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Secretory Phospholipases A_2 in Plants

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Secreted phospholipases ($sPLA_2$s) in plants are a growing group of enzymes that catalyze the hydrolysis of *sn-2* glycerophospholipids to lysophospholipids and free fatty acids. Until today, around only 20 $sPLA_2$s were reported from plants. This review discusses the newly acquired information on plant $sPLA_2$s including molecular, biochemical, catalytic, and functional aspects. The comparative analysis also includes phylogenetic, evolutionary, and tridimensional structure. The observations with emphasis in *Glycine max* $sPLA_2$ are compared with the available data reported for all plants $sPLA_2$s and with those described for animals (mainly from pancreatic juice and venoms sources).

Keywords: secretory phospholipase A_2, interfacial catalysis, auxin, *Glycine max*, phospholipase–membrane interaction

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INTRODUCTION

For more than a century experiments were performed with $sPLA_2$s enzymes, being used as lipid model enzymology and as paradigms for the formalism of interfacial catalysis (Dennis et al., 2011). The phospholipase A_2 (PLA_2, EC 3.1.1.4) superfamily is a broad and growing group of enzymes that stereo specifically catalyzes the cleavage at the *sn-2* acyl ester bond from diacyl-phospholipid liberating lysophospholipid and free fatty acid. In plants, secreted PLA_2 ($sPLA_2$) represents one type of phospholipase A_2 whose lipid products mediate a variety of cellular processes, including growth, development, defense, and stress responses (Stahl et al., 1998, 1999; Kim et al., 1999; Lee et al., 2003; Ryu, 2004; Mansfeld, 2009; Chen et al., 2011). Although numerous $sPLA_2$ genes have been identified in plants, little is known about these enzymes in opposition to their insect, animal or human counterparts (Burke and Dennis, 2009). $sPLA_2$ is best known from mammals where several $sPLA_2$s have been identified in the last 25 years (Murakami et al., 2011). Moreover, many $sPLA_2$s were found in sources as venoms from snakes, scorpions, bee, etc.; from microorganisms as bacteria and yeasts, as components of pancreatic juices, where it occurs abundantly and has a digestive role; arthritic synovial fluid; and in many different mammalian tissues (Valentin et al., 1999; Schaloske and Dennis, 2006; Burke and Dennis, 2009; Murakami et al., 2010, 2011). Additionally, for the first time, we have recently described the interfacial properties of purified recombinant $sPLA_2$s from *Streptomyces violaceoruber* (Yunes Quartino et al., 2015) and from *Glycine max* (Mariani et al., 2012, 2015b), i.e., the optimal surface lipid packing conditions (interfacial quality) in which a $sPLA_2$ can hydrolyze phospholipid in an organized membrane. This point, no less important for interfacial enzymes, was also addressed comparatively in the present review.

Glycine max (Soybean), in addition to being one of the most widely used oil crop grain in the world, possesses valuable contributions to health due to its high nutritional level. Lipids, proteins and other valuable bioactive components such as: phospholipids (known as lecithin), hormones,

and antioxidants are present in soybean (Messina, 1999; Choi and Rhee, 2006). The industrial use of sPLA$_2$s from animal pancreas extracts and microbes, especially in food production, has a long tradition (Guo et al., 2005; De Maria et al., 2007). One of the targets in the future may be the utilization of sPLA$_2$ from plants for enzymatic processing to stereospecifically obtain lysoderivatives. This alternative has been recently recognized to satisfy food regulation requirements such as Kosher and Halal (Havinga, 2010). However, no sPLA$_2$s from plants have now been yet available for industrial application (Mansfeld, 2009).

Secreted PLA$_2$s are low MW calcium dependent enzymes (12–18 kDa) (Schaloske and Dennis, 2006). From a perusal revision of sequence data, almost all sPLA$_2$s from plants and animals contain a signal sequence. So, in the general secretion way after removal of the N-terminal signal peptide in the endoplasmic reticulum (ER), they are secreted into the extracellular space in a either mature or pre-protein form (Fujikawa et al., 2005; Lee et al., 2005; Mansfeld et al., 2006). Although sPLA$_2$s are recognized to be secreted proteins, a few of them were reported to act intracellularly prior or during secretion (Mounier et al., 2004; Shridas and Webb, 2014). Until now, the pre-protein form would be exclusive for animals (see **Table 1**).

Important common features shared for all sPLA$_2$s are the presence of: (i) one HIS residue at the catalytic domain for nucleophilic attack at the *sn-2* acyl ester bond of the glycerol backbone, (ii) requisite of calcium for full activity (μM-mM), and (iii) exceptionally heat-stable enzymes. sPLA$_2$s also contain a domain, named PA$_2$c, with the highly conserved Ca^{2+} binding loop (YGKYCGxxxxGC) and the active site motif (DACCxxHDxC), where the HIS/ASP pair was found to be highly well conserved in both animal and plants sPLA$_2$s. At least, two characteristics are of great interest in the structure of all sPLA$_2$s: the catalytic site and the interfacial recognition surface (IRS). All sPLA$_2$s have the same architecture (about 55% of identity) at the catalytic site level (HIS-ASP) (Lee et al., 2005) but differ in the amino acid residues that conform the IRS region (Berg et al., 2001) sharing only 15% of identity in the amino acid sequence.

In the presence of reducing compounds such as β-mercaptoethanol or dithiothreitol (DTT) the activity is affected or abolished by disrupting the protein structure (reduction of disulfide bridges) (Stahl et al., 1998). They also show high resistance to organic solvents, acidic conditions and high temperatures (they are even more resistant in the presence of Ca^{2+}). A common procedure to confirm the catalytic mechanism is by checking if the activity is chemically canceled by the alkylation of HIS localized in the catalytic triad HIS/ASP/X (where X may be either HIS, SER, or ASP) induced by p-bromophenacylbromide (BPB) (Minchiotti et al., 2008). A resume of the general characteristics comparing animals from plants sPLA$_2$ is shown in **Table 1**.

Fatty acids produced by the hydrolysis carried out by sPLA$_2$s, such as oleic (1:18) or arachidonic (4:20) acid, are sources of energy reserve. Furthermore, arachidonic acid can function as intracellular second messenger or as precursor of eicosanoids inflammation mediators, if is the extracellular product of the reaction catalyzed by secreted phospholipase as occurs for human synovial fluid (Baynes and Marek, 2004). The other product of the action of sPLA$_2$, the lysophospholipid is important in cell signaling and remodeling or membrane perturbations (Khan et al., 1995). In contrast, in plants the jasmonic acid and its related compounds are important hormones involved in plant defense reaction against microbial pathogens, herbivores and UV light damaging as well as senescence mechano-transduction (Schaller, 2001).

In the past years, significant advances have been made toward understanding the role of these enzymes in normal cellular and tissue homeostasis or function particularly in mammals (Rhee and Bae, 1997; Assmann and Shimazaki, 1999; Williams, 1999; Liscovitch et al., 2000; Murakami et al., 2015) but, the more recent data reported for plant sPLA$_2$s are rather scarce. Therefore, this review focuses on recently acquired information on all sPLA$_2$ from plants reported until now with emphasis in *Gm*sPLA$_2$s identified in *G. max* (soybean), comparing them with the more relevant published data for several sPLA$_2$s obtained from different sources. A comparative description with respect to the sequence characterization, biochemical, molecular, and functional aspects of sPLA$_2$s enzymes was done.

TABLE 1 | General characteristics presented by calcium dependent sPLA$_2$s from animals to plants.

Properties/ characteristics	Animals	Plants
Intracellular second messenger	PL → arachidonic acid → prostaglandins and leukotrienes	PL → linoleic acid → jasmonic acid
Main metabolic pathway	Eicosanoid pathway	Octadecanoid pathway
Secreted as zymogen	Some	NR
Catalytic triad	ASP/HIS/ASP	ASP/HIS/X (X = ASN or SER or HIS)
MW (kDa)	12–18	∼14
Cysteines	8–14	12
Disulphide bridges	4–7	6
Calcium requirement[a]	mM[b]	μM-mM

PL, phospholipid; NR, no reported. [a]Minimum of required Ca^{2+} concentration for full activity. [b]Regarding to the general mM requirement for reported sPLA$_2$ from animal source, it has been described one exception for a sPLA$_2$ isolated from venom of the marine snail Conus magus (McIntosh et al., 1995). This exception for sPLA$_2$ was also remarked by Six and Dennis (2000).

SECRETORY PHOSPHOLIPASES A$_2$ IN PLANTS

In comparison with the animal sPLA$_2$, the knowledge generated for sPLA$_2$ from plants is still limited, even though when recombinant enzymes from plants have been recently expressed in *Escherichia coli* and yeast and characterized. Some studies about enzyme activities have been reported in more or less

TABLE 2 | sPLA$_2$s from plants, accession numbers, N-terminus characteristics and purification/recombinant process applied.

Source	Name	Purification	N-terminal[a]	Accession number	Group XI	References
A. thaliana	*At*sPLA$_2$-α	cDNA	Recombinant	At2g06925	B	Mansfeld and Ulbrich-Hofmann, 2007
	*At*sPLA$_2$-β	cDNA	Mature	At2g19690	A	Lee et al., 2003
(arabidopsis)	*At*sPLA$_2$-γ	cDNA	Mature	At4g29460	A	Bahn et al., 2003
	*At*sPLA$_2$-δ	cDNA	NR	At4g29470	A	Bahn et al., 2003; Ryu et al., 2005
R. communis	*Rc*sPLA$_2$α	cDNA	Recombinant	XM002523613	B[b]	Bayon et al., 2015
(castor bean)	*Rc*sPLA$_2$β	cDNA	Recombinant	XM002514118	B[b]	Bayon et al., 2015
C. sinensis	*Css*PLA$_2$α	cDNA	Recombinant	GU075396	B[b]	Liao and Burns, 2010
(orange)	*Css*PLA$_2$β	cDNA	Recombinant	GU075398	A[b]	Liao and Burns, 2010
D. caryophillus (carnation)	*Dc*sPLA$_2$	cDNA	NR	AF064732	B	Kim et al., 1999
U. glabra[c] (elm)	*Ug*sPLA$_2$	Seeds	Purified	NR	NR	Stahl et al., 1998
G. max	*Gms*PLA$_2$-XIA-I	cDNA	Mature	BT092274	A	Mariani et al., 2012
(soybean)	*Gms*PLA$_2$-XIA-II	NR	NR	BT094641	A	Mariani et al., 2012
	*Gms*PLA$_2$-XIB-I	NR	NR	BT095220	B	Mariani et al., 2012
	*Gms*PLA$_2$-XIB-II	cDNA	Mature	BT091171	B	Mariani et al., 2015b
	*Gms*PLA$_2$-XIB-III	NR	NR	BT099163	B	Mariani et al., 2012
L. usitatissimum	*Lus*PLA$_2$-I	cDNA	Fusion Protein	KU361324	B	Gupta and Dash, 2017; Gupta et al., 2017
(flax)	*Lus*PLA$_2$-II	cDNA	Fusion Protein	KU361325	A	Gupta and Dash, 2017; Gupta et al., 2017
P. somniferum (opium)	*Ps*sPLA$_2$	cDNA	Recombinant	KU900749	B	Jablonicka et al., 2016
O. sativa	*Oss*PLA$_2$-I	Seeds	PPfE	AJ238116	A	Lee et al., 2005
(rice)	*Oss*PLA$_2$-II	cDNA	Mature	AJ238117	B	Stahl et al., 1999; Guy et al., 2009
	*Oss*PLA$_2$-III	NR	NR	AAK50122	B	Lee et al., 2005
N. tabacum	*Nt*1PLA$_2$	cDNA	Recombinant	AB190177	A	Fujikawa et al., 2011
(tobacco)	*Nt*2PLA$_2$	Extract	PPfE	AB190178	B	
L. esculentum (tomato)	*Les*PLA$_2$	NR	NR	AI487873	B	Lee et al., 2005; Verlotta et al., 2013
T. durum	*Td*sPLA$_2$I	cDNA/LE	PPfE	JX021445	A	Verlotta et al., 2013
(durum wheat)	*Td*sPLA$_2$II	cDNA/LE	PPfE	JX021446	B	Verlotta et al., 2013
	*Td*sPLA$_2$III	cDNA	Recombinant (6× His-TdsPLA$_2$III)	JX021447	B	Verlotta et al., 2013; Verlotta and Trono, 2014
	*Td*sPLA$_2$IV	cDNA/LE	PPfE	JX021448	B	Verlotta et al., 2013
Z. mays (maize)	*Zms*PLA$_2$[d]	NR	NR	EU968759	B	Mariani et al., 2012

