superfamily has three subtypes, i.e., phospholipase A_1 (PLA_1), phospholipase A_2 (PLA_2), and patatin-like PLA (pPLA). PLA_1 and PLA_2 catalyze the hydrolysis of membrane glycerophospholipids at their sn-1 or sn-2 positions, respectively, while pPLA shows activity at both positions. In this chapter, we will describe the current classifications of PLA_1s and PLA_2s that have been identified in plants, especially in *Arabidopsis*, and recent advances in our understanding of how these PLAs are involved in various cellular signaling pathways.

2 Phospholipase A_2

Based on the functional, structural, and catalytic properties of lipolytic enzymes, the PLA_2 superfamily can generally be divided into five principal families in animals, categorized as secretory PLA_2s ($sPLA_2$), cytosolic PLA_2s ($cPLA_2$), Ca^{2+}-independent PLA_2s ($iPLA_2$s), platelet-activating factor acetyl hydrolases (PAF-AHs), and lysosomal PLA_2s (Schaloske and Dennis 2006). Only low molecular weight secretory PLA_2s have been reported in plants, although patatin-like PLAs have been identified, which are similar to the $iPLA_2$s or $cPLA_2$ but exhibit both PLA_1- and PLA_2-like activity (Lee et al. 2005; Ryu 2004).

2.1 $sPLA_2$ Grouping, Expression, and Localization

Four and three secretory PLA_2 paralogs were identified in the *Arabidopsis* and rice genomes, respectively. Based on their primary structures, plant $sPLA_2$s are equivalent to animal group XI $sPLA_2$s, which are further divided into groups XIA and XIB. The former group includes $AtsPLA_2$-α, $OsPLA_2$-α, and $OsPLA_2$-β, while the latter group includes $AtsPLA_2$-β, $AtsPLA_2$-γ, $AtsPLA_2$-δ, and $OsPLA_2$-γ (Lee et al. 2005; Schaloske and Dennis 2006; Singh et al. 2012).

$AtsPLA_2$s exhibit different spatial and temporal expression patterns. *$AtsPLA_2$-α* and *AtsPLA2-β* are expressed in all sporophytic tissues. Unlike *$AtsPLA_2$-α*, *$AtsPLA_2$-β* is strongly expressed in actively growing young tissues and pollen. *$AtsPLA_2$-γ* and *$AtsPLA_2$-δ* are exclusively expressed in pollen. While *$AtsPLA_2$-β* is expressed during all stages of pollen development, *$AtsPLA_2$-γ* is expressed at low levels during the early stage of pollen formation and is strongly expressed at the mature stage, and *$AtsPLA_2$-δ* is expressed at the mature pollen stage (Bahn et al. 2003; Kim et al. 2011b; Lee et al. 2003).

Studies of the subcellular localizations of plant $sPLA_2$ have been performed using fused fluorescence proteins. These studies provide important insights into the functions of these proteins in plants. Early experiments involving transient expression analysis of green fluorescence protein (GFP) in onion epidermal cells showed

that AtsPLA$_2$-β and AtsPLA$_2$-γ are secreted into the cell wall/extracellular space (Bahn et al. 2003; Lee et al. 2003), but this localization pattern is regarded as a result of the masking of the ER retention signal (KTEL) by that of the C terminal GFP fusion protein of AtsPLA$_2$-β. More recently, Seo et al. (2008) reexamined the subcellular localization of AtsPLA$_2$-β with N terminal GFP fusion protein of AtsPLA$_2$-β and observed that it indeed localizes to the ER in *Vicia faba* guard cells, and the result was supported in AtsPLA$_2$-β:YFP transgenic plants (Lee et al. 2010). Transient expression of *AtsPLA$_2$-γ:YFP* and *AtsPLA$_2$-δ:YFP* in tobacco leaf epidermal cells demonstrated that AtsPLA$_2$-γ localizes to the ER and Golgi, while AtsPLA$_2$-δ localizes to the ER and is found in the pollen of transgenic plants (Kim et al. 2011b). Subcellular localization of AtsPLA$_2$-α revealed a rather complicated localization pattern unlike that of its paralogs. An analysis of *AtsPLA$_2$-α:DsRed2* transgenic *Arabidopsis* plants suggests that AtsPLA$_2$-α localizes to the Golgi in root tissues (Lee et al. 2010). In another study, AtsPLA$_2$-α exhibited different localizations in a time-dependent manner when this gene was introduced into *Arabidopsis* seedlings and tobacco leaves via *Agrobacterium*-mediated transient transformation (Froidure et al. 2010). The AtsPLA$_2$-α:YFP signal was detected in cytoplasmic vesicles around the nucleus 36 h after inoculation and was detected at the extracellular spaces outside of the cells at 48 h after inoculation. AtsPLA$_2$-α was partially localized to the cell nucleus when it was co-expressed with AtMYB30.

