Activation of phospholipase PLA_2 actvity in *Ricinus communis* leaves in response to mechanical wounding

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In order to investigate the defense response in castor bean (*Ricinus communis*) against predators, we analyzed the effect of mechanical wounding upon the phospholipase A_2 (PLA_2) activity of leaf extracts. Time course experiments revealed that the highest levels of increased PLA_2 activity (*ca.* two fold) occurred 15 min and 60 min after injury. The induced activities demonstrated high sensitivity towards aristolochic acid (10 mM), a PLA_2 inhibitor. Based on SDS-PAGE analysis, the PLA_2 activity induced 15 min after wounding migrated with a molecular mass of 40 kDa and was denoted $RcPLA_2$ I. The protein activity induced 60 min after wounding, $RcPLA_2$ II, migrated with a molecular weight of 14 kDa. Furthermore its N-terminal sequence shared homology with PLA_2 from elm and rice. The PLA_2 enzymes were purified to near homogeneity by a combination of gel filtration and electro-elution of protein bands after native PAGE.
Key words: cellular defense, castor bean, plant signaling, phospholipase A_2, *Ricinus communis*

Com o objetivo de avaliar a resