Phospholipase A in Plant Immunity

Susana Rivas and Thierry Heitz

Abstract In addition to their structural role as major components of cellular membranes, lipids and lipid-derived molecules act as important regulators of plant cell signaling. The roles of lipid deacylating enzymes in the control of plant growth, development, and responses to stress are becoming increasingly evident. Following perception of attack by pathogenic microbes or herbivorous insects, plant phospholipase A (PLA) activity has been linked to the activation of defense signaling and appears to play crucial roles during the establishment of plant immune responses through, for example, the production of defense signals such as jasmonates and other oxylipins. Evidence for nonenzymatic modes of action of PLA is also emerging. In this chapter, we review current data on plant PLA proteins, and their involvement in plant defense responses to microbes and insects, before discussing new ways to decipher PLA-dependent processes.

Keywords Lipid acyl hydrolase • Galactolipase • Oxylipin • Lipid profiling • Plant immunity • Phospholipase A

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1 Introduction

In plants, recognition of pathogenic microbes triggers a signal transduction cascade that leads to the transcriptional reprogramming of the plant cell. In the case of resistant plants, this signaling cascade directs the activation of plant immune responses that are frequently associated with the development of the so-called hypersensitive response (HR), a form of programmed cell death that contributes to plant resistance by restricting pathogen growth to the inoculation site (Mur et al. 2008; Coll et al. 2010).

Rapid accumulation of reactive oxygen species (ROS), changes in ion fluxes, activation of protein kinase cascades, and production of defense molecules, such as phytolexins, are some of the early signaling events that have been described following pathogen perception by plant cells. In addition, a growing body of evidence indicates that phospholipases and phospholipid-related molecules, such as the secondary signal messanger molecule phosphatidic acid (PA), play essential roles during the establishment of plant disease resistance (Andersson et al. 2006a; Testerink and Munnik 2011). This suggests that phospholipids are much more than just structural components of biological membranes. Moreover, a great variety of lipid-related components including glycerolipids, sphingolipids, fatty acids, oxylipins, jasmonates, and sterols are also increasingly involved in the regulation of defense-related signaling (Shah 2005; Kachroo and Kachroo 2009).

In contrast to phospholipases C and D that act on the polar head of phospholipids, phospholipase A catalyzes the hydrolysis of phospholipids into lysophospholipids and free fatty acids, either at the *sn*-1 (PLA_1) and/or *sn*-2 position (PLA_2) of glycerolipids (Matos and Pham-Thi 2009). Free fatty acids may be oxidized by lipoxygenases (LOX) or an α-dioxygenase (α-DOX) resulting in the biosynthesis of oxylipins and jasmonic acid (JA), which play important roles during plant defense signaling as mentioned above (Blee 2002; Browse 2009; La Camera et al. 2009), and lysophospholipids are also bioactive molecules. In addition, plant PLA_2 activity has been linked to ROS production (Chandra and Low 1995) as well as efflux of vacuolar protons, which triggers a pH-dependent signal for the biosynthesis of phytoalexins (Viehweger et al. 2006). Galactolipids being the major components of plant cell membranes, plant lipid acyl hydrolases (LAHs) hydrolyze phospholipids but are also able to act on galactolipids, several characterized LAHs even showing preferential galactolipase (GLA) activity in vitro. Numerous LAH (bearing PLA, GLA, or triglyceride lipase, TGL activities) have been identified in plants but, in many cases, knowledge of their real in vivo substrates and cleavage specificities awaits deeper characterization.

In Arabidopsis, PLA proteins have been grouped in three main subfamilies (Table 1): small secretory PLAs with PLA_2 activity (*At*sPLA_2s), patatin-like proteins with both PLA_1 and PLA_2 activities (pPLAs), and lipase-like PLA_1s (DAD1-like, for Defective in Anther Dehiscence1-like) (Grienenberger et al. 2010). In recent years, the extensive use of molecular approaches related to lipid signaling has led to the characterization of new enzymes and to the identification of new proteins with putative lipase/esterase signatures (Matos and Pham-Thi 2009).

Table 1 Examples of LAH proteins with reported roles in responses to microbial infections or wounding

LAH family[a]	Member name	AGI	Subcellular localization	Stress inducing transcriptional regulation	Activity	Effect on lipid profile during defense	In vivo function in immunity	References
Secretory PLA_2 (4)	$AtsPLA_2$-α	At2g19690	Golgi, apoplast, nucleus	Bacterial infection	PLA_2, not required for AtMYB30 regulation	ND	Interacts with and represses AtMYB30-mediated HR	Froidure et al. (2010), Lee et al. (2010), Jung et al. (2012)
α/β hydrolase	SOBER1	At4g22300	ND	ND	PLA, carboxylesterase	Reduced PA levels	Suppression of HR	Cunnac et al. (2007), Kirik and Mudgett (2009)
Patatin (10)	pPLA-IIα	At2g26560	Cytosol	Fungal and bacterial infection	GLA > PLA	Increased 2OH-FA levels	Host cell death execution	La Camera et al. (2005, 2009)
	pPLA-IIIß	At3g54950	Membrane-bound	Fungal and bacterial infection	PLA, GLA, AcylCoA thioesterase	ND	ND	La Camera et al. (2005), Li et al. (2011)
	pPLA-I	At1g61850	Cytosol, chloroplast	ND	GLA > PLA	Basal JA production	Antifungal resistance	Yang et al. (2007)
	NtPAT1, NtPAT3		Cytosol, chloroplast	Viral, fungal, and bacterial infection	GLA > PLA	ND	Host cell death execution, resistance to TMV	Dhondt et al. (2000)

