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Identification of a secretory phospholipase A_2 from *Papaver somniferum* L. that transforms membrane phospholipids

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ABSTRACT

The full-length sequence of a new secretory phospholipase A_2 was identified in opium poppy seedlings (*Papaver somniferum* L.). The cDNA of poppy phospholipase A_2, denoted as *pspla*$_2$, encodes a protein of 159 amino acids with a 31 amino acid long signal peptide at the N-terminus. *Ps*PLA$_2$ contains a PLA$_2$ signature domain (PA2c), including the Ca^{2+}-binding loop (YGKYCGxxxxGC) and the catalytic site motif (DACCxxHDxC) with the conserved catalytic histidine and the calcium-coordinating aspartate residues. The aspartate of the His/Asp dyad playing an important role in animal sPLA$_2$ catalysis is substituted by a serine residue. Furthermore, the *Ps*PLA$_2$ sequence contains 12 conserved cysteine residues to form 6 structural disulfide bonds. The calculated molecular weight of the mature *Ps*PLA$_2$ is 14.0 kDa. Based on the primary structure *Ps*PLA$_2$ belongs to the XIB group of PLA$_2$s. Untagged recombinant *Ps*PLA$_2$ obtained by expression in *Escherichia coli*, renaturation from inclusion bodies and purification by cation-exchange chromatography was characterized *in vitro*. The pH optimum for activity of *Ps*PLA$_2$ was found to be pH 7, when using mixed micelles of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and Triton X-100. *Ps*PLA$_2$ specifically cleaves fatty acids from the *sn*-2 position of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and shows a pronounced preference for PC over phosphatidyl ethanolamine, -glycerol and -inositol. The active recombinant enzyme was tested *in vitro* against natural phospholipids isolated from poppy plants and preferably released the unsaturated fatty acids, linoleic acid and linolenic acid, from the naturally occurring mixture of substrate lipids.

1. Introduction

In recent years, an appreciation of phospholipids as more than just passive structural components of biological membranes has grown. Phospholipids play important roles in many biochemical and physiological processes in plants, including cell-to-cell communication or responses to external stimuli. Phospholipid-derived molecules with roles in plant signaling and communication can be generated by phospholipases (Canonne et al., 2011). While phospholipases C and D have been extensively characterized over the past decades (Janda et al., 2015; Zhao, 2015), there is still a lack of information about phospholipases A_2 (PLA$_2$s). PLA$_2$ (phosphatide 2-acylhydrolase, EC 3.1.1.4) catalyzes the stereospecific hydrolysis of membrane glycerophospholipids at the *sn*-2 position to release free fatty acids (FFAs) and lysophospholipids (LPLs). Even though PLA$_2$s are rather small proteins, there is a striking structural diversity among representatives of the PLA$_2$ superfamily. Based on amino acid sequences, molecular weight and biochemical properties, PLA$_2$s are classified into five groups: cytosolic Ca^{2+}-dependent PLA$_2$s (cPLA$_2$s) and Ca^{2+}-independent PLA$_2$s (iPLA$_2$s); secretory PLA$_2$s (sPLA$_2$s); lysosomal PLA$_2$s and platelet-activating factor acylhydrolases (Six and Dennis, 2000). In plants, only two groups of PLA$_2$ have been identified: the low-molecular-weight sPLA$_2$, and the less specific patatin-like PLA with a combined PLA$_2$ and PLA$_1$ activity (Lee et al., 2005). No cPLA$_2$ is known to be present in plants. Within the last years full sequences of putative sPLA$_2$ cDNAs have been cloned from rice (Ståhl et al., 1999), carnation flowers (*Dianthus caryophyllus* L. cv. Degio) (Kim et al., 1999), tobacco (*Nicotiana tabacum*) (Fujikawa et al., 2005, 2012), orange (*Citrus sinensis*) (Liao and Burns, 2010), durum wheat (*Triticum durum*) (Verlotta et al.,

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Abbreviations

AtsPLA_2	secretory phospholipase A_2 from *Arabidopsis thaliana*
BIA	benzylisoquinoline alkaloid
CsPLA_2	secretory phospholipase A_2 from *Citrus sinensis*
DcPLA_2	secretory phospholipase A_2 from *Dianthus caryophyllus*
DOPC	1,2-dioleoyl-*sn*-glycero-3-phosphocholine
$cPLA_2$	cytosolic phospholipase A_2
FAME	fatty acid methylester
FFA	free fatty acid
GC	gas chromatography
GmsPLA_2	secretory phospholipase A_2 from *Glycine max*
$iPLA_2$	Ca^{2+}-independent phospholipase A_2
IPTG	isopropyl β-D-1-thiogalactopyranoside
LPC	lysophosphatidylcholine
LPI	lysophosphatidylinositol
LPL	lysophospholipid
MJ	methyl jasmonate
NtPLA_2	secretory phospholipase A_2 from *Nicotiana tabacum*
OsPLA_2	secretory phospholipase A_2 from *Oryza sativa*
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PLA_1	phospholipase A_1
PLA_2	phospholipase A_2
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol
PsPLA_2	secretory phospholipase A_2 from *Papaver somniferum*
PtPLA_2	secretory phospholipase A_2 from *Populus trichocarpa*
RcPLA_2	secretory phospholipase A_2 from *Ricinus communis*
$sPLA_2$	secretory PLA_2
TdsPLA_2	secretory phospholipase A_2 from *Triticum durum*
TLC	thin layer chromatography
VvPLA_2	secretory phospholipase A_2 from *Vitis vinifera*
ZmPLA_2	secretory phospholipase A_2 from *Zea mays*