[a]*Mature, without extra amino acids at the N-terminus after heterologous expression. PPfE, when the enzyme was Partially Purified from Extracted from a plant organ (partial purification, less than 90% purity). LE, leaves extract. Recombinant is indicated when, according to the reported data, it is not known if the expression assayed is in mature form or contain any tags in the final purified recombinant form (no clearly indicated in the original paper).* [b]*Classified in this review from proper alignment of the reported sequences. NR, not reported.* [c]*For sPLA$_2$ from Ulmus glabra (elm) it was assigned as UgsPLA$_2$ since in the original describing paper (Stahl et al., 1998) was named as sPLA$_2$ without initial letters of identification.* [d]*Named as ZmsPLA$_2$ in this review.*

crude preparations (Moreau and Morgan, 1988; Mukherjee, 1990; Minchiotti et al., 2008; Murakami et al., 2011).

The first sPLA$_2$ purified to homogeneity, sequenced and characterized from plants, was the sPLA$_2$ from elm seed endosperm (*Ulmus glabra*) in 1998 (Stahl et al., 1998). Later in 1999, two cDNAs encoding sPLA$_2$ (sPLA$_2$-I and-II) were isolated from shoots of rice (*Oryza sativa*) and characterized (Stahl et al., 1999). cDNAs full sequences coding for putative sPLA$_2$s were obtained from flowers of carnation (*Dianthus caryophyllus*) (Kim et al., 1999). These later clones from carnation and rice have not been further characterized to demonstrate that they encode functional enzymes. With progress in genome sequencing projects, more sPLA$_2$s have been identified: in tomato (Lee et al., 2005) and outbreaks of castor bean (*Ricinus communis*) (Domingues et al., 2007). Four isoforms of sPLA$_2$ from *Arabidopsis thaliana* have been also isolated, called *At*sPLA$_2$-α, -β, -γ, and -δ (Bahn et al., 2003; Lee et al., 2003, 2005; Mansfeld and Ulbrich-Hofmann, 2007; Seo et al., 2008), which have been expressed (Ryu et al., 2005; Mansfeld and Ulbrich-Hofmann, 2007) two isoforms have been studied in tobacco (*Nicotiana tabacum*) (Dhondt et al., 2000; Fujikawa et al., 2005, 2011) and orange (*Citrus sinensis*) (Liao and Burns, 2010). Three cDNA from durum wheat (*Triticum durum*) were isolated and two of them studied in detail (Verlotta et al., 2013; Verlotta and Trono, 2014). A novel sPLA$_2$ from opium (*Papaver somniferum*) was purified and characterized (Jablonicka et al., 2016) and two

sPLA$_2$ from flax (*Linum usitatissimum*) were studied in detail (Gupta and Dash, 2017; Gupta et al., 2017). Moreover, one gene was reported for tomato (*Lycopersicon esculentum*) (Lee et al., 2005) and one gene for maize (*Zea mays*) found in UniProt and mentioned in (Mariani et al., 2012). From our laboratory, five *G. max* phospholipases A$_2$ were reported (Mariani et al., 2012), and two of them (*Gms*PLA$_2$-XIA-I and -XIB-II) were cloned, expressed in *E. coli*, further purified from inclusion bodies and the activity was evaluated using organized lipid systems such as mixed micelles and monomolecular films as substrates (Mariani et al., 2015b).

Table 2 summaries the different enzymes found in plants, their origin and source, GenBank accession numbers and the subgroup at which they belong to within the XI group of the PLA$_2$ superfamily.

RECOMBINANT vs. NATIVE sPLA$_2$s PROTEINS: ROLE OF THE INTACT N-TERMINAL PRESERVATION

Usually the N-terminus region of sPLA$_2$ has an alpha helix domain which forms one wall of the channel through which the hydrophobic substrate entries as reported for groups I and II sPLA$_2$s enzymes (according to Dennis, 1973b). Thus, in the case of pancreatic enzyme (group I), when the zymogen is converted into the active form by removing a short portion of the N-terminus, the remaining N-terminal helix is now able to be involved in the binding interfacial membrane (Scott et al., 1990). This would be affected by the extension of seven amino acids at the N-terminus in the zymogen (pro-enzyme) preventing the binding to lipid interfaces. Crystallographic evidence suggests that the zymogen has a more flexible N-terminus compared to the mature protein (van Deenen, 1971).

The effect of an extra amino acid on the N-terminus of pancreatic sPLA$_2$ can be critical, for example, if it is of hydrophobic nature (van Scharrenburg et al., 1984). This was observed in the pioneering work of deHaas group, showing that the extension of an amino acid (doubling of the terminal ALA of the mature form) caused a decrease in enzyme catalysis to phosphatidylcholine (PC) short chain substrate presented as micelles or when the substrate was arranged as a lipid monolayer (Slotboom et al., 1977). Furthermore, in the case of porcine pancreatic enzyme, an absolute free amino terminal is required (Dijkstra et al., 1984).

In a recent work with a sPLA2 from group II of *Crotalus atrox* venom, the importance of a native N-terminus was also evident. By using chemically modified enzyme the authors concluded that N-terminal region plays a mechanistic role in catalysis and acts as a surface-active component of the complex interfacial catalytic site (Randolph and Heinrikson, 1982). This structural requirement is also found in other sPLA$_2$ expressed in bacteria, such as human sPLA$_2$ from synovial fluid (Marki and Hanulak, 1993). It was observed that, when expressing a sPLA$_2$ in *E. coli*, the initial MET is not removed from the protein that had an ASN at position 1 in the sequence. This is because the bacterial aminopeptidase does not catalyze the removal of the initial MET if it is followed by ASN. The lipolytic activity of this protein was very low relative compared with the expressed correct N-terminus mature form (Othman et al., 1996). Similarly, another study reported that the protein with an extra MET at its N-terminus had the same pH optimum and prefered substrate compared to the one with native end (without MET), but the activity was drastically reduced (Marki and Hanulak, 1993). Bacterial aminopeptidases remove initial MET efficiently when the amino acid in position 2 of the mature sequence is little and without charge (such as ALA, GLY, SER), but fail when the residue is voluminous and charged as ASN (Hirel et al., 1989). Othman et al. (1996) have substituted the ASN by ALA to express the recombinant protein thus allowing the removal of the initial MET by the bacteria and avoiding a subsequent step of chemical or enzymatic cleavage.

A similar observation was made in sPLA$_2$ mutants from Taiwan cobra (Anderson and Dufton, 1997). The addition of a MET at the N-terminus generates structural distortions, and it was postulated that affects the active site through hydrogen bonds network. Moreover, an extra MET decreases the activity with respect to the enzymes with native end (Chiou et al., 2008). Some reports suggest that the N-terminal helix of groups I and II sPLA$_2$s acts as a regulatory domain that mediate the interfacial activation (Qin et al., 2005).

The correct design of the heterologous expression of the cloned enzyme is crucial because the recombinant protein must be generated with the correct native N-terminus, without any additional amino acid extension, since any modification or extension of the N-terminus in sPLA$_2$ can severely alter the catalytic properties (van Scharrenburg et al., 1984; Othman et al., 1996). This is also valid for any additional N-terminal tag (such as HIS-Tag, frequently used in molecular biology protocols to express recombinant proteins). Both facts make the recombinant protein act as a zymogen like pre-protein.

In this sense, the sPLA$_2$s obtained from *G. max* were expressed without N-terminal extension (Mariani et al., 2012, 2015b) by using the pHUE vector system that utilizes the ubiquitin fusion technique (Catanzariti et al., 2004), which allows easy purification and high yield of recombinant proteins (see **Figure 1**). The *E. coli* pHUE vector permits the expression of a particular protein as HIS-tagged ubiquitin fusion. Then, the HIS-tag-ubiquitin-sPLA$_2$ fusion is further processed by the deubiquitylating enzyme used to cleave off the fusion to obtain the protein of interest free of any N-terminal extension (**Figure 1**).

In the particular case of the mature protein *Gms*PLA$_2$-XIB-II, the LEU amino acid at the N-terminus was mutated to an ALA, to optimize the chance of obtaining the correct refolding as previously recommended (Kohler et al., 2006). In *At*sPLA$_2$-α, it was shown that an uncleaved signal peptide of the pre-processed forms produced a significant suppression of activity compared with the corresponding mature protein form (Ryu et al., 2005). Moreover, other sPLA$_2$s from plants were expressed without the signal peptide (Mansfeld et al., 2006;

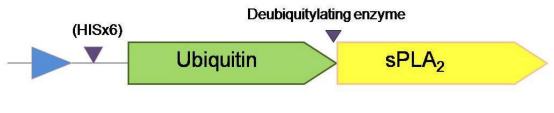

FIGURE 1 | Schematic representation of the system applied for *Gms*PLA$_2$s expression and purification. Triangles indicate the position where is the polyHIS-tag motif and the position where the deubiquitylating enzyme cut in order to release the protein with its native N-terminus without any extension.

Guy et al., 2009). In animals, a correct and functional sPLA$_2$ from *Bothrops diporus* was produced without any extra extension at the N-terminus (Yunes Quartino et al., 2012). Using the ubiquitin/deubiquitinase system, in the latter case, it was clearly shown that the recombinant protein had the same interfacial catalytic profile when compared to the native one (Yunes Quartino et al., 2015).

As sPLA$_2$ activity is very sensitive to N-terminus modifications, in **Table 2** we include all sPLA$_2$s from plants known until today and the process originally reported to obtain the final protein (either proteins purified from plant extracts, in a mature recombinant form or with an additional tag). It should be noted that not all reported information disclosed the sequence of phospholipase A$_2$ either cloned or purified.

However, similar to its counterpart in animals, sPLA$_2$s from plants have N-terminal signal peptides that were predicted to direct protein secretion into the extracellular or intracellular space (Bahn et al., 2003; Lee et al., 2003). It is noteworthy that some sPLA$_2$s from plants have the sequences KTEL, KFEL, and KLEL at the C-terminal which are similar to the endoplasmic reticulum retention sequences KDEL and HDEL reported for animals (Matsushima et al., 2003). Even when this putative KxEL endoplasmic reticulum (ER) retention sequence (Pagny et al., 2000; Seo et al., 2008) is present in some plant sPLA$_2$, the biochemical significance is still unknown (see **Supplementary Figure S1**).

TABLE 3 | Sequence characteristics of the *Gms*PLA$_2$s[a].

Name	Full-length cDNA (nt)	Open reading frame (ORF) (nt)	Residues of native protein with signal peptide	Residues of mature protein
*Gms*PLA$_2$-XIA-I	789	417	138	114
*Gms*PLA$_2$-XIA-II	875	417	138	115
*Gms*PLA$_2$-XIB-I	826	474	157	128
*Gms*PLA$_2$-XIB-II	762	471	156	128
*Gms*PLA$_2$-XIB-III	821	477	158	128

[a]*In* ***Supplementary Figure S5*** *it is shown the complete sequence with the N-terminal region with the signal peptide and the putative theoretical site of cleavage for all sPLA$_2$ reported for plants. Signal peptides for each sequence was determined by using the signalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP-3.0/).*

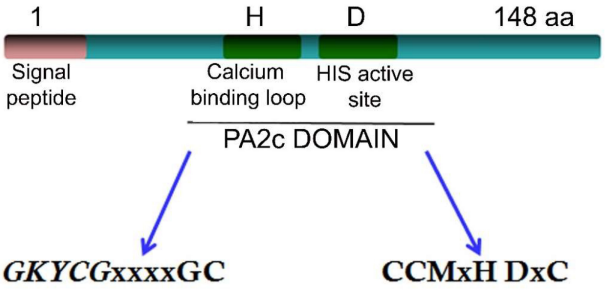

FIGURE 2 | Schematic representation of the *Gms*PLA$_2$ gene.

*Gms*PLA$_2$s GENE FAMILY, CLASSIFICATION, AND DOMAIN STRUCTURE

In *G. max*, five sPLA$_2$s isoforms were identified (Mariani et al., 2012), named as *Gms*PLA$_2$-XIA-I, *Gms*PLA$_2$-XIA-II, *Gms*PLA$_2$-XIB-I, *Gms*PLA$_2$-XB-II, and *Gms*PLA$_2$-XIB-III. Detailed information about the genes and proteins are shown in **Table 3**. As indicated above, the extension of the N-terminus of the mature protein is crucial for the activity, we show in **Supplementary Figure S5** all the sequences of the sPLA$_2$s of known plants with their signal sequence and their point of theoretical cut using the programs available online.

Moreover, the genes encoding for *Gms*PLA$_2$-XIA-I and *Gms*PLA$_2$-XIB-I are located in chromosome I, *Gms*PLA$_2$-XIA-II y *Gms*PLA$_2$-XIB-II are positioned in chromosome 7 and the gene of *Gms*PLA$_2$-XIB-III is located in chromosome 8. Whereas *Gms*PLA$_2$-XIB-I, *Gms*PLA$_2$-XIB-II, and *Gms*PLA$_2$-XIB-III possess three introns and four exons, the genes of *Gms*PLA$_2$-XIA-I and *Gms*PLA$_2$-XIA-II have two introns and three exons, respectively (see Supplementary Material in Mariani et al., 2012). These facts are indicative that during the course of evolution events of divergence and duplication might have occurred as it was suggested previously for *Ats*PLA$_2$s (Lee et al., 2005).

All sPLA$_2$s sequences found in plants hold a PA2c (SMART accession number SM00085[1]) domain that contains the highly conserved Ca^{2+}-binding loop (YGKYCGxxxxGC) (see **Figure 2**). The active site motif (DACCxxHDxC) that holds the highly conserved HIS/ASP pair (Laigle et al., 1973) corresponds to position 49/50 for *Gms*PLA$_2$-XIA-I, 47/48 for *Gms*PLA$_2$-XIA-II and 62/63 for *Gms*PLA$_2$-XIBs whereas for *Ats*PLA$_2\alpha$ and *Ats*PLA$_2\gamma$, it corresponds to the position 62/63 and 7/48, respectively (Mansfeld et al., 2006).

However, there is a dissimilarity that remains unclear in the HIS/ASP of the catalytic dyad in sPLA$_2$s from plants compared with those found in animals (Mansfeld et al., 2006). It was proposed that water molecules assist in the Ca^{2+} coordination

[1]http://smart.embl-heidelberg.de/

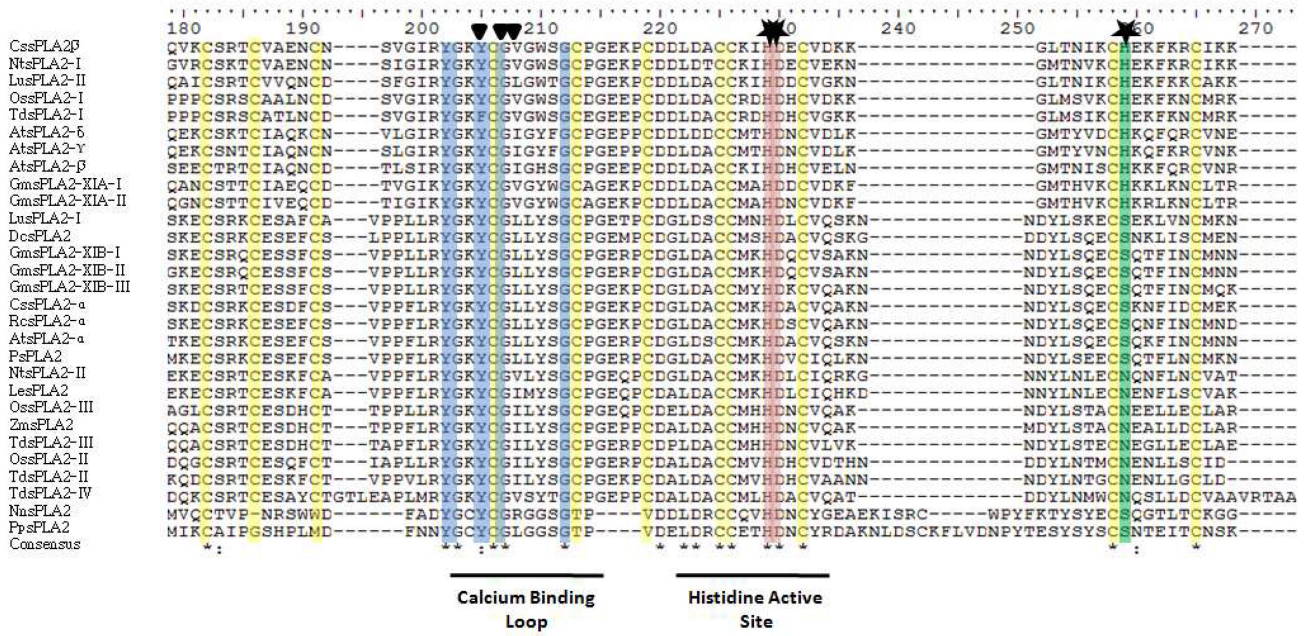

FIGURE 3 | Sequence alignment of the sPLA$_2$s reported from plants: analysis of the deduced amino acid sequences. The alignment was performed by using the Clustal X v2.1 program (http://www.clustal.org) and edited with jalview program. The triangles denote the amino acid residues involved in binding Ca^{2+} and stars denote amino acid residues putatively involved in catalysis. In yellow, CYS residues are marked. For abbreviation code of plants sPLA$_2$ see **Table 2**. Two animals sPLA$_2$ are included at the bottom of the figure for comparison: *Nns*PLA$_2$ from *Naja naja* (cobra) venom and *Pps*PLA$_2$ from pig pancreatic juice (*Sus scrofa*). The accession numbers of sPLA$_2$ indicated in this figure are provided as **Supplementary Appendix S1**.

at the HIS48-ASP49 active site in bovine pancreatic *bps*PLA$_2$ (Bahnson, 2005), the roles of ASP99 in this sPLA$_2$ (Kumar et al., 1994), and ASP64 in bee venom sPLA$_2$ (West et al., 2013) were also claimed to take part in the hydroxyl-imidazole-carboxylate motif (Annand et al., 1996). However, for sPLA$_2$ plant enzymes this important catalytic residue is replaced by an HIS or an ASN residue in enzymes from group XIA and by a SER or an ASN in those enzymes belonging to group XIB (Mansfeld et al., 2006) as shown in the alignment in **Figure 3**. Mansfeld et al. (2006) demonstrated that SER, ASN, or HIS in plant sPLA$_2$s may fulfill the catalytic role assigned to ASP in animal's sPLA$_2$s (Mansfeld et al., 2006). Sequence alignment also reveals that, contrary to *Oss*PLA$_2$ isoforms, the ASP residue of the highly conserved HIS/ASP catalytic dyad of the animal counterpart is replaced by an HIS residue in the durum wheat *Tds*PLA$_2$ isoform I, and by an ASN residue in all of the others durum wheat sPLA$_2$ isoforms (Verlotta et al., 2013), see **Figure 3** for more details of others sPLA$_2$ from plants. Even though the comparison showed low homology among them within the overall amino acid sequences, both the catalytic site and the Ca^{2+} binding loop are highly conserved (**Figure 3**). Other relevant conserved residues within the Ca^{2+} binding loop are the two TYR and two GLY residues which are involved in the hydrogen bonding network reported for both animal and plant sPLA$_2$s (Lee et al., 2005). A more perusal view of this domain offers additional information. The more conserved domain YGKYCG seems not to be exclusive, a change in the second TYR residue was observed for *Tds*PLA$_2$-I changing to YGKFCG. Also, the following hydrophobic domain mainly formed by the LL pair may be VL, IL, IM, VS, IG, or VG (see **Figure 3**). However, the putative role of these differences on calcium affinity or phospholipase activity was not elucidate yet.

The mature proteins of both groups XIA and XIB contain 12 CYS residues (**Figure 3**) known to form six structural disulfide bonds that also are present in the same position as other known sPLA$_2$s from plants (Mansfeld et al., 2006). CYS residues are essential for secreted sPLA$_2$s and it has been shown to play a relevant role in the structural stability in mature sPLA$_2$s (Six and Dennis, 2000; Mariani et al., 2015b).

The HIS residue (at position 49 in *Gms*PLA$_2$-XIA-I, 47 in *Gms*PLA$_2$-XIA-II and at 62 in *Gms*PLA$_2$-XIB-II, -II, and -III) was suggested to play a crucial role in the nucleophilic attack at the *sn-2* bond in the glycerol backbone of phospholipids for all sPLA$_2$s (Six and Dennis, 2000; Berg et al., 2001; Burke and Dennis, 2009).

All plants sPLA$_2$s are low MW enzymes (12–18 kDa) with the exception of *Css*PLA$_2$β from Citrus that has an unexpected high MW (**Table 4**). The theoretical isoelectric points (*pI*) for each sPLA$_2$ are shown also in **Table 4**. As it can be observed, four of the putative *Gms*PLA$_2$s are rather acidic or neutral (*Gms*PLA$_2$-XIA-I, *Gms*PLA$_2$-XIB-I, -II, and -III) as reported for sPLA$_2$s isolated from *Bothrops diporus* venom (de Haas et al., 1968; Daniele et al., 1997). Acidic sPLA$_2$s were also reported for some enzymes found in the Crotalinae subfamily (dos Santos et al., 2011) and those found in rice (isoforms I and III) (Lee et al., 2005).