(continued)

Table 1 (continued)

LAH family[a]	Member name	AGI	Subcellular localization	Stress inducing transcriptional regulation	Activity	Effect on lipid profile during defense	In vivo function in immunity	References
DAD1 (12)	DAD1	At2g44810	Chloroplast	Expressed in anthers; wound-induced in leaves	PLA > GLA > TGL	JA production in anthers	Late JA production in wounded leaves	Ishiguro et al. (2001)
	DGL (PLA1-Iα-1)	At1g05800	Lipid bodies	Wound-induced in leaves	GLA, PLA		Minor role in wound-induced JA biosynthesis ?	Hyun et al. (2008), Ellinger et al. (2010)
	PLA1-Iγ1	At1g06800	Chloroplast	Fungal and bacterial infection	GLA, PLA, TGL	Contributes to wound-induced JA accumulation	ND	Seo et al. (2009), T. Heitz, unpublished
	PLA1-Iγ2	At2g30550	Chloroplast	Fungal and bacterial infection	GLA, PLA, TGL	ND	ND	Seo et al. (2009), T. Heitz, unpublished
	NaGLA1		Chloroplast	Repressed by wounding and infection	ND	Supplies FAs for JA and divinyl-ether synthesis	ND	Kallenbach et al. (2010), Bonaventure et al. (2011)
Related to fungal lipases	PAD4	At3g52430	Nucleo-cytoplasmic shuttling	Bacterial infection, SA treatment	ND	ND	Immune regulator	Wiermer et al. (2005), Feys et al. (2001)
	EDS1	At3g48090	Nucleo-cytoplasmic shuttling	Bacterial infection, SA treatment	ND	ND	Immune regulator	Wiermer et al. (2005), Feys et al. (2001)

GDSL (108)	GLIP1	At5g40990	Extracellular	*A. brassicicola*, ethylene	Esterase	ND	Signaling; antifungal activity	Oh et al. (2005), Kwon et al. (2009)
	GLIP2	At1g53940	ND	SA	Esterase	ND	Antifungal, antibacterial resistance	Lee et al. (2009)

FA fatty acid, *GLA* galactolipase, *HR* hypersensitive response, *JA* jasmonic acid, *PA* phosphatidic acid, *PLA* phospholipase A, *SA* salicylic acid, *TGL* triacylglycerolipase, *TMV* Tobacco Mosaic Virus, *ND* not determined. All examples are from Arabidopsis except NaGLA from *Nicotiana attenuata* and NtPAT1 and NtPAT3 from *Nicotiana tabacum*

[a]Number of genes in Arabidopsis

Here we summarize our current knowledge about plant LAH proteins that have been found to play a role in the regulation of plant immunity.

2 Secretory PLAs

Plant sPLA$_2$ genes encode proteins with N terminal signal peptides that are of low molecular weight (13–18 kDa) after secretion. sPLA$_2$s present a highly conserved Ca^{2+}-binding loop and an active site motif with a conserved His/Asp dyad. The mature proteins contain 12 cysteine residues that can form six disulfide bonds to stabilize the protein's three-dimensional structure (Ryu 2004).

In Arabidopsis, the secretory PLA$_2$ family (*At*sPLA$_2$) consists of four isoforms named *At*sPLA$_2$-α, *At*sPLA$_2$-β, *At*sPLA$_2$-γ, and *At*sPLA$_2$-δ (Ryu 2004). *AtsPLA$_2$-γ* and *AtsPLA$_2$-δ*, which are exclusively expressed in pollen and localize to the endoplasmic reticulum (ER) and Golgi apparatus, are involved in pollen germination and tube growth (Kim et al. 2011b). *AtsPLA$_2$-β*, which is expressed in different tissues and localizes to the ER, mediates cell elongation, shoot gravitropism, stomatal opening, and pollen development and likely acts as a downstream component of auxin signaling (Lee et al. 2003; Seo et al. 2008; Kim et al. 2011b). Interestingly, the amino acid sequence, enzymatic properties, and expression pattern of *At*sPLA$_2$-α are distinguishable from those of the other three *At*sPLA$_2$ isoforms, suggesting that *At*sPLA$_2$-α may play a distinct role that differs from that of the other *At*sPLA$_2$s (Ryu 2004).

AtsPLA$_2$-α is expressed in diverse plant organs (Kim et al. 2011b) and is localized to Golgi-associated vesicles (Lee et al. 2010). *At*sPLA$_2$-α was reported to modulate PIN protein trafficking to the plasma membrane (and therefore regulation of auxin transport) in Arabidopsis root cells (Lee et al. 2010). A recent report showed that *AtsPLA$_2$-α* gene expression is controlled in a developmental stage- and tissue-specific manner (Jung et al. 2012). The subcellular localization of *At*sPLA$_2$-α appears to be dependent on the leaf developmental stage. Indeed, in premature young leaves of transgenic Arabidopsis plants expressing an *At*sPLA$_2$-α-RFP fusion protein, *At*sPLA$_2$-α was localized at the Golgi apparatus, whereas in mature leaves, it was primarily detected in the apoplast (Jung et al. 2012). Apoplasts are important sites for the interaction between plant cells and invading bacteria. In agreement with this notion, translocation of *At*sPLA$_2$-α to the apoplast was found to be enhanced after plant inoculation with avirulent bacteria, suggesting that *At*sPLA$_2$-α may participate to the plant defense response that is mounted in the apoplast.