2013) and soybean (*Glycine max*) (Mariani et al., 2012). In the Arabidopsis genome, genes for four isoforms of $sPLA_2$ have been identified, denoted *Ats*PLA_2-α, *Ats*PLA_2-β, *Ats*PLA_2-γ, and *Ats*PLA_2-δ (Lee et al., 2005). Putative $sPLA_2$s have been purified from elm seeds (*Ulmus glabra*) (Ståhl et al., 1998) and rice (*Oryza sativa*) (Ståhl et al., 1999). Based on their deduced primary structures, plant $sPLA_2$ have been classified into groups XIA and XIB (Mansfeld et al., 2006; Six and Dennis, 2000).

FFA and LPLs, the enzymatic products of PLA_2 catalysis, can serve as precursors of signaling messengers involved in various physiological and pathological processes. In animals, arachidonic acid released from membrane lipids by PLA_2 action is metabolized into prostaglandins and leukotrienes that play an important role in inflammation (Clark et al., 1995). For plants, LPLs, such as lysophosphatidylcholine, have been demonstrated to have a role in host-symbiont communication during plant-microbe interactions in soil, suggesting that PLA_2s are important players in plant-microbe interactions (Drissner et al., 2007). FFAs released by PLA_2s in plants can be precursors for the enzymatic or chemical production of a multitude of oxylipins with signaling and defensive functionalities (Mosblech et al., 2009). Moreover, PLA_2s have also been reported to be biologically active in the control of various biological processes in plants, such as cell growth (Scherer et al., 2007), cell elongation (Lee et al., 2003), stomatal opening (Seo et al., 2008) or defence and stress responses (Canonne et al., 2011). From the avaliable data it can be concluded that PLA_2s have a role in lipid signaling events mediating plant defense responses.

Opium poppy belongs to the world's oldest and most prominent medicinal plants due to the production of therapeutically important secondary metabolites, in particular benzylisoquinoline alkaloids (BIAs). Opium – the dried latex obtained from unripe poppy seed pods – is the source of analgesic morphine, the cough suppressant codeine, the vasodilator papaverine, the potential anticancer drug noscapine, or the antimicrobial agent, sanguinarine (Beaudoin and Facchini, 2014). It is a paradox, that much more is known about the pharmacological effects of BIAs than about their biological role in the poppy plants in which they naturally occur. Secondary metabolites are not essential in maintaining normal physiological processes in plants; however, they might be important under special conditions, for instance during plant defence reactions (Hagel and Facchini, 2013) or for communication between plants and their microbiotic surroundings. Several BIAs are known to be involved in chemical defence responses against microorganisms and herbivores (Schmeller et al., 1997). For instance, the production of sanguinarine was rapidly induced in suspension cultures of *Papaver somniferum* L. (Holková et al., 2010) or *Eschscholtzia californica* Cham. after elicitation with the necrotrophic fungal pathogen, *Botrytis cinerea*, or with methyl jasmonate (MJ), respectively (Kollárová et al., 2014). Plants accumulate jasmonates in response to many biotic and abiotic stresses, particularly after herbivore attack and wounding and, therefore, jasmonate signaling might be involved in the control of defense responses (Okada et al., 2015), in poppy possibly including the production of BIAs.

Until now, opium poppy is the only commercial source for morphine, codeine and semi-synthetic derivatives such as oxycodone and naltrexone (Beaudoin and Facchini, 2014), even though several protocols for the chemical synthesis of morphine and its derivatives have been published (Rinner and Hudicky, 2012) and their production via microbial biomanufacturing seems to be promising (Fossati et al., 2015; Thodey et al., 2014). Despite all these efforts, the extraction of morphinan from poppy plants as a natural resource is still far more economical. Therefore, the study of signaling pathways controlling the biosynthesis of alkaloids in poppy plants remains a current topic of research in the field of plant alkaloid metabolism.

It has been shown that the production of BIA in poppy plants is stimulated by exposure of the plants to exogenous stresses, such as wounding (Mishra et al., 2013). From other plant model systems, such as Arabidopsis, it is known that the same wounding stress triggers responses that are mediated by lipid signaling cascades involving phospholipases (Mosblech et al., 2008; Munnik, 2001). To understand the molecular players involved in the control of alkaloid accumulation, we have started to analyze possible connections between phospholipid signaling and plant defence response mechanisms in opium poppy. A better understanding of the players and principles of regulation of BIA biosynthesis might be the basis for the future genetic modification of opium poppy to optimize BIA production. It is therefore important, to analyze the background of