A more recent study shows that subcellular localization of AtsPLA$_2$-α is dependent on the developmental stage of the leaf tissue. Fluorescence signals are present primarily at the Golgi apparatus in premature young leaves in transgenic AtsPLA$_2$-α:RFP plants, while these signals are detected primarily in the apoplasts in mature leaves (Jung et al. 2012). Also, translocation of AtsPLA$_2$-α to the apoplast is stimulated by bacterial infection in premature young leaves. In contrast, the signal of GFP:OssPLA$_2$-α merges with that of an endoplasmic marker in onion epidermal cells, which indicates that the transiently expressed OssPLA$_2$-α localizes to the ER (Singh et al. 2012). RFP fusion protein, but not GFP and YFP, is relatively stable in the apoplasts. Thus, it is desirable to use RFP fusion protein rather than GFP or YFP fusion proteins for apoplastic localization studies.

2.2 *sPLA$_2$ Functions*

Plant PLA$_2$s have been implicated in important physiological processes such as development, senescence, biotic and abiotic stress responses, and the induction of secondary metabolite accumulation (Lee et al. 2005; Mansfeld 2009; Ryu 2004; Wang 2001). Lysophosphatidylcholine (LPC) and linolenic acid, which are the products of PLA$_2$, induce a decline in pH and accelerate the elongation of corn coleoptiles (Yi et al. 1996). Also, the PLA$_2$ inhibitors aristolochic acid and manoalide inhibit the auxin-induced pH decrease and coleoptile elongation. These results suggest that PLA$_2$ is activated by auxin, and its products induce

acidification of the apoplast by activating the H^+ pump through the signal transduction pathway of protein kinase, which in turn promotes corn coleoptile elongation (Yi et al. 1996). This hypothesis was supported by Lee et al. (2003) using transgenic plants. These authors reported that *AtsPLA$_2$-β* transcripts are induced by auxin treatment. In addition, RNA interference-mediated silencing (RNAi) of *AtsPLA$_2$-β* expression retards cell elongation, while overexpression of *AtsPLA$_2$-β* promotes cell elongation. Moreover, *AtsPLA$_2$-β* overexpressors exhibit faster stomatal opening than wild type, and AtsPLA$_2$-β-RNAi plants exhibit delayed light-induced stomatal opening, which can be rescued by exogenous application of LPC or lysophosphatidylethanolamine (LPE). Also, exogenous applications of LPC or LPE enhance stomatal opening in wild-type plants (Seo et al. 2008). In contrast, AtsPLA$_2$-β, AtsPLA$_2$-γ, and AtsPLA$_2$-δ are involved in pollen development and pollen germination (Kim et al. 2011b). *AtsPLA$_2$-β* may play a more vital role in pollen development, while *AtsPLA$_2$-γ* and *-δ* may function in pollen germination and pollen tube growth. Also, pollen germination is inhibited by the application of sPLA$_2$ inhibitors and is recovered by exogenous application of LPE but not of LPC or lysophosphatidic acid (LPA). These results indicate that LPE in particular is a key signal molecule in pollen germination and tube growth (Kim et al. 2011b).

Both AtsPLA$_2$-α-RNAi *Arabidopsis* plants and *Arabidopsis* seedlings treated with the sPLA$_2$ inhibitor ONO-RS-082 exhibit significantly disrupted plasma membrane (PM) localization of PINs in the root tissues, causing internal PIN compartments to form (Lee et al. 2010). Application of exogenous LPE restores the PM localization of PINs in an *AtsPLA$_2$-α* mutant and in ONO-RS-082-treated seedling. These results indicate that AtsPLA$_2$-α modulates PIN-FORMED protein trafficking to the PM in *Arabidopsis* roots, thereby revealing that sPLA$_2$ also plays an important role in intracellular membrane trafficking in plants (Lee et al. 2010). AtsPLA$_2$-α was also suggested to be as a negative regulator of the defense response through its interaction with AtMYB30, a transcription factor that functions in the hypersensitive response in short-day conditions (Froidure et al. 2010). Intriguingly, the regulation does not depend on the enzymatic activity of AtsPLA$_2$-α but on the just physical binding of AtsPLA$_2$-α to A+MYB30.

Aristolochic acid reduces root elongation and causes radial swelling of the root tip caused by microtubule disorganization (Gardiner et al. 2008). This indicates that PLA$_2$ is involved in microtubule organization and anisotropic growth. Another study employing aristolochic acid suggests that PLA$_2$ plays a critical role in programmed cell death induced by misexpression of fatty acid elongation, likely involving the exchange of very long chain fatty acids (VLCFAs) between phospholipids and the acyl-CoA pool (Reina-Pinto et al. 2009). PLA$_2$ is involved in salt stress-induced LPA production in the unicellular green alga *Chlamydomonas* (Meijer et al. 2001). LPA accumulates in *Chlamydomonas* under conditions of salt and nonionic hyperosmotic stress. The fact that LPA is generated by PLA$_2$ was recently confirmed using differential ^{32}P-radiolabeling experiments (Arisz and Munnik 2011).

3 Phospholipase A1

Plant PLA$_1$s are classified based on the presence of N terminal stretches, sequence similarities in the catalytic region, and substrate specificity. These PLA$_1$s include group I, II, and III PLA$_1$, phosphatidic acid-specific PLA$_1$ (PA-PLA$_1$), and lecithin: cholesterol acyltransferase-like PLA$_1$ (LCAT-PLA$_1$) in *Arabidopsis* and group I and II PLA$_1$ and PA-PLA$_1$ in rice (Chen et al. 2011; Singh et al. 2012).

3.1 Class I PLA$_1$s

Class I PLA$_1$s include seven PLA$_1$ genes in *Arabidopsis* and eight in rice. Class I PLA$_1$s are defined by the presence of a putative N terminal chloroplast-targeting signal (Chen et al. 2011; Seo et al. 2009). All class I PLA$_1$s have a GXSXG motif in the lipase class 3 domain and catalytic triad (Ser, Asp and His residues). All GFP-class I PLA$_1$ fusion proteins localize to the chloroplast (Ellinger et al. 2010; Grienenberger et al. 2010; Hyun et al. 2008; Ishiguro et al. 2001; Seo et al. 2009). However, a second opinion about the subcellular localization of AtPLA$_1$-Iα1 has recently emerged (Ellinger et al. 2010). In this study, AtPLA$_1$-Iα1 was co-localized with cytoplasmic lipid bodies instead of localizing to chloroplasts.

Defective in Anther Dehiscence1 (DAD1, *AtPLA$_1$-Iβ1*) was the first reported PLA$_1$ enzyme in *Arabidopsis*. This enzyme preferentially hydrolyzes phosphatidylcholine (PC) at the sn-1 position and regulates anther dehiscence in flowers by releasing linolenic acid for the initial step of JA biosynthesis (Ishiguro et al. 2001). DONGLE (DGL, *AtPLA$_1$-I*α1) and DAD1 are necessary and sufficient for JA production (Hyun et al. 2008). DGL plays a specific role in maintaining basal JA content under normal conditions. During wounding, DGL was shown to be required for the rapid JA burst in the early phase, and DAD1 was shown to play a role in the late phase of JA production. However, the initial step in the biosynthesis of wound- and pathogen-induced JA production remains controversial. Ellinger et al. (2010) suggest that *AtPLA$_1$-Iγ1* is a novel target gene for the manipulation of jasmonate biosynthesis and that, in addition to DAD1 and AtPLA$_1$-Iγ1, as yet unidentified enzymes with sn-1 and sn-2 hydrolase activity are involved in stress-induced JA formation, indicating that there is functional redundancy within the lipase family.