A role for intracellular *At*sPLA$_2$-α in the regulation of plant defense has been reported. Golgi-associated *At*sPLA$_2$-α partially relocalized to the plant cell nucleus after co-expression with the Arabidopsis MYB transcription factor AtMYB30 (Froidure et al. 2010). AtMYB30 is a positive regulator of defense- and cell death-associated responses through the activation of the lipid biosynthesis pathway that leads to the production of very long chain fatty acids [VLCFAs; (Raffaele et al. 2008)]. AtMYB30 and *At*sPLA$_2$-α physically interact in the plant cell nucleus, leading to repression of AtMYB30-mediated transcriptional activity. Physical or

functional interactions have been previously suggested between cytoplasmic PLA_2s ($cPLA_2$) and proteins involved in transcriptional regulation in animals (Flati et al. 1996; Pawliczak et al. 2002; Tashiro et al. 2004b). In agreement with the finding that transcriptional activation of VLCFA-related genes by AtMYB30 is required to mount an efficient defense response during bacterial infection (Raffaele et al. 2008), the protein interaction between AtMYB30 and *At*sPLA$_2$-α correlates with the negative regulation of Arabidopsis HR and defense responses (Froidure et al. 2010). Together, these data highlight the importance of dynamic nucleocytoplasmic protein trafficking for the regulation of the transcriptional activation related to defense (Rivas 2012).

Interestingly, *At*sPLA$_2$-α nuclear targeting, interaction with AtMYB30, repression of AtMYB30 transcriptional activity, and repression of HR development appeared to be independent of *At*sPLA$_2$-α enzymatic activity (Froidure et al. 2010). Therefore, it was proposed that *At*sPLA$_2$-α may control AtMYB30-mediated response through the physical interaction between the two proteins (preventing AtMYB30 from activating its targets) rather than through a lipid signal produced by *At*sPLA$_2$-α. This assumption is in agreement with the hypothesis formulated for animal $cPLA_2$, which is able to interact not only with B-Myb but also with additional nuclear proteins that stimulate apoptosis, strongly supporting the notion that $cPLA_2$ plays additional roles in the nucleus other than its enzymatic activity at the nuclear membrane (Sheridan et al. 2001; Tashiro et al. 2004a). Moreover, new functions have been attributed to $sPLA_2$s that do not require enzymatic activity in animal cells. For example, it has been reported that $sPLA_2$s bind to and possibly signal through cell surface molecules (Lambeau and Lazdunski 1999; Granata et al. 2005; Triggiani et al. 2006; Boyanovsky and Webb 2009). In addition to acting as lipolytic enzymes, $sPLA_2$s may serve as high-affinity ligands for cell surface receptors (Cupillard et al. 1997; Kundu and Mukherjee 1997; Lambeau and Lazdunski 1999; Higashino et al. 2002). $sPLA_2$s are also able to bind proteoglycans with high affinity due to their overall positive charge (Rosengren et al. 2006). Although our knowledge of the cellular functions of plant PLAs is limited, it is tempting to speculate that, similar to animal PLAs, functions of plant PLAs may extend beyond those related to their catalytic activities.

3 The Arabidopsis PLA_2-Type Protein SOBER1

A well-characterized example of a PLA_2-type protein that is involved in plant defense responses is the Arabidopsis *α/β* hydrolase SOBER1 (*S*uppressor *O*f Avr*B*sT-*E*licited *R*esistance*1*). AvrBsT is a type III effector from *Xanthomonas campestris* pv. *vesicatoria* that is predicted to encode a Cys protease. In an attempt to identify lines resistant to *Xanthomonas* expressing AvrBsT, the natural variation existing among Arabidopsis ecotypes was surveyed. Only the ecotype Pi-0 was found to develop an HR in response to AvrBsT (Cunnac et al. 2007). In addition, Pi-0 is resistant to *Pseudomonas syringae* pathovar *tomato* strain DC3000 expressing AvrBsT [*Pst* DC3000 (AvrBsT)] and resistance was found to be due

to a loss-of-function mutation in SOBER1, suggesting that SOBER1 inhibits AvrBsT-triggered phenotypes in Arabidopsis (Cunnac et al. 2007). Members of the SOBER1 protein family possess phospholipase and carboxylesterase activity with diverse substrate specificities. SOBER1-dependent suppression of the HR in Pi-0 suggested that it might hydrolyze a plant lipid or precursor required for HR induction. Indeed, Pi-0 leaves infected with *Pst* DC3000 (AvrBsT) accumulated higher levels of PA compared to leaves infected with virulent *Pst* DC3000, in a Phospholipase D (PLD)-dependent manner (Kirik and Mudgett 2009). Overexpression of SOBER1 in Pi-0 led to reduction of PA levels and inhibition of the HR. Moreover, SOBER1 was found to hydrolyze PC (phosphatidylcholine) but not PA or lysoPC in vitro, indicating that the enzyme displays PLA_2 activity (Kirik and Mudgett 2009). Chemical inhibition of PLA_2 activity in leaves expressing SOBER1 resulted in HR in response to *Pst* DC3000 AvrBsT, suggesting that SOBER1 PLA_2 activity suppresses PLD-dependent production of PA in response to AvrBsT elicitation. Although the mechanism by which AvrBsT leads to PA accumulation in Arabidopsis Pi-0 remains to be determined, this work indicated an important role for SOBER1 in the regulation of PA levels generated in plants in response to bacterial inoculation.