TABLE 4 | Molecular weight, isoelectric point, and specific activity of different sPLA$_2$s from plants.

Origin	Name	Mature protein MW (kDa)	*pI*	Reported activity (μmol min^{-1} mg^{-1} protein) and substrate	References
A. thaliana	*Ats*PLA$_2$-α	14.2	7.7	16.7 (DOPC)	Lee et al., 2005; Mansfeld and Ulbrich-Hofmann, 2007
	*Ats*PLA$_2$-β	16.3	8.2	0.53 (PC)	Lee et al., 2003, 2005
(arabidopsis)	AtsPLA$_2$-γ	17.5	8.3	NR	Bahn et al., 2003; Lee et al., 2005
	AtsPLA$_2$-δ	18.0	7.7	NR	Lee et al., 2005
D. caryophillus (carnation)	*Dc*sPLA$_2$	12.4	6.9	NR	Lee et al., 2005
R. communis (castor bean)	*Rc*sPLA$_2$α	14	6.3[a]	52.3 pmol min^{-1} mg^{-1} ([^{14}C]18:1-PC)	Bayon et al., 2015
C. sinensis (orange)	*Css*PLA$_2$α	17.1	6.9[a]	0.013[d] arachidonoyl Thio-PC	Domingues et al., 2007; Liao and Burns, 2010
	*Css*PLA$_2$β	31.6	8.1[a]	0.013[d] arachidonoyl Thio-PC	Liao and Burns, 2010
U. glabra (elm)	*Ug*sPLA$_2$	13.9	NR	90 (PCPC)	Stahl et al., 1998; Lee et al., 2005
G. max	*Gm*sPLA$_2$-XIA-I	12.3	6.9	0.44 (DLPC)	Mariani et al., 2012, 2015b
(soybean)	*Gm*sPLA$_2$-XIA-II	12.6	7.4	NR	Mariani et al., 2012, 2015b
	*Gm*sPLA$_2$-XIB-I	13.9	5.7	NR	Mariani et al., 2012, 2015b
	*Gm*sPLA$_2$-XIB-II	13.9	5.7	0.25 (DLPC)	Mariani et al., 2012, 2015b
	*Gm*sPLA$_2$-XIB-III	14	6.8	NR	Mariani et al., 2012, 2015b
L. usitatissimum	*Lus*PLA$_2$-I	17.9	6.7	∼2 (PC$_{LIN}$)	Gupta and Dash, 2017
(flax)	*Lus*PLA$_2$-II	15.7	8.8	∼2.7 (PC$_{LIN}$)	Gupta and Dash, 2017
P. somniferum (opium)	*Ps*sPLA$_2$	14	6.9	∼7 (DOPC)	Jablonicka et al., 2016
O. sativa (rice)	*Oss*PLA$_2$-I[b]	12.9	7.9	145 (*sn1*-palmitoyl-*sn2*-[^{14}C]caproyl-PC)	Stahl et al., 1999; Lee et al., 2005
	*Oss*PLA$_2$-II[b]	13.8	5.5	145 (*sn1*-palmitoyl-*sn2*-[^{14}C]caproyl-PC)	Stahl et al., 1999; Lee et al., 2005; Guy et al., 2009
	*Oss*PLA$_2$-III	13.5	4.8	NR	Lee et al., 2005
N. tabacum	*Nt*1PLA$_2$	17.0	8.57	1.2 (POPC)	Fujikawa et al., 2005
(tobacco)	*Nt*2PLA$_2$	12.7	6.8[a]	NR	Lee et al., 2005
L. esculentum (tomato)	*Les*PLA$_2$	13.9	6.9	NR	Lee et al., 2005
T. durum	*Td*sPLA$_2$s-I	∼14[c]		1.55[d] (PC$_{LIN}$)	Verlotta et al., 2013
(durum wheat)	*Td*sPLA$_2$s-II	∼15.7[c]		1.55[d] (PC$_{LIN}$)	Verlotta et al., 2013
	*Td*sPLA$_2$s-III	∼13.9	4.5[a]	3.2 (PC$_{LIN}$)	Verlotta and Trono, 2014
	*Td*sPLA$_2$s-IV	∼17[c]		1.55[d] (PC$_{LIN}$)	Verlotta et al., 2013
Z. mays (maize)	*Zm*sPLA$_2$	14.3[a]	5.43[a]	NR	This review

[a]*Indicates the pI or MW calculated by using the on line interface https://web.expasy.org/compute_pi/.* [b]*The mixed enzyme activity was determined from an 8-day-old rice shoots extract (Stahl et al., 1999).* [c]*Corresponds to full length sequence (Verlotta et al., 2013).* [d]*The enzyme activity of all isoforms (per gram of dry extract) was determined from a direct orange (Liao and Burns, 2010) and wheat (Verlotta et al., 2013) extract. NR, not reported.*

On the other hand, the expected *pI* of *Gm*sPLA$_2$-XIA-II is slightly alkaline similar to those of all the sPLA$_2$s found in *Arabidopsis* (Lee et al., 2005) and *Papaver somniferum* (Jablonicka et al., 2016); whereas other sPLA$_2$s have a *pI* almost neutral as those found for carnation and tomato (Lee et al., 2005).

The functional role of the diverse *pI*s found in different sPLA$_2$s has not clearly been elucidated yet.

Another relevant domain information is that the enzymes from the different subgroups differ in the third Ca^{2+} coordinating amino acid, being a GLY residue in subgroup XIA or LEU residue in subgroup XIB (see **Figure 3**). The ASP located upstream in the sequence of the common HIS/ASP catalytic dyad found in animal sPLA$_2$s does not correlate in the counterpart found in plants. Instead of this additional ASP residue, the plant enzymes that belong to group XIA contain an HIS residue, and the enzymes belonging to group XIB contain either a SER or an ASP residue (Mansfeld et al., 2006). The functional role of these differences

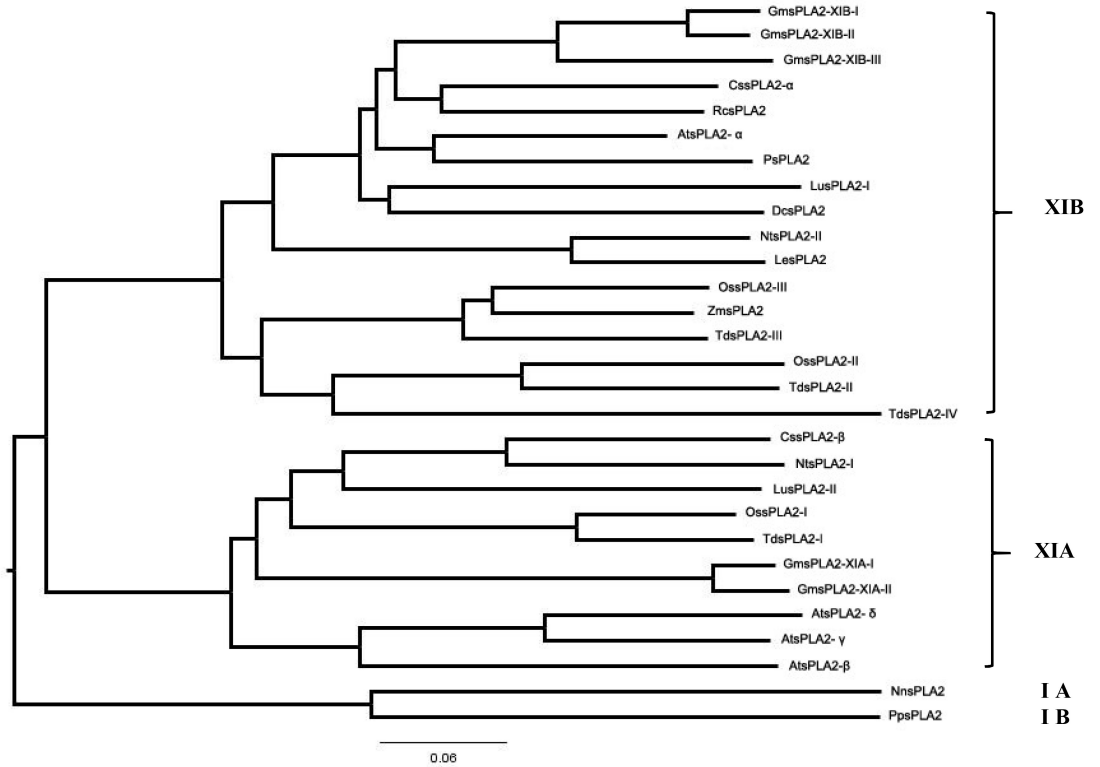

FIGURE 4 | Phylogenetic tree of the deduced amino acid sequences of all reported plant sPLA$_2$s. The program used to perform the sequence alignment was Clustal X (version 2.0) and to produce phylogenetic analysis the phylogenetic tree generation methods from the ClustalW2 package was used (on line tool http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/). To draw the tree we used the Figtree v.1.4.4 program (http://tree.bio.ed.ac.uk/software/figtree/). The accession numbers of sPLA$_2$ indicated in this figure are provided as **Supplementary Appendix S1**.

with regard to the catalytic properties has not been completely elucidated yet.

*Gm*sPLA$_2$s CLASSIFICATION IN THE sPLA$_2$ SUPERFAMILY

Secretory phospholipases in plants superfamily is composed of multiple members represented by multiple isoforms distinguishable by their structural, catalytic and physiological characteristics. sPLA$_2$ are within the most populated group of PLA$_2$ in nature which in turn is classified into 15 subgroups (Six and Dennis, 2000). In this context, the plant sPLA$_2$s were classified into a separate group (group XI) (Meneghetti and Maggio, 2013), which, in turn, could be subdivided into two categories named XIA and XIB because of differences in MW and deviating sequences in the N- and C-terminal regions of the mature enzyme (Six and Dennis, 2000).

Figure 4 shows the phylogenetic classification into the two subgroups of all the sPLA$_2$s from plants known until now. This way, *Gm*sPLA$_2$-XIA-I and -II are taking part of group XIA, which includes *At*sPLA$_2$-γ, *At*sPLA$_2$-β, *At*sPLA$_2$-δ, *O. sativa* isoform I, *N. tabacum* isoform I, *T. durum* isoform I, *C. sinensis* isoform β, and *L. usitatissimum* isoform II. Whereas two of the enzymes of *G. max* correspond to the subgroup XIA, three are grouped in the subgroup XIB (Mariani et al., 2012) named as *Gm*sPLA$_2$-XIB-I, -II, and -III together with *At*sPLA$_2$-α, *O. sativa*-II, -III, and -IV, *D. caryophillus*, *N. tabacum* isoform II, *Z. maize*, *R. communis* isoform α, *P. somniferum*, *T. durum*-II, -III, and -IV, *L. esculentum*, *C. sinensis* isoform α, and *L. usitatissimum* isoform I.

The data show a close evolutionary relationship among all sPLA$_2$s from plants (see **Figure 4**). The highest level of similarity in amino acid sequences was observed between *Gm*sPLA$_2$-XIA-I and *Gm*sPLA$_2$-XIA-II, being of 95.5%, whereas between *Gm*sPLA$_2$-XIB-I and *Gm*sPLA$_2$-XIB-II the level of similarity is of 94.5% (Mariani et al., 2012) (see **Supplementary Figure S2**). Moreover, between *Le*sPLA$_2$ and *Nt*sPLA$_2$-II the level of similarity is of 90.4% and between *Td*sPLA$_2$-I and *Os*sPLA$_2$-I, *Gm*sPLA$_2$-II and *Gm*sPLA$_2$-III, *Ps*PLA$_2$ and *Rc*sPLA$_2$-α and *At*sPLA$_2$-δ and *At*sPLA$_2$-γ the levels of similarity are of 89.9, 87.3, 83.7, and 82.7%, respectively.

TRIDIMENSIONAL STRUCTURE

Although the sPLA$_2$ sequences from different sources differ significantly, the tridimensional structures have many features

in common. There are more than 40 sPLA$_2$s entries in the Protein Data Bank (PDB[2]) from all sources. Native and complex structures of sPLA$_2$s simulated with mimic substrate have helped to identify the catalytically important residues involved in the active site (Pan et al., 2002).

The tridimensional structure of many sPLA$_2$s, such as porcine pancreas or bee venom (Dijkstra et al., 1984; Scott et al., 1990), has been elucidated by X-ray crystallography which revealed a common, rigid and highly conserved region with a similar tridimensional architecture compared with those from plants. The active site is not directly accessible to the aqueous phase and is within a rather local hydrophobic environment denoted as "*i-face*" that allows the interaction with the substrate in its monomeric form (Dijkstra et al., 1981). The putative residues involved in the "*i-face*" of some sPLA$_2$ from plants are shown in **Table 5**.

One of the first sPLA$_2$ "*i-face*" identified was for the secreted pig pancreatic enzyme (Bai et al., 2008). In *Gm*sPLA$_2$-XIA-I the residues found in the putative "*i-face*" are VAL18, GLY19, VAL28, HIS49, HIS64, LEU101, ALA102, ILE103, LEU104, LEU105, and LEU108. **Table 5** shows the putative amino acids proposed to be in contact with the membrane for different sPLA$_2$s enzymes.

The binding of sPLA$_2$ to the membrane is energetically favorable (**Table 6**) and, keeping in mind that most of the residues situated in the *i-face* are hydrophobic, the overall domain constitutes a hydrophobic environment that surrounds the active catalytic site. Hydrophobic side chains of the residues forming the "*i-face*" would be able to partition to the hydrophobic core, which allows the anchoring of the enzyme to the membrane, excluding water molecules in the region surrounding the active site and the diffusion of the substrate to the pocket of the active site to be hydrolyzed. The general molecular conformation proposed for plants sPLA$_2$ is in agreement with the general vision proposed for secreted phospholipases of animal source (Scott et al., 1990).

Physically, the soluble sPLA$_2$ protein must penetrate the phospholipid interface to exert its action. Therefore, the successful binding surface is located where the substrate is a prerequisite in the catalytic cycle, and this property can determine some specific characteristics of the enzyme activity. However, there are a limited number of charged residues in the flat topography of the "*i-face*" (see **Table 5**) that could modulate further interaction with the interface of the substrate in a way which has not been fully elucidated yet (Jain and Berg, 2006).

It is important to note that even when the energetic to membrane binding is favorable according to the available on-line calculation program (Lomize et al., 2012) used for some sPLA$_2$s, the residues involved in the "*i-face*" differ for the same enzyme if a different approach is used instead (compare **Tables 5**, **6**).

To date, only few structures corresponding to sPLA$_2$s from plants were reported in the PDB or in the Protein Model Database (PMDB[3]) and correspond to *O. sativa* (rice) isoform II (PBD 2WG7), which belongs to the group XIB, and its tertiary structure was recently determined by X-ray crystallography to 2.0 Å resolution (Guy et al., 2009). Moreover, homology modeling and molecular dynamics were used to elucidate the structure of sPLA$_2$ isoform α from *Arabidopsis* (Mansfeld et al., 2006) but its PDB is not available. The predicted models of *Lus*PLA$_2$s proteins were elucidated and submitted to PMDB identified as PM0080416 (*Lus*PLA$_2$-I) and PM0080415 (*Lus*PLA2-II) (Gupta and Dash, 2017). The structure of *Gm*sPLA$_2$-XIA-I was modeled by using homology modeling and molecular dynamics (Mariani et al., 2012) and also *Gm*sPLA$_2$-XIB-II by using a similar methodology (see **Figure 5**, modeled structures in PDB format were not uploaded in the PDB). The data corresponding to *Pig pancreatic* (*Sus scrofa*), *Naja naja* (Indian cobra), *Naja sagittifera* (Andaman cobra venom) are also indicated in **Table 6** for comparison in order to include sPLA$_2$ able to hydrolyze aggregate lipids structured in a high packing organization, as it occurs with sPLA$_2$ from cobra venom, or only at low packing as it certainly happens with sPLA$_2$ from pig pancreas (see below and **Table 10**).

The structure of rice sPLA$_2$ shows that the half N-terminal chain contains mainly structured loops, including the conserved calcium binding loop domain together with two short anti-parallel β-strands. The half C-terminal is folded into three anti-parallel α-helix, in which two of them are highly conserved among others sPLA$_2$s, containing the crucial catalytic HIS residue and the calcium binding/coordinating ASP residues (Guy et al., 2009). This overall general folded conformation seems to be shared by almost all known sPLA$_2$ from plants. The complete putative mature structure of *Gm*sPLA$_2$-XIA-I protein was reported using homology modeling and molecular dynamics simulations (Mariani et al., 2012). The most mobile regions are the N- and C-terminal, followed by the loops in residues 74–85, 53–62, 34–37 that connect, respectively, the last two helices, the first with the second helix, and the last beta-sheet with the first helix (see **Figure 5**). As other sPLA$_2$s in the family, the dominant secondary structure is the α-helix, with only a small portion of beta sheet with abundant regions containing turns and bends. The observations indicate that the terminal helix is rather a dynamic region and has three principal conformations: one fully helical, other with the last seven residues in coil, and the third one with a kink plus coil (Mariani et al., 2012). As noted before, this behavior can be attributed to a low number of hydrophobic

[2]https://www.rcsb.org/

[3]http://srv00.recas.ba.infn.it/PMDB/main.php

TABLE 5 | Reported and proposed amino acid residues involved in the "*i-face*" of several sPLA$_2$.

sPLA$_2$ name	Proposed amino acids in the *i-face*	References
*Pp*sPLA$_2$ (Group IB)	L^2, W^3, R^6, L^{19}, M^{20}, L^{31}, and Y^{69}	Kuipers et al., 1991
*Bp*sPLA$_2$	L^2, W^3, F^5, I^9, F^{22}, $L^{31,63-65}$, and Y^{69}	Yu et al., 1999b
*Gm*sPLA$_2$-XIA-I	V^{18}, G^{19}, V^{28}, H^{49}, H^{64}, L^{101}, A^{102}, I^{103}, L^{104}, L^{105}, and L^{108}	Mariani et al., 2012
*Gm*sPLA$_2$-XIB-II	F^{25}, S^{27}, L^{31}, V^{112}, A^{116}, L^{119}, V^{123}, L^{124}, and P^{127}	Obtained by using the on-line platform OPM (Lomize et al., 2012)
*Os*sPLA$_2$-II	A^{29}, P^{30}, V^{65}, Y^{72}, and L^{41}	Guy et al., 2009

Pp, P. pancreas; Bp, B. pancreas; Gm, G. max; Os, O. sativa.

TABLE 6 | Orientation of different sPLA$_2$s at the membrane interface.

Protein	Depth/hydrophobic thickness	$\Delta G_{transfer}$ (kcal/mol)	Tilt angle	Embedded residues
*Gms*PLA$_2$-XIB-II[a]	2.8 ± 0.9 Å	−10.4	71 ± 4°	F^{25}, S^{27}, L^{31}, V^{112}, A^{116}, L^{119}, V^{123}, L^{124}, and P^{127}
*Gms*PLA$_2$-XIA-I[b]	1.0 ± 2.8 Å	−1.0	69 ± 20°	P^{114}
*Oss*PLA$_2$-II[c]	4.1 ± 0.5 Å	−10.6	71 ± 2°	G^{3}, L^{6}, A^{25}, L^{28}, Y^{30}, G^{31}, I^{116}, R^{120}, and D^{121}
*Lus*PLA$_2$I[d]	4.1 ± 0.6 Å	−7.1	86 ± 3°	F^{27}, A^{29}, V^{30}, P^{32}, and L^{33}
*Lus*PLA$_2$II[d]	1.9 ± 1.2 Å	−5.5	86 ± 26°	F^{24} and L^{102}
Naja sagittifera[e] *Nss*PLA$_2$	1.6 ± 0.4 Å	−4.3	85 ± 2°	D^{20} and K^{65}
Naja naja[f] *Nss*PLA$_2$	3.6 ± 0.3 Å	−6.7	86 ± 2°	Y^{3}, W^{19}, W^{61}, and F^{64}
Pig pancreatic[g] *Pps*PLA$_2$	2.4 ± 2.5 Å	−1.9	32 ± 16°	L^{64}

[a]*Structure were modeled in a similar way than that described for GmsPLA$_2$-XIA-I in Mariani et al. (2012), the PMDB accession number is PM0082160.* [b]*Structure modeled in Mariani et al. (2012), the PMDB accession number is PM0082161.* [c]*Obtained from PBD 2WG7.* [d]*PMDB identified as PM0080416 (LusPLA$_2$-I) and PM0080415 (LusPLA$_2$-II).* [e]*Crystal structure of Naja sagittifera was reported in Jabeen et al. (2005). PDB 1MH8.* [f]*Naja naja NnsPLA$_2$. PDB 1A3D.* [g]*Pig pancreatic PpsPLA$_2$. PDB 1PIR.*

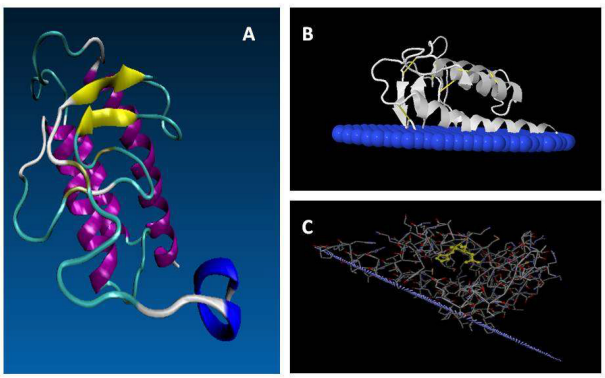

FIGURE 5 | Putative mature structure of GmsPLA$_2$-XIA-I. **(A)** Proposed structure from homology modeling of GmsPLA2-XIA-I (Mariani et al., 2012). Yellow: beta sheet strand; magenta: alpha-helix; blue: C-terminal; cyan: turns; white: coils. **(B,C)** Molecular simulation of the interaction between *Gms*PLA$_2$-XIB-II with the membrane interface, simulated with the OPM (Orientation of Proteins in Membrane) database online service (opm.phar.umich.edu/; see Lomize et al., 2012). In panel **(B)** blue: interfacial membrane; white: protein. **(C)** Light purple represents the interfacial membrane; the sticks denote the protein amino acids with the H/D dyad highlighted in yellow.

contacts of this region, a high aqueous exposed area and the presence of a highly flexible GLY98 residue (Mariani et al., 2012).