3.2 Class II PLA$_1$s

Class II PLA$_1$s comprise four PLA$_1$ genes in *Arabidopsis* and three in rice (Chen et al. 2011; Singh et al. 2012). Based on their sequence homology, PLA$_1$s from other species are also included in class II, including *Dclipase* from *Dianthus caryophyllus* (carnation) (Hong et al. 2000), *LeLID1* from *Lycopersicon esculentum*

(tomato) (Matsui et al. 2004) and *CaPLA$_1$* from *Capsicum annuum* (hot pepper) (Seo et al. 2007). Class II PLA$_1$s lack N terminal signal peptides and are predicted to localize to the cytosol. Cytosolic localization of AtPLA$_1$-IIδ and DAD1-like Seedling Establishment-related Lipase (AtDSEL, AtPLA$_1$-IIγ) were confirmed through GFP fusion protein studies (Kim et al. 2011a; Lo et al. 2004). *AtPLA$_1$-IIδ* expression is induced by treatment with sublethal levels of UV-B. Plants with suppressed *AtPLA$_1$-IIδ* expression via antisense technology exhibit increased tolerance to sublethal levels of UV-B stress and are unable to upregulate the expression of *pathogenesis-related protein 1* (*PR-1*) in response to UV-B treatment. These results indicate that AtPLA$_1$-IIδ is capable of deesterifying membrane phospholipids and is induced in response to UV-B irradiation (Lo et al. 2004). Recombinant AtDSEL expressed in *Escherichia coli* (*E. coli*) shows a preference for 1,3-diacylglycerol and 1-monoacylglycerol over PC, which suggests that AtDSEL is a sn-1-specific lipase.

AtDSEL overexpressors are defective in post-germinative seedling growth on medium lacking an exogenous carbon source; this phenotype is rescued by the addition of sucrose to the growth medium. By contrast, *atdsel-1* and *atdsel-2* exhibit a mildly fast-growing phenotype in the absence of an exogenous carbon source. *AtDSEL*-overexpressors retained numerous peroxisomes and oil bodies in their 5-day-old cotyledons, while these organelles are exhausted in wild-type or mutant cotyledons. These results suggest that AtDSEL is involved in the negative regulation of seedling establishment by inhibiting the breakdown of storage oils (Kim et al. 2011a). *Dclipase* transcript levels increase just as carnation flowers begin to senesce, and the expression of this gene is also induced by ethylene treatment. Southern blot analysis confirmed that these flowers contain a single copy of *Dclipase*. *Dclipase* is predicted to be involved in mediating the onset of senescence (Hong et al. 2000). Recombinant LeLID1 protein exhibits high activity against triacylglycerols (TAGs) with long acyl chains but little activity against PC or monogalactosyldiacylglycerol. Transcript levels of *LeLID1* increase rapidly in seeds during germination, reaching a maximum level just before cotyledon opening, followed by a rapid decrease. Low levels of *LeLID1* expression are detected in flowers and fruits, while none can be detected in roots. LeLID1 is thought to function as a lipase during lipid mobilization to liberate fatty acids from TAG's stored in oil bodies (Matsui et al. 2004).