4 Patatin-Related Phospholipases (pPLA)

Patatins were identified and then isolated some decades ago as abundant storage proteins in potato tubers (Park et al. 1983), which are at the origin of the generic name given to this group of closely related proteins (Hendriks et al. 1991). These proteins are responsible for most of the soluble LAH activity extracted from this organ, acting in vitro on galactolipids or phospholipids (with low or no positional specificity) but not on triglycerides (Racusen 1984; Andrews et al. 1988). Later on, LAH activities purified from plant leaves were found to be related to patatins, and patatin-encoding genes appeared to be inducible by various environmental stresses such as microbial infection, drought, or senescence. The nomenclature of plant patatins was recently updated, namely by introducing the acronym "pPLA" (Scherer et al. 2010).

While patatins are suspected to accumulate in potato tubers for defensive functions in addition to their presumed nitrogen storage function, the formal demonstration of such a hypothesis is still missing. Two reports have described direct activities on insect and microbial aggressors. An early study established that potato tuber patatin inhibits Southern corn rootworm larval growth and that this activity is sensitive to serine hydrolase inhibitors (Strickland et al. 1995). Similarly, a patatin-related protein was purified from a somatic hybrid between potato and a wild relative and displayed inhibitory activity in *Phytophthora* spore germination assays (Sharma et al. 2004).

The first in-depth study of a non-tuber patatin in a context of induced innate immunity was initiated by purification of LHA activity in soluble extracts of

tobacco leaves reacting hypersensitively to *Tobacco Mosaic Virus* (TMV) (Dhondt et al. 2000). Two hypersensitive response (HR)-induced genes (*NtPAT*) were cloned that encode highly active LAH with preferential galactolipase over phospholipase A activity. Their early expression was correlated with appearance of cell death symptoms, upregulation of oxylipin biosynthetic genes, and jasmonic acid (JA) accumulation. Further investigation using ß-megaspermin, a necrotizing protein elicitor from *Phytophthora megasperma*, to spatially dissect the LAH response showed that *NtPAT*-encoded LAH activity was transiently stimulated in elicitor-treated tissues that undergo programmed cell death and later on in living tissue immediately surrounding the collapsed zone (Dhondt et al. 2002). However, JA accumulation only occurred in tissue committed to cell death and therefore uncoupling NtPAT activity from JA biosynthesis. *NtPAT* expression was induced in tobacco leaves by viral, bacterial, and fungal pathogens, all triggering different forms of host cell death. Transgenic tobacco plants overexpressing NtPAT3 exhibited an altered response to TMV infection manifested by decreased necrotic lesion areas. In an independent study, using elicitin- and bacteria-induced HR, Cacas et al. (2005) proposed that the combined action of 9-lipoxygenase and *NtPAT*-encoded galactolipase is sufficient to bring about programmed cell death during HR by converting plastid-released fatty acids into toxic hydroperoxides. A similar situation was described in cotton leaves where co-expression of a patatin galactolipase with a 9-LOX precedes *Xanthomonas campestris*-mediated HR symptoms, pointing to a possible conservation of an oxylipin-based cell death pathway across the plant kingdom. The coupling of different LAHs with oxylipin biosynthetic pathways seems to be evolutionary ancient, as lipolytic activities trigger oxylipin defense upon wounding or epiphytic growth in red algae (Lion et al. 2006).

Patatin genes are ubiquitous in plant genomes and although they have only been investigated thoroughly in a few species, stress-inducible patatins can be identified in most plant species where large-scale transcriptional data are available, including rice (Singh et al. 2012), wheat (Desmond et al. 2008), tomato, or maize. Inducible patatin genes and LAH activity have been associated with other physiological processes. For example, in cowpea, the extent of galactolipid breakdown upon drought stress correlates with drought sensitivity, galactolipase activity, and patatin gene induction (Matos et al. 2001).

The patatin family has been characterized in recent years in Arabidopsis, where a family of ten *pPLA* gene members has been defined and classified into three subclasses, according to gene structure and overall similarity (Holk et al. 2002; La Camera et al. 2005; Scherer et al. 2010). There is evidence that at least one member of each subclass is involved in plant defense mechanisms. pPLAI, the single member of subclass I, is an atypical patatin as its sequence resembles $iPLA_2\alpha$, a member of the animal so-called PNPLA protein family displaying a patatin domain (Kienesberger et al. 2009). pPLAI also possesses leucine-rich and Armadillo repeats that suggest regulatory interactions with protein partners. When assayed in vitro, pPLAI exhibits preferential galactolipase over phospholipase activity and hydrolyzes both oxylipin-containing and unoxidized galactolipids (Yang et al. 2007). However, the in planta relevance of this observation is unknown, as

lipid profiling of *Botrytis*-infected leaves did not evidence alterations in mono- or di-galactosyldiacylglycerol hydrolysis between wild-type and *pplaI*-deficient plants. Despite *pPLAI* transcription being stable upon biotic stress, *pplaI* mutants were more sensitive to *Botrytis* infection, but their JA levels were not altered. Instead, basal JA levels were reduced compared to WT, although how this change conditions antifungal resistance is unclear (Yang et al. 2007). Several studies showed that upon infection or wounding, Arabidopsis plants accumulate large amounts of bound oxylipins, namely, the JA-precursor 12-oxo-phytodienoic acid, that are esterified to galactolipids (Stelmach et al. 2001; Andersson et al. 2006b; Buseman et al. 2006). Also, the induction by bacterial infection of novel and abundant galactolipid accumulation bearing fragmented and/or peroxidized fatty acids was reported (Zoeller et al. 2012).