The active site of the sPLA$_2$ protein contains a crucial calcium ion cofactor commonly present in other plant sPLA$_2$s (Mansfeld et al., 2006; Guy et al., 2009) that is important in the catalytic mechanism and is a requisite for full enzyme activity. The HIS-ASP pair constitutes the active center and the calcium binding loop (see **Figure 3**) is essential for the proper function of the enzyme (Scott et al., 1990). All sPLA$_2$s catalyze the hydrolysis through the same mechanism: an abstraction of a proton from a water molecule followed by a nucleophilic attack on the *sn-2* bond position of the diacylglycerophospholipids (Jorgensen et al., 1983; Berg et al., 2001). NMR structural studies of porcine pancreas sPLA$_2$ show that the N-terminus is flexible with no defined structure in solution, unlike what it was evidenced by crystallography. It was hypothesized that this flexibility in solution would be related to the near null activity against monomeric substrate form [more unstructured unbound state (van den Berg et al., 1995)].

ENZYMATIC PROPERTIES OF PLANTS sPLA$_2$s

Optimum Conditions for Plants sPLA$_2$s Catalysis

The sPLA$_2$s from *N. tabacum* and elm have optimum pH in the range of 8–10 and 8–9, respectively (Stahl et al., 1999; Fujikawa et al., 2005). In *Arabidopsis* the optimum pH ranges for the activities are pH 6–11, 6–7, 7–9, and 8–9 for *At*sPLA2-α, -β, -γ, and -δ, respectively (Lee et al., 2005). Nevertheless, a similar situation was found for almost all the sPLA$_2$s found in plants or animals. The pH optimum was at around 7 for *Gms*PLA$_2$-XIA-I and -XIB-II (see **Table 7**), when using mixed micelles of DLPC:Triton X-100 as substrate in presence of calcium 10 mM. The optimum pH for pancreatic sPLA$_2$ was reported to be 8 (de Haas et al., 1968; Fujikawa et al., 2005) similar to that reported for bee venom (Daniele et al., 1997). For human non-pancreatic PLA$_2$ optimum pH is in between 8 and 10 (Kramer et al., 1989). However, it should be mentioned that different substrates (including different aggregation presentation of substrate) have been used to determine optimum pH for the different sPLA$_2$s reported in the literature.

Only few sPLA$_2$s were investigated about the optimum temperature and stability. *Gms*PLA$_2$s-XIA-I and -XIB-II demonstrate to be very stable when increasing the temperature (Mariani et al., 2015b) as previously determined by using an sPLA$_2$ homogenate (Minchiotti et al., 2008). This proves that these enzymes are highly resistant to temperature denaturation due in part to the disulfide bridges that are postulated to be involved in the stability of sPLA$_2$s (Berg et al., 2009; Murakami et al., 2010). **Table 7** shows the optimal temperature reported for several sPLA$_s$ from plants.

The optimum calcium concentrations for activity of *Gms*PLA$_2$-XIA-I and -XIB-II are in the micromolar range using DLPC:Triton X-100 mixed micelles as substrates (**Table 7**). This micromolar calcium requirement is rather unusual for sPLA$_2$s enzymes that mostly possess millimolar requirement

TABLE 7 | Optimum requirements deduced for catalytic activity of the different sPLA$_2$s found in plants.

Source	Name	pH	Calcium requirement*	T (°C)	References
A. thaliana	*At*sPLA$_2$-α	6–11	mM	30–40	Lee et al., 2005; Mansfeld et al., 2006; Mansfeld and Ulbrich-Hofmann, 2007
	*At*sPLA$_2$-β	6–7	>0.5 mM	30	Lee et al., 2003, 2005
(arabidopsis)	*At*sPLA$_2$-γ	7–9	>0.5 mM	30	Bahn et al., 2003; Lee et al., 2005
	*At*sPLA$_2$-δ	8–9	>0.5 mM	NR	Lee et al., 2005
R. communis (castor bean)	*Rc*sPLA$_2$α	8	10 mM	30	Bayon et al., 2015
C. sinensis	*Css*PLA$_2$α	7.4	10 mM	25	Liao and Burns, 2010
(orange)	*Css*PLA$_2$β	7.4	10 mM	NR	Liao and Burns, 2010
D. caryophillus (carnation)	*Dc*sPLA$_2$	NR	NR	NR	–
U. glabra (elm)	*Elm*sPLA$_2$	8–9	10–15 mM	30	Stahl et al., 1998
G. max (soybean)	*Gm*sPLA$_2$-XIA-I	6–7	>1 mM	40–60	Mariani et al., 2015b
	*Gm*sPLA$_2$-XIA-II	NR	NR	NR	–
	*Gm*sPLA$_2$-XIB-I	NR	NR	NR	–
	*Gm*sPLA$_2$-XIB-II	6–7	>1 mM	40–60	Mariani et al., 2015b
	*Gm*sPLA$_2$-XIB-III	NR	NR	NR	–
L. usitatissimum (flax)	*Lus*PLA$_2$-I	9	1 mM	NR	Gupta and Dash, 2017
	*Lus*PLA$_2$-II	9	1 mM	NR	Gupta and Dash, 2017
P. somniferum (opium)	*Ps*PLA$_2$	7	NR	37	Jablonicka et al., 2016
O. sativa (rice)	*Oss*PLA$_2$-I	8	10 mM	30	Stahl et al., 1999; Guy et al., 2009
	*Oss*PLA$_2$-II	8	10 mM	30	Stahl et al., 1999; Guy et al., 2009
	*Oss*PLA$_2$-III	NR	NR	NR	–
N. tabacum (tobacco)	*Nt*1sPLA$_2$	8–10	<1 mM	37	Fujikawa et al., 2005, 2011
	*Nt*2sPLA$_2$	–	–	–	NR
L. esculentum (tomato)	*Les*PLA$_2$	NR	NR	NR	NR
T. durum	*Td*sPLA$_2$-I	9	>2 mM	25	Verlotta et al., 2013
(durum wheat)	*Td*sPLA$_2$-II	9	>2 mM	25	Verlotta et al., 2013
	*Td*sPLA$_2$-III	9	1 mM	25	Verlotta et al., 2013; Verlotta and Trono, 2014
	*Td*sPLA$_2$-IV	9	>2 mM	25	Verlotta et al., 2013
Z. mays (maize)	*Zm*sPLA$_2$	–	–	–	NR

**For Arabidopsis sPLA$_2$s-β, -γ, and -δ, a μM requirement was reported without specifying the precise concentration. NR, not reported.*

(Six and Dennis, 2000). Moreover, the same behavior was observed for the activities of *At*sPLA$_2$-β, -γ, and -δ (Lee et al., 2005). It is important to remark that none of these secreted enzymes (either from animals or plants) exhibit activity in absence of calcium. Particularly, for *At*sPLA$_2$-α the activity augmented as the calcium concentration increased up to 10 mM and for elm sPLA$_2$ the range of calcium concentration for optimal activity was around 10–15 mM $CaCl_2$ (Stahl et al., 1998; Lee et al., 2005; Mansfeld and Ulbrich-Hofmann, 2007). However, to achieve 50% of maximal enzyme activity a concentration of 0.5 mM $CaCl_2$ was sufficient, at least, for these two latter enzymes. The maximal activity for sPLA$_2$ from *N. tabacum* was detected above 1 mM $CaCl_2$. This behavior is similar to that observed for the most animal sPLA$_2$s, which require millimolar concentrations of Ca^{2+} and have no activity in the absence of this cation (Six and Dennis, 2000; Fujikawa et al., 2005). Even when it is evident the molecular differences among the enzymes in the sPLA$_2$ family, the absolute requirement of Ca^{2+} for hydrolysis is indicative that all of them share a common mechanism for lipid hydrolysis. For durum wheat sPLA$_2$ the activity continuously increased as Ca^{2+} concentration increased with a plateau close to 2–4 mM $CaCl_2$, even though a 300 μM $CaCl_2$ was sufficient to reach 50% of the maximal activity (Verlotta et al., 2013) (see **Table 7**).

The differences in the activity reported from many authors for plant sPLA$_2$s is not easy to compare in absolute terms. Usually the reported activity values are informed as specific activities (μmol of hydrolyzed lipid.min^{-1}.mg of protein^{-1}) and this quantity may be affected by many factors. Among the main factors that can affect the sPLA$_2$ activity can be mentioned (i) inherent deficiencies in the folding of recombinant enzymes, (ii) additional tags at the N-terminus, and (iii) the lack of standardization of substrate offered to the enzyme (lipid monolayers, micelles,

SUVs, presence of detergents mixed with the lipid substrate, etc.). However, taking into account these precautions the activity of the reported enzymes could be compared although different substrates and systems were used in the assays (see **Table 4**).

Conformational Stability of sPLA$_2$s

It is known that CYS residues are essential for the structural stability of sPLA$_2$ and it has been shown to play an important role in the structural stability of the mature enzyme (Six and Dennis, 2000; Welker et al., 2011). In animals, sPLA$_2$s contain between 10 and 16 CYS that have the potential to form 5–8 intramolecular disulfide bridges (Schaloske and Dennis, 2006). In contrast, all sPLA$_2$s reported from plants have 12 CYS that can form 6 disulfide bridges (see **Table 1**). It is known that some sPLA$_2$ from animals (especially type I and II), are rather stable upon heating compared with cytoplasmic cPLA$_2$ (Mazereeuw-Hautier et al., 2000). Resistance to heating for sPLA$_2$ from plants was reported for some enzymes indicating a similar behavior to that observed for animal sPLA$_2$. The structural stability for durum wheat sPLA$_2$ was demonstrated by the resistance to high temperatures (87% of the activity was retained after treatment of the crude leaf extract at 100°C for 15 min), see (Verlotta et al., 2013). Recombinant *At*sPLA$_2\alpha$ and *At*sPLA$_2\beta$ retained 80–95% of their activities following 5 min treatment in boiling water (Lee et al., 2005), and a similar result was obtained for sPLA2 purified from elm seeds (Stahl et al., 1998). Moreover, for *Gm*sPLA$_2$-XIA-I and *Gm*sPLA$_2$-XIB-II preserved the activity after heating 5 min at 80°C (Mariani et al., 2015b).

The main reason for the scarceness of information on recombinant plant sPLA$_2$s may be attributed to the low expression yields obtained with the different protocols currently used and the strong propensity of the recombinant enzymes to aggregate (Mansfeld et al., 2006). The generally lower yields of the purified enzymes from inclusion bodies might be an indication for a higher fraction of misfolded and/or aggregated protein after the renaturation process. This may be the reason of different V_{max} or specific activity values obtained when studying kinetic parameters in sPLA$_2$ recombinant enzymes from plants (see **Table 9**).

In bee venom sPLA$_2$ (*Bv*sPLA$_2$), it was reported that the formation of disulfide bonds is not essential for correct re-folding of the protein and an active enzyme form can be reobtained even from the completely denatured and reduced state (Welker et al., 2011). It is known that, in contrast to the seven disulfide bonds present in porcine pancreas enzyme (*Pp*sPLA$_2$), all five disulfide bonds of *Bv*sPLA$_2$ are essential for conformational stability and contribute to the activity (Welker et al., 2011). In the case of bacterial sPLA$_2$ from *Streptomyces violaceoruber*, it possesses only two disulfide bridges (Sugiyama et al., 2002) which were sufficient to be active comparable to animal or plant sPLA$_2$s (Yunes Quartino et al., 2015).