3.3 Class III PLA$_1$

There is only one class III PLA$_1$ in the *Arabidopsis* genome and none in the rice genome (Chen et al. 2011; Singh et al. 2012). Recombinant DAD1-like acylhydrolase (AtDLAH, AtPLA$_1$-III) displays a stronger preference for 1-lysophosphatidylcholine, 1-monoacylglycerol, and phosphatidic acid than for PC, which indicates that AtDLAH is an sn-1-specific acylhydrolase. AtDLAH exclusively localizes to the mitochondria. Seeds of *Arabidopsis AtDLAH*

overexpressors are more tolerant to accelerated-aging treatment than wild type, and thus these seeds have higher germination rate than wild-type seeds. By contrast, *atdlah* knockout mutant seeds are susceptible to accelerated-aging conditions. These results suggest that AtDLAH plays an important role in *Arabidopsis* seed viability (Seo et al. 2011).

4 PA-PLA$_1$

SGR2 in *Arabidopsis* is classified as a PA-PLA$_1$ based on the similarity of its domain structures to that of a mammalian PA-PLA$_1$. One gene of PA-PLA$_1$ was found to be present in the *Arabidopsis* and rice genome. The fusion protein of AtPA-PLA$_1$ and GFP localizes to the membranes of vacuoles and small organelles. However, the localization of OsPA-PLA$_1$ was predicted to be nuclear using in silico tools. AtPA-PLA$_1$ mutants exhibit abnormal gravitropism in inflorescence stems and hypocotyls and irregularly shaped seeds. Mutant analysis suggests that AtPA-PLA$_1$ is involved in a vacuolar membrane system that affects the early step of shoot gravitropism. Microarray data and quantitative RT-PCR in rice show that *OsPA-PLA$_1$* transcripts are upregulated in response to salt and drought stress (Kato et al. 2002; Morita et al. 2002; Singh et al. 2012). In animals, LPA is a phospholipid mediator with multiple biological roles, functions via interactions with G protein-coupled seven-transmembrane receptors (GPCRs), and is implicated in various human diseases. While two pathways for LPA production have been identified in animal cells, little is known about the function of LPA at the molecular level. It was demonstrated that LPA produced by hair follicle-specific PA-PLA$_1$ is an important signaling molecule for hair follicle development, which functions by modulating epidermal growth factor receptor signaling (Aoki et al. 2008; Chen et al. 2011; Inoue et al. 2011). However, PLA$_1$ activity was not detected with recombinant AtPA-PLA$_1$ proteins expressed in *E. coli*. Moreover, GPCRs are not found in plants. Elucidating the mechanisms of PA-PLA$_1$ function in plants will be challenging.

5 LCAT-PLA1

During a search for plant genes encoding enzymes involved in sterol esterification by free acids, At3g03310 was identified as lecithin:cholesterol acyltransferase (LCAT) (Noiriel et al. 2004). This gene has sequence homology with the recently discovered gene encoding phosphatidylcholine:diacylglycerol acyltransferase from *Saccharomyces cerevisiae* (*ScPDAT*). PLA$_1$ activity was first revealed by LCAT or PDAT assays, and the gene encoding PLA$_1$ was designated *AtLCAT-PLA$_1$* (Chen et al. 2011; Noiriel et al. 2004). Yeast expressing *AtLCAT-PLA$_1$* accumulates TAG

(Noiriel et al. 2004). Thus, AtLCAT-PLA$_1$ may play a role in acyl-editing during TAG formation (Bates et al. 2009; Lu et al. 2009) but not in signaling.

6 Conclusions

Recent discoveries have significantly advanced our understanding of the biochemical and genetic requirements of distinct phospholipid signaling in plants. However, several unanswered questions still remain. For example, which isoforms of the PLA families are activated in response to specific external stimuli? What are the downstream targets of the lipid signals that are generated by PLA? These targets may be lysophospholipid-specific receptors, protein kinases, mitogen-activated protein kinase, or other signaling enzymes. And what are the upstream regulators of PLA activation? These regulators may take the form of receptors, activators, inhibitors, or hormones. The next coming years will likely produce significant advances towards the elucidation of PLA-mediated membrane phospholipid signaling in plants at both the biochemical and genetic levels. Moreover, the construction of knockout mutants and activation tagging lines, as well as the analysis of rapid protein activation without involving gene expression, will help clarify the phospholipid-derived signaling cascades.

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