Subclass II of patatin-related PLAs (pPLAII) comprises five genes in Arabidopsis that are the closest by organization and sequence to classical potato tuber patatins and also generally comprise the isoforms known so far in other plant species (Scherer et al. 2010). Subclass I and II contain the canonical GTSTG esterase box that is part of the catalytic center. Transcript profiling identified pPLAIIα/PLP2 as the only isoform of subclass II whose expression was upregulated in response to necrotizing bacteria or fungi (La Camera et al. 2005), and this induction is dependent on JA signaling in response to *Botrytis*. Overexpression of Arabidopsis pPLAIIα in petunia has independently conducted to similar observations (Zahn 2005). *pPLAIIα* was also reported to be responsive to numerous stimuli in Arabidopsis (Narusaka et al. 2003), including drought stress (Matos et al. 2008) where it may contribute to galactolipid breakdown. Functional analysis of pPLAIIα in various pathosystems has shown that its expression increases the severity of symptoms and favors colonization of leaves by fungal and bacterial pathogens. pPLAIIα-deficient plants are more resistant to *Botrytis* or avirulent *Pseudomonas syringae* infection whereas overexpressing plants display enhanced cell death symptoms and elevated pathogen multiplication (La Camera et al. 2005). These results were supported by altered sensitivity of engineered plants to the cell death-inducing herbicide paraquat and point a primary role of pPLAIIα in promoting host cell death execution. Conversely, a positive impact of pPLAIIα expression on resistance was recorded when plants were inoculated with the obligate pathogen *Cucumber Mosaic Virus* whose multiplication is counteracted by plant cell death (La Camera et al. 2009). Finally, pPLAIIα effect on developmentally triggered cell death was examined, eliminating the variable contribution of pathogen virulence factors to the plant response. Silencing or overepression of pPLAIIα in the lesion mimick mutant background *vascular associated disease* (*vad1*) reduced or enhanced, respectively, the extent and kinetics of leaf damage. Collectively, these results illustrate the multifaceted contribution of the pPLAIIα to the execution of leaf cell death triggered by various microbial attackers, or by the plant itself.

To gain insight into the metabolic changes related to *pPLAIIα* expression, broad-spectrum oxylipin profiling was performed in pPLAIIα-modifed, *Botrytis*-infected plants. The results indicated that pPLAIIα manipulation does not significantly alter fungus-induced accumulation of free C18 fatty acids and jasmonates, but that it

modulated α-dioxygenase-derived oxylipin abundance, which was previously linked to cell death promotion (La Camera et al. 2009). This analysis suggests that in response to microbes, pPLAIIα does not seem to act as a signaling enzyme, but rather catalyzes late, nonspecific hydrolysis of membrane lipids that commits tissue to cell death. The exact mechanism by which pPLAIIα affects the cell death process is still uncertain, as is the precise nature of endogenous substrate(s), because no extensive phospho- and galacto-lipid profiling of pathogen-challenged modified plants is available for this pPLA. Blocking genetically branches of oxylipin pathways in plants displaying pPLAIIα-enhanced cell death may inform if cellular collapse relies on specific oxylipin synthesis.

The subclass III of patatins contains four members in Arabidopsis that share less overall similarity to potato tuber patatin (Holk et al. 2002; La Camera et al. 2005; Scherer et al. 2010) and are distinguished by a variant esterase motif where the central reactive serine typical of serine hydrolases is lacking, resulting in a GXGXG motif. Until recently, there was no formal evidence of enzymatic activity for this class of patatins, but characterization of pPLAIIIß clearly revealed LAH (with a preference for *sn*-2 position) and acyl-CoA thioesterase activities, whereas misexpression of pPLAIIIß affects plant growth and morphology (Li et al. 2011). Transcript profiling identified pPLAIIIß as the only subclass III isoform that is upregulated upon *Botrytis* or *Pseudomonas* infection (La Camera et al. 2005). However, pPLAIIIß mutant or overexpressing plants appeared unaffected in their resistance to these pathogens, and there is therefore no clear evidence of pPLAIIIß involvement in plant innate immunity.

Interestingly, lipolytic proteins with limited patatin-resembling domains evolved in animals with important functions in fat storage mobilization (Kienesberger et al. 2009) or in pathogenic bacteria as virulence factors (Banerji and Flieger 2004; Lang and Flieger 2011). Three Arabidopsis loci encode proteins with a minimal patatin domain. Two of them (SDP1 and SDP1-like) are related to the yeast triacylglycerol lipase TGL4, but with very limited resemblance to canonical pPLA, and are responsible for most triglyceride breakdown upon post-germinative seedling growth (Scherer et al. 2010).

When relatively stable and usually soluble patatin-encoded LAH activity is detectable in total protein extracts from plant tissues, it is typically very difficult to biochemically monitor other PLAs, because of their weaker or less stable/soluble activity. Consequently, other LAHs have been poorly accessible to direct purification in tissues/organs, such as leaves, that are not specialized in lipolysis. Alternative approaches, including genetics, are then suitable, although its has been difficult to rationally design phenotypic screens that specifically target the isolation of PLA mutants.