In sPLA$_2$ from *A. thaliana*, the removal of disulfide bonds increased the proteolytic susceptibility of the native proteins whereas the stability decreased (Mansfeld et al., 2014). Regarding *Gm*sPLA$_2$s, it was demonstrated that the calcium ion also contributes to keep the protein folded in its native structure. This effect was observed by two independent assays using dynamic simulations and intrinsic fluorescence experiments (Mariani et al., 2012, 2015b).

The comparison of the data obtained on *bovine pancreatic* sPLA$_2$, *bee venom* sPLA$_2$, and *porcine pancreatic* sPLA$_2$ with those obtained on sPLA$_2$s from plants suggests that conserved disulfide bonds in those homologous proteins are important to keep the conformational architecture and stability. However, with the recompiled information, it is almost clear that not all the disulfide bridges are needed for the protein to be active, but are necessary for a protein correct folding.

INTERFACIAL CATALYSIS ACTIVATION

Phospholipids are constituents of biological membranes, so a very important prerequisite step to perform the lipolytic action of sPLA$_2$ is the interaction with the amphipathic nature of these interfaces; and in turn, determine the catalytic properties of the organized substrate (Jain and Berg, 2006). The interfacial binding step is crucial for enzymatic action of sPLA$_2$, and it is mediated by a region of the protein often referred to as *i-face* (see above), also reported as *IRS, the interfacial recognition site* (Tatulian, 2001). The *i-face* or IRS is not a proper "site" or a flat face, it is rather a 3D domain with the confluence of several residues that crowns and precedes the catalytic site, giving an adequate environment for the catalysis, and also help keeping the enzyme attached to the membrane where the hydrolytic reaction takes place. The proper intimate contact of the *i-face* of sPLA$_2$s with the interface is essential to provide the substrate access to the active site. Interfacial activation is a concept that means an adequate contact between the catalytic active site and the *i-face* modulating the catalytic activity (Scott et al., 1990; Tatulian et al., 2005; Jain and Berg, 2006; Winget et al., 2006). The binding and kinetic characteristics of interfacial catalysis by sPLA$_2$ depend upon the organization and dynamics of the interface. The overall rate of catalytic turnover is not only determined by the kinetics at the interface, but also by the *binding/desorption equilibrium kinetics* of the enzyme with the interface (Ramirez and Jain, 1991). Hence, the hydrolysis of the organized substrate can occur in two extreme distinct modes: (i) in the *scooting mode* of catalysis, that requires that the enzyme remains bound at the interface between several catalytic turnover cycles and, (ii) in the pure *hopping mode*, where the binding and the desorption of the bound enzyme occur during each catalytic turnover cycle leading to a jumping mechanism (Jain et al., 2009) (see **Supplementary Figure S4** for more details and a schematic description of both mechanism of lipids hydrolysis induced by sPLA$_2$).

A few mode of interfacial catalysis for sPLA$_2$s has been reported. Moreover, in plants, we were the only in studying the catalytic mode till today. The enzyme studied in order to determine the mode of catalysis was the sPLA$_2$ from *G. max* (*Gm*sPLA$_2$-XIA-I) (Mariani et al., 2015b). Whereas pancreatic sPLA$_2$ presents a scooting mode of catalysis when using anionic lipids (Berg et al., 1991), it presents a hopping mode of catalysis if the specific experimental conditions are changed to

zwitterionic lipids (Scott et al., 1994). In our hands, *Gm*sPLA$_2$-XIA-I acts in the hopping mode against zwitterionic lipids (Mariani et al., 2015b).

Hydrolysis Using Micelles as Substrate Membrane Model System

There have been some reports in the literature regarding sPLA$_2$ activity against different substrates and in different conditions. For some sPLA$_2$, it has been demonstrated that the hydrolysis rate is sensitive to the surface charge density of the lipid aggregates (Volwerk et al., 1986). Several kinetics studies on pancreatic as well as snake venoms and plants phospholipases have been reported in which lipid phase transition, lipid membrane curvature, and composition may modulate the lipolysis (Wilschut et al., 1978; Bell and Biltonen, 1989; Bell et al., 1996; Leidy et al., 2004). However, it should not be forgotten, that sPLA$_2$ has optimum of lipid packing for hydrolysis, i.e., that some enzymes have the ability to hydrolyze lipid in a low packing organization (low lateral pressure in lipid monolayers more compatible with micelles) but others also have optimum condition of hydrolysis at high lateral pressure in monolayers compatible with liposomes or biological membranes (Ramirez and Jain, 1991; Yunes Quartino et al., 2015).

Usually, short-chain zwitterionic phospholipids have been employed as substrates in single component systems (de Haas et al., 1971; Wells, 1972) or, for the case of long-chain phospholipids, they were mixed with neutral detergents (Dennis, 1973b; Yu et al., 1999a; Mansfeld and Ulbrich-Hofmann, 2007). Moreover, the activity is generally increased when the lipid substrate forms mixed micelles in presence of detergents (Dennis, 1973a; Dennis et al., 1981). The effect of enzyme immobilization on the sPLA$_2$ kinetics was also reported (Madoery et al., 1999). Description and kinetics properties of sPLA$_2$ from plants have been more frequent in their recombinant counterpart after appropriate expression, purification, and folding protocols (Bahn et al., 2003; Ryu et al., 2003; Fujikawa et al., 2005; Mansfeld and Ulbrich-Hofmann, 2007; Mariani et al., 2012) compared with their equivalent found in animals sPLA$_2$s. The reason of this is due to the relative high amounts of the latter proteins found in their respective natural sources (venoms and pancreatic juice) and, therefore it allows an efficient purification of the mature forms of sPLA$_2$. However, few studies using purified plant enzymes were reported from elm seeds (Stahl et al., 1998) and of *G. max* (Minchiotti et al., 2008).

Mammalian and plant enzymes differed in head group specificity. While some mammalian sPLA$_2$s show high activity on anionic phospholipids (Ghomashchi et al., 1991; Bezzine et al., 2002), sPLA$_2$s from plants preferred zwitterionic phospholipids (Mansfeld and Ulbrich-Hofmann, 2007; Mansfeld, 2009; Mariani et al., 2015b). In **Table 8** we summarize the substrate lipid preference (head group or acyl chain) differences observed in some sPLA$_2$s from plants reported in the literature (see also **Table 9** additional kinetic data).

Table 9 shows the K_m and V_{max} values determined and reported for sPLA$_2$s from different sources. As shown, we can infer that the values of V_{max} could be sensitive to both the lipid substrate used in the assays and the interfacial quality of the surface in which the substrate is inserted.

Phospholipid Hydrolysis Using Langmuir Monolayers as Membrane Model System

The influence of substrate lipid packing on sPLA$_2$s activities was studied for numerous authors using Langmuir-lipid monolayers performed at different surface pressures using almost exclusively sPLA$_2$ from animal sources (Yunes Quartino et al., 2015). Moreover, to study the catalytic activity at the air-water interface the lipid monolayer technique in the "zero order" regime was used since the surface pressure is kept constant during the reaction (Panaiotov and Verger, 2000; Yunes Quartino et al., 2012) (see **Supplementary Figure S3** for a schematic representation of this experimental system).

The optimum surface pressure of these enzymes to hydrolyze the lipids of the membranes differed with the origin of the sPLA$_2$ (Ramirez and Jain, 1991; Mariani et al., 2015b; Yunes Quartino et al., 2015). *Gm*sPLA$_2$s were the first sPLA$_2$s from plants to be studied with respect to their interfacial characteristics. **Table 10** shows the optimum pressure determined for different sPLA$_2$s. The optimum for plants *Gm*sPLA$_2$s seems to fall intermediate in between the values of "pancreatic like" enzymes that have high activity against micelles structured lipids rather bilayers (lipolytic ratio lower than 0.1) compared with toxic venom sPLA$_2$s (lipolytic ratio higher than 1) that can hydrolyze intact cell membranes such as erythrocytes (Demel et al., 1975). Then, it may be concluded that sPLA$_2$s from plants would have a more ubiquitous functionality, since they can be active *in vitro* against a rather wide range of curvature radio of structured lipid substrates (less sensitivity to the supramolecular organization).

Auxin Effect Over sPLA$_2$ Activity

Studies of plant sPLA$_2$s demonstrated that auxins play important roles in signal transduction regulating cellular processes and probably they are implicated in phospholipid signaling (Wang, 2001; Ryu et al., 2005; Scherer et al., 2010). At the cellular level, auxins control cell division, growth, extension, and differentiation (Davies, 1995). At the whole plant level, auxins play an essential role in processes such as apical dominance, lateral/adventitious root formation, tropisms, fruit set and development, vascular differentiation, and embryogenesis (Friml, 2003). A rapid increase in sPLA$_2$ activity was first verified by treating isolated microsomes and cell cultures with auxins (Scherer and Andre, 1989; Scherer, 1990; Andre and Scherer, 1991; Scherer, 1992; Scherer and Andre, 1993; Scherer, 1996) and microsomes isolated from hypocotyls segments (Blanchet et al., 2008b). However, as the molecular mechanism of the putative effect of auxins over sPLA$_2$s is unknown we have investigated whether these phytohormone have any direct effect over the enzyme by using simple *in vitro* assays.

Secretory phospholipases, like other lipolytic enzymes, are interfacial active proteins, since they access from water to the interface of the insoluble organized substrate to carry

TABLE 8 | Substrate preference of different $sPLA_2$s from plants.

Origin	Name	*sn*-specificity	Fatty acid preference	Head group selectivity	References
A. thaliana (arabidopsis)	*At*sPLA_2-α *At*sPLA_2-β	*sn-2 sn-2*	Linoleic Palmitic>linoleic	PC>PE (baja)>PG>PI PE (low)	Lee et al., 2005; Mansfeld and Ulbrich-Hofmann, 2007; Lee et al., 2005
	*At*sPLA_2-γ	*sn-2*	Linoleic>palmitic-oleic	PE (high)	Bahn et al., 2003; Lee et al., 2005
	*At*sPLA_2-δ	*sn-2*	Palmitic-oleic>linoleic	PE (high)	Bahn et al., 2003; Lee et al., 2005
D. caryophillus (carnation)	*Dc*sPLA_2	NR	NR	NR	NR
R. communis (castor bean)	*Rc*sPLA2α	*sn-2*	Palmitic>ricinoleic	PC	Bayon et al., 2015
C. sinensis (orange)	*Cs*sPLA2α	*Deducted sn-2*	NR	NR	Liao and Burns, 2010
	*Cs*sPLA2β	*Deducted sn-2*	NR	NR	
U. glabra (elm)	*Ug*sPLA_2	*sn-2*	Oleic (C8–C12)	NR	Stahl et al., 1998; Lee et al., 2005
G. max	*Gm*sPLA_2-XIA-I	*sn-2*	Lauroil	PC	Mariani et al., 2015b
(soybean)	*Gm*sPLA_2-XIA-II	*Deducted sn-2*	NR	NR	Mariani et al., 2015b
	*Gm*sPLA_2-XIB-I	*Deducted sn-2*	NR	NR	Mariani et al., 2015b
	*Gm*sPLA_2-XIB-II	*sn-2*	Lauroil	PC	Mariani et al., 2015b
	*Gm*sPLA_2-XIB-III	*Deducted sn-2*	NR	NR	Mariani et al., 2015b
L. usitatissimum	*Lu*sPLA_2-I	*Deducted sn-2*	NR	NR	Gupta and Dash, 2017
(flax)	*Lu*sPLA_2-II	*Deducted sn-2*	NR	NR	Gupta and Dash, 2017
P. somniferum (opium)	*Ps*sPLA_2	*sn-2*	Linolenic	PC>PE	Jablonicka et al., 2016
O. sativa (rice)	*Os*sPLA_2-I	*sn-2*	NR	PC	Stahl et al., 1999;
	*Os*sPLA2-II	*sn-2*	NR	PC	Lee et al., 2005
N. tabacum	*Nt*1sPLA_2	*sn-1/sn-2*	NR	PC	Fujikawa et al., 2011
(tobacco)	*Nt*2sPLA_2	*sn-2*	NR	PC	Fujikawa et al., 2011
L. esculentum (tomato)	*Le*sPLA_2	*sn-2*	NR	PC	Narvaez-Vasquez et al., 1999
T. durum	TdsPLA_2-I	*sn-2*	*Non-specified*	PC	Verlotta et al., 2013
	TdsPLA_2-II	*sn-2*	*Non-specified*	PC	
(durum wheat)	TdsPLA_2-III	*sn-2*	Palmitic	PC	Verlotta and Trono, 2014
	TdsPLA_2-IV	*sn-2*	*Non-specified*	PC	Verlotta et al., 2013

NR, not reported.