5 DAD1-Like LAHs

This LAH gene family was identified by forward genetics when Arabidopsis T-DNA mutant collections were screened for male sterility phenotypes. One of the lines, *deficient in anther dehiscence 1* (*dad1*), is impaired in flower and anther opening and pollen maturation and hence its sterility (Ishiguro et al. 2001). The inactivated gene was identified as encoding a plastidial PLA_1 that initiates JA synthesis in stamens to regulate water transport and late stage development processes in anthers. This finding was the first description of a specific LAH that meets the properties expected from a LAH feeding fatty acids to the plastid-localized early steps of JA synthesis. *DAD1* is expressed in filaments rather than in anthers of wild-type stamens, suggesting that water flux is controlled by JA in filaments. Phylogeny searches showed that DAD1 is one of a 12-member gene family encoding putative lipolytic enzymes. Their catalytic region contains an esterase (GXSXG) motif and these proteins are somehow related to fungal triacylglycerolipases, with which they share *sn-1*-specific PLA and galactolipase activities. These predicted proteins were grouped into three subclasses according to the structure of their N-termini and overall similarity. Subclass I contains seven members including DAD1 that bear predicted transit peptides for targeting to plastids. Subclass II members lack this feature and may reside in the cytosol. The single subclass III member has an N terminal peptide typical for mitochondrial targeting. A nomenclature for this *PLA_1* gene family has been defined (Ryu 2004; Ellinger et al. 2010). Organelle targeting has been experimentally confirmed for some class I members in chloroplasts (Padham et al. 2007; Seo et al. 2009; Ellinger et al. 2010; Grienenberger et al. 2010) and for subclass III AtDLAH (or $AtPLA_1$-III) in mitochondria (Seo et al. 2011). Lipolytic activities of the seven subclass I proteins have also been reported, and in vitro assays of recombinant proteins allowed distinguishing three patterns of substrate preference for these enzymes. The first one displays PLA-only activity, the second PLA and galactolipase activities, and the third deacylates phosphatidylcholine, galactolipids, and triglycerides (Seo et al. 2009). The catalytic diversification in this structural family is consistent with distinct patterns of chloroplast labeling when expressing LAH-GFP fusions (Grienenberger et al. 2010). This differential distribution may reflect association of the enzymes with subchloroplastic compartments such as the envelope, thylakoids, and plastoglobules that are enriched in phospholipids, galactolipids, and triglycerides, respectively. These LAH thus seem to play distinct roles in plastidial lipid homeostasis.

The conclusive characterization of $DAD1/PLA_1$-Iβ1 in JA biosynthesis for anther and pollen development fostered additional functional studies to explore the possibility that other family members may initiate JA synthesis in other organs/situations, for example, under stress conditions. The initial enzymatic steps of JA synthesis from linolenic acid up to its precursor 12-oxo-phytodienoic acid are localized in the chloroplast (Schaller and Stintzi 2009), making the seven subclass I enzymes good candidates to feed fatty acid precursors into the JA pathway in

leaves. DAD1 deficiency was initially reported not to affect JA accumulation after mechanical leaf wounding mimicking attacks by herbivorous insects (Ishiguro et al. 2001). Later on, the sequential cooperation for JA biosynthesis between DAD1 and a closely related galactolipase, DGL (also named PLA_1-Iα-1), was described (Hyun et al. 2008). An Arabidopsis activation line that overexpresses DGL displays stunted growth, accumulates high levels of JA, and displays strong resistance to *Alternaria brassicicola*, a necrotrophic fungus for which resistance is mediated through the JA/ethylene defense pathway. No data on fungal resistance are available for DGL-deficient lines. According to the authors, ectopic expression of other Arabidopsis *PLA_1* genes did not result into elevated JA levels. DGL was proposed to play a role in maintaining basal JA levels and for early accumulation while DAD1 would participate in the later phases. These conclusions that DAD1/PLA_1-Iß1 and DGL/PLA_1-Iα-1 are necessary and sufficient for JA accumulation were debated in another study (Ellinger et al. 2010) that failed to confirm diminished basal or early-induced JA levels in DGL-deficient or *dad1* plants upon wounding or *Pseudomonas* infection. Also, detailed localization studies showed that DGL is associated with lipid bodies rather than with chloroplasts. A reduction by half in the wound-induced JA levels was evidenced in the single mutant *PLA_1-γ 1*. This impairement could be accentuated to about 30 % of the wild-type basal and injury-induced JA accumulation in a quadruple mutant in four *DAD1*-like genes (including *PLA_1-γ1*), showing that in Arabidopsis, different lipases are partially redundant and may act during different phases of the response to account for the full JA burst. In search of novel players to catalyze this step, 14 additional genes encoding putative lipolytic enzymes with predicted plastidial transit peptides were identified in the Arabidopsis genome, but analysis of mutant lines in 11 of these genes showed normal basal and wound-induced JA levels (Ellinger et al. 2010). This suggests that in chloroplasts, many lipases do not present the adequate activity, association with lipids, or enzymatic partners to channel linolenic acid into JA biosynthesis. Three other *PLA_1-I* genes, *PLA_1-γ1*, *PLA_1-Iγ2*, and *PLAIII*, were examined in the context of antimicrobial defense and found to be induced by *Pseudomonas* or *Botrytis* attack (Grienenberger et al. 2010). However, mutant lines in these genes did not exhibit a clear defect in microbe-induced JA levels or in pathogen resistance. Therefore, strong evidence from PLA_1-deficient Arabidopsis lines demonstrating the role of these enzymes in antimicrobial defense is still lacking. Other Arabidopsis genes in the PLA_1 family have been linked to roles in leaf senescence [PLA_1-α2 (Padham et al. 2007)], seed viability [mitochondria-targeted PLA_1-III (Seo et al. 2011)], or negative regulation of seed storage oil mobilization [PLA_1-IIγ (Kim et al. 2011a)].

Interestingly, the functional analysis of *PLA_1-I* genes in inducible defense in the wild tobacco relative *Nicotiana attenuata* provided more clear data. Three DGL-DAD1 homologs named GLA (for glycerolipase A) have been identified in *N. attenuata*. Although *GLA1* was the only isoform whose mRNA abundance was downregulated upon wounding (Kallenbach et al. 2010), its silencing clearly and specifically attenuated the accumulation dynamics of jasmonates and some other oxylipins. *GLA1*-silenced plants were reduced by 50–65 % in their content in JA

and JA-Ile in leaves or roots that were wounded or treated with insect elicitor (Bonaventure et al. 2011). In the same material, *sn-1*- and *sn-2*-specific lysophospho- and lysogalacto-lipid levels showed a similar reduction, in line with the in vitro *sn-1*-specific activity on phospholipids, galactolipids, and triglycerides determined for recombinant GLA1 (Kallenbach et al. 2010). In contrast, *GLA1*-silenced plants display normal levels of JA and JA-Ile in flowers and are fully fertile. Similarly, JA levels of leaves infected by the fungus *Fusarium oxysporum* or by the oomycete *Phytophthora parasitica* var. *nicotianae* were unaffected as compared to the different plant genotypes. Impact on other branches of the lipoxygenase pathway (Feussner and Wasternack 2002; La Camera et al. 2004) was also investigated. Green leaf volatiles that are hydroperoxide lyase cleavage products released upon wounding were not modified. However, *GLA1* silencing was found to affect the early metabolism of divinyl ethers upon infection of leaves by *P. parasitica* (Bonaventure et al. 2011). These results indicate that in vivo, the plastidial lipase GLA1 is post-transcriptionally recruited to act on different lipid classes and provides C18 fatty acid substrate to initiate JA synthesis or modulate other oxylipins in some specific situations. This reinforces the idea that biosynthesis of a given oxylipin may require different lipases depending on the stimulus and the plant organ. Conversely, a given lipase can affect the abundance of different types of oxylipins, pointing to a growing complexity of lipid hydrolysis networks. Understanding how these critical events are regulated and interconnected is a major challenge for future biochemical/cellular studies.

6 Other Predicted Lipases

6.1 The EDS1/PAD4/SAG101 Regulatory Node

In Arabidopsis, EDS1 (*E*nhanced *D*isease *S*usceptibility*1*) has emerged as a central immune regulator (Aarts et al. 1998; Wiermer et al. 2005; Garcia and Parker 2009). In association with its sequence-related partners PAD4 (*P*hyto*A*lexin *D*eficient*4*) and SAG101 (*S*enescence-*A*ssociated *G*ene*101*), EDS1 forms an essential regulatory node that controls both low-level basal immunity to virulent pathogens and effector-triggered activation of intracellular nucleotide-binding leucine-rich repeat (NB-LRR) proteins, triggering the HR (Feys et al. 2001, 2005). EDS1 presents different molecular and spatial in planta associations with PAD4 and SAG101, and these complexes have distinct signaling activities (Feys et al. 2001, 2005). Indeed, EDS1 homodimers are restricted to the cytoplasm whereas EDS1-PAD4 heterodimers accumulate in both the cytoplasm and the nucleus, and EDS1-SAG101 complexes are confined to nuclei. Importantly, dynamic interactions between nuclear and cytoplasmic pools of EDS1 are important in directing defense signaling (Feys et al. 2005; Garcia et al. 2010).

Based on primary-sequence analysis, the N terminal domain of EDS1 has homology to proteins of the α/β-hydrolase fold family, including three residues that comprise a serine hydrolase catalytic triad (Ser-Asp-His) (Ollis et al. 1992). EDS1 shares this overall homology and a unique C terminal "EP" (EDS1-PAD4-specific) domain with PAD4 and SAG101 (He and Gan 2002; Wiermer et al. 2005). Although SAG101 lacks the canonical catalytic triad, weak acyl esterase activity was detected for recombinant SAG101 expressed in *Escherichia coli* (He and Gan 2002). However, no enzymatic activity has been detected in the case of EDS1 or PAD4 and their biochemical mode of action remains unknown. Although the lipase homologies of these proteins might be misleading in terms of catalytic activity, these domains appear to be conserved in all plant EDS1 and PAD4 orthologs examined, and it has been suggested that they may play a structural rather than an enzymatic role during plant immunity (Wiermer et al. 2005). EDS1 and PAD4 are required to transduce reactive oxygen species-derived signals leading to cell death during photooxidative stress and immune responses, suggesting the involvement of a redox component necessary for EDS1 signal transduction (Muhlenbock et al. 2008; Straus et al. 2010). Based on these observations and the lack of enzymatic activity, it has been also proposed that that EDS1 and its partners may help traffick rather than hydrolyze oxygenated lipids inside the cell (Wiermer et al. 2005).