TABLE 9 | Kinetic parameters for some reported plant sPLA$_2$.

Origin	K_m (mM)	V_{max} (μmol.min^{-1} mg^{-1})	Lipid substrate used	References
*Gms*PLA$_2$-XIA-I	0.23	10.2	DLPC	Mariani et al., 2015b
	17.9	13.9	DLPG	Mariani et al., 2015b
*Gms*PLA$_2$-XIB-II	0.07	19.7	DLPC	Mariani et al., 2015b
	1.1	6.7	DLPG	Mariani et al., 2015b
*Ats*PLA$_2$-α	5.7	29.8	DOPC	Mansfeld and Ulbrich-Hofmann, 2007
*Td*sPLA$_2$	0.43	1.43 U.g^{-1}[a]	PC	Verlotta et al., 2013
Reported for animal sPLA$_2$s	0.18–3.2	NR	DOPC	Mansfeld and Ulbrich-Hofmann, 2007
*Pp*sPLA$_2$[b]	3.7	2	diC8-PC	Kuipers et al., 1991

K_m is expressed as specific activity. V_{max} is expressed as μmol.min^{-1}.mg^{-1}. [a]Expressed in Units per gram of dry leaves extract. [b]Pig pancreatic sPLA$_2$.

TABLE 10 | Parameters determined for different sPLA$_2$s using monomolecular films of DLPC.

Phospholipase A_2 origin	Optimum surface pressure	Substrate	Lipolytic ratio $LR_{(20/10)}$	References
*Gms*PLA$_2$-XIA-I	13	DLPC	0.45	This review
*Gms*PLA$_2$-XIB-II	16	DLPC	0.25	This review
B. diporus sPLA$_2$-I	11	DLPC	∼0	Yunes Quartino et al., 2015
B. diporus sPLA$_2$-II	12	DLPC	∼0	Yunes Quartino et al., 2015
M. fulvius-12	9–10	DLPC	0.07	Fernandez et al., 2017
Pig pancreas *Pp*sPLA$_2$	9	DLPC	0.08	Yunes Quartino et al., 2015
Bee venom *Bv*sPLA$_2$	18	DLPC	1.1	Yunes Quartino et al., 2015
*B. diporus Bd*sPLA$_2$-III	20	DLPC	1.3	Yunes Quartino et al., 2015
*B. asper Bs*sPLA$_2$-III	18	DLPC	1.3	Yunes Quartino et al., 2015
*N. naja Nn*sPLA$_2$	17	DLPC	1.5	Yunes Quartino et al., 2015
*N. m. mossambica Nm*sPLA$_2$	18	DLPC	1.6	Yunes Quartino et al., 2015
M. fulvius-17	19–20	DLPC	1.7	Fernandez et al., 2017

out the lipid hydrolysis. For this reason, the activity of the enzyme is directly modulated at the interface by the supramolecular organization of the substrate summarized in the concept of "*interfacial quality*" [e.g., the physical state of the lipids, proper lateral packing, modulation by non-substrate lipids, "membrane lateral defects," among others (Verger et al., 1978; Daniele et al., 1996; Jain and Berg, 2006; Blanchet et al., 2008a; Campagnoli et al., 2008; Fico et al., 2008; De Tullio et al., 2013)].

The stimulation effect of auxins over recombinant sPLA$_2$s from *G. max* is rather an interfacial effect. Despite porcine pancreas sPLA$_2$ presents low identity with the known reported sPLA$_2$s from plant sources, shows a significant similarity in the active site and calcium binding loop regions (Mansfeld and Ulbrich-Hofmann, 2007), making it an acceptable model for comparison. Using mixed micelles was determined that the effect of auxins on sPLA$_2$ stimulation depends on the concentration of the phytohormones employed with an optimal effect around 100 μM [the maximum perturbing effect (Mariani et al., 2015a)]. The hypotheses states that a direct action over sPLA$_2$ enzyme molecule or a synergic effect on the micelle surface doing more favorable the interface for lipolysis occurs. Both phytohormones IAA (indole 3-acetic acid) and IPA (indole 3-propionic acid) were active toward both type of sPLA$_2$, either coming from plant or pancreatic sPLA$_2$, suggesting that there is not a direct specific enzyme-phytohormone interaction involved. So, the effect of auxins can be attributable to changes in the interfacial quality of the organized substrate rather than a direct effect over the enzyme (Mariani et al., 2015a). The molecular details by which the particular mixed interfaces formed by auxins/phospholipids may modulate the sPLA$_2$ activity, regardless of the enzyme origin, remain to be elucidated. However, to ascertain the interfacial hypothesis of auxins over the action of sPLA$_2$ we further analyzed the surface properties of two auxins: IAA and IPA, i.e., the capability of these phytohormones to partition into lipid interfaces (Mariani et al., 2015a). Both IAA and IPA did not show any affinity toward lipid-clean interfaces (self-adsorption to water surface) but, very importantly, both auxins showed *the ability to penetrate lipid interfaces* forming stable and insoluble monolayers with phospholipids. This capability to form mixed lipid-auxin interfaces allowed the activation of two recombinants *Gms*PLA$_2$s and pancreatic sPLA$_2$ (Mariani et al., 2015a). The interfacial activation exerted by auxins was, regardless of sPLA$_2$ source, supporting the theory that at the action is at lipid-auxin interface and not a direct effect over the enzyme (Mariani et al., 2015a).

POTENTIAL INDUSTRIAL APPLICATION AND PERSPECTIVES

The application of biotechnology, particularly enzymes in industrial processes, is continuously growing due to its minimal environmental impact, since they produce non-toxic waste

substances and consume little energy (Warner, 2005). Natural and modified phospholipids have been extensively used in food industry, cosmetics, pharmaceuticals and agriculture (Guo et al., 2005). Therefore, in the production of these "modified phospholipids," secreted phospholipases obtained mostly from microorganisms or mammals have been used by the industry either for refined oils, dairy products, baked goods and other health food industries (De Maria et al., 2007; Wang et al., 2012). As sPLA$_2$ enzyme catalyze the stereospecific hydrolysis at the chiral carbon (*sn-2*) of glycerophospholipids converting them to lysoderivatives, the enzymatic bioconversion is the only selective pathway for obtaining *sn*-2-lysophospholipids. Lysophospholipids have a greater bioemulsifiers capability and have been applied in food and pharmaceutical industries (Stafford and Dennis, 1988). In this regard, most sPLA$_2$s used are from animal pancreas (porcine or bovine) or venoms (bee, snake) since they are enzymes easily isolated in large quantities relatively to the low cost and they are commercially available (de Haas et al., 1968; De Maria et al., 2007). However, products of animal source, are rejected by many customers for religious reasons or risk of viral or prion contamination. Moreover, the use of enzymes from animal sources in processes for obtaining food additives may be incompatible with certain international regulations, which is not accepted in certain fields of application, since they do not meet the requirements of current international food standards. This is the reason why the industrial production of vegetable sPLA$_2$s may become desirable. Nevertheless microbial sPLA$_2$s are being accepted, sPLA$_2$s from plant would be an advantage because its putative natural specificity (Lee et al., 2005; Mansfeld, 2009).

In the last decade, research has focused on the study of the still little known vegetable sPLA$_2$s (Wang, 2001). Important advances have taken place in the identification, classification, biochemical characterization and functional analysis of plant sPLAs. Recent progress in understanding the biochemical and functional properties of plant sPLAs paves the way for approval of them for commercial use and various applications. Several sPLA$_2$s have shown great potential as a target in the field of plant biotechnology, and molecular and catalytic diversity of plant sPLA$_2$s shows that the phospholipases are of increasing value for biotechnology applications.

The possibility of using plant phospholipases in food processing would be an advantage, from the point of view of food regulations. Considering the large production of soybean in the world, it is of great interest to study the properties of its lipolytic enzymes in terms from of both agronomic and biotechnology point of views (Rönner, 2003; Hermida, 2005). Moreover, it should be noted that in the purification process of soybean oil, a byproduct named "gum" is a material enriched in phospholipids (about 65% of dry weight), which is usually used in animal's food production or, after drying, it is sold as *soybean lecithin*. The hydrolytic products obtained by the action of sPLA$_2$ over soybean lecithin, the lysophospholipids (lysoderivatives), are widely used as emulsifiers (Henderson et al., 1995; Dashiell, 2001).

Recently, sPLA$_2$s were tested as catalysts for the synthesis of phospholipids with defined fatty acids by transesterification of lysophospholipids (Mansfeld, 2009). Furthermore, plant sPLA$_2$s showed to be distinctive from animals due to differences in substrate selectivity regarding the polar head and the acyl chains of glycerophospholipids (Lee et al., 2005). The potential properties of plant sPLAs would open new horizons to the engineering of biocatalysts.

The plant sPLA$_2$s is expected to have advantages over from animals regarding the performance or the incorporation of polyunsaturated fatty acids such as linoleic acid in egg PC for food production. Therefore, the processes for the production of phospholipids with fatty acids are not common and special performance requirements are desirable. Often, small differences in primary or 3D structure result in differences in the catalytic properties, which can be of great importance in biocatalytic applications. However, despite their enormous potential, plant enzymes have not been yet considered for industrial application. This could be attributed to the limited availability of these enzymes, recently discovered and characterized. Besides, these enzymes are much less abundant in the natural environment and no plant enzymes are available commercially.

Over 100 years, experiments with members of the sPLA$_2$ superfamily have been carried out and kinetic and structural characterization established sPLA$_2$ as an important model of interfacial enzymology. The future of this promising enzymes seems to be very exciting, leading to find out specific inhibitors of them, and further elucidating plants sPLA$_2$'s roles in cellular processes, along with potential uses in the industry.

AUTHOR CONTRIBUTIONS

MM and GF conceived the main idea, designed the general format of this manuscript, created the tables, and carried out the final corrections of this manuscript. MM prepared the figures and drafted this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00861/full#supplementary-material

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