6.2 Other Structural Families

There are at least two more large gene families structurally related to lipases, with members that have been clearly linked to innate immunity, but whose glycerolipase activity remains unclear. The first one is the so-called GDSL family that displays a conserved motif (PFAM 00657) present in the catalytic site and that is distinct from the widespread GxSxG serine hydrolase motif discussed earlier. Structural analysis suggests a flexible active site that can accommodate various substrates and characterized members display lipase, protease, esterase, or even acyltransferase activities (Volokita et al. 2011; Kikuta et al. 2012). Therefore, it is not known to which extent some isoforms are true lipolytic enzymes or even if some act on glycerolipids (Li-Beisson et al. 2010). Arabidopsis has 108 GDSL members, but none has been described as displaying PLA activity. In most described studies, poor enzyme characterization was conducted, using synthetic short-chain *p*-nitro-phenyl esters as substrates. Still, two genes have been identified with functions in plant defense. GLIP1 was purified as a protein secreted by salicylic acid-treated cultured cells (Oh et al. 2005). *GLIP1* gene expression is induced by fungal infection and *glip1* mutant plants are susceptible to *Alternaria* infection. GLIP1 has direct fungitoxic activity but may also act in systemic signaling as its expression conditions the presence in phloem exudates of a defense-inducing activity (Kwon et al. 2009). A closely related member called GLIP2 seems to affect both auxin signaling and resistance to the necrotrophic bacterium *Erwinia carotovora* (Lee et al. 2009).

Finally, a pepper GDSL ortholog is a positive effector of defense against biotrophic microbial infections (Hong et al. 2008), suggesting that this class of enzymes may affect a conserved step in defense against pathogens with different colonization strategies.

The second family called PRLIP was first identified in Arabidopsis (Jakab et al. 2003) and similar sequences were found in rice and grapevine (Szalontai et al. 2012). Predicted PRLIP proteins present features of serine hydrolases/esterases and class 3 lipases but again no true lipidic substrate is known. These sequences are grouped into two clades, one of which with constitutive expression is found in all angiosperms with available sequences, the second being taxon-specific and comprising defense signal- and pathogen-induced members.

7 Conclusions and Perspectives

Although interesting advances have been recently seen in the description of the properties of diverse phospho- or galacto-lipases, our understanding of their roles in innate immunity remains largely fragmentary. We are still far from understanding how and where different lipid hydrolysis events are orchestrated by the recruitment of the numerous players at specific subcellular sites.

Two main problems whose resolution will need the implementation of more powerful strategies based on novel technologies are behind the slow progress in the functional characterization of LAHs in recent studies. First, most reverse genetic studies involve pre-identified gene families in plants or in other organisms where candidate proteins exhibit recognizable features of esterases/lipases, such as in rice (Singh et al. 2012). Gene annotation can sometimes be misleading and has conducted many investigators to regard as lipolytic some protein candidates that likely use non-acyl-lipidic substrates. Conversely, many true lipolytic enzymes have likely been missed because of their "noncanonical" primary structures and remain to be discovered. This functional gap between insufficient gene annotation and in-depth characterization could be partly bridged by screening candidates at the protein rather than transcript level, for example, by applying activity-based proteomics. This approach allows to visualize enzymes in complex proteomes by incubation with fluorescent chemical probes that covalently label active sites (Birner-Gruenberger and Hermetter 2007). Mass spectrometry-based identification of labeled proteins then provides access to functional studies. This strategy proved highly successful in the global analysis in animal models of storage lipid hydrolysis such as adipocytes (Schittmayer and Birner-Gruenberger 2009) and has started to be used in plant pathology. For example, this technique was applied to the *Arabidopsis–Botrytis* interaction and led to the identification of 45 Arabidopsis serine hydrolases of which only 9 had been characterized previously and also of fungus-derived cutinases and lipases (Kaschani et al. 2009). Similar experiments repeated with probes specific for PLA/GLA activities have the potential to reveal

unknown lipolytic enzymes, including some that are activated to establish immunity independently of their transcriptional upregulation.

A second challenge is to determine where a given PLA acts in the cell and on which substrate. Images of fluorescently tagged PLA provide a first indication as to where hydrolysis may take place, but they are generally acquired in heterologous expression systems and do not account for the cellular changes and damage that occur when immune reponses are being deployed in infected tissue. In this context, the ultimate aim would be to visualize kinetically and with high-resolution individual lipolytic events in living cells. Before this is feasible, we must rely on global lipid profiling approaches where impressive analytical progress has been recently made. Early studies attempted to link the activation or deficiency of a given LAH with changes in lipid or presumed hydrolysis product abundance, but the analytic resolution was limited to the separation of global lipid classes and frequently did not correlate with the modification of lipid composition. A major pitfall is that many phospholipids are present in several membrane systems across the cell, potentially masking the discrete action of individual LAHs at a defined subcellular location. In vitro-determined lipolytic activities can also be misinterpreted as to the real substrate that is hydrolyzed in vivo. Improved protocols largely based on electrospray ionization-mass spectrometry, either by direct infusion of coupled to liquid chromatography, allowed the separation, identification, and in some cases quantification of many lipid molecular species (Welti and Wang 2004). In further recent methodological developments, hundreds of glycerolipid species differing by their polar head and acyl chains were characterized (Ibrahim et al. 2011; Vu et al. 2012; Zoeller et al. 2012). Furthermore, in response to wounding or bacterial infection, the appearance of dozens of novel oxidized or fragmented galactolipid species was reported. This enhanced analytic resolution will allow a much deeper metabolic examination of LAH-modified lines with increased possibility of identifying subtle changes in their lipid contents. Alterations in organelle- or membrane-specific (lyso)-lipid species can even provide precise information on real in vivo substrate(s). Finally, the emergence of MS-imaging techniques on tissues or isolated organelles opens another door to the visualization of PLA product formation (Horn and Chapman 2012). The combination of expanded genetic resources with more systematic biochemical and analytical tools should significantly contribute to increase our understanding about how PLAs impact the adaptation of plants to their ever changing environment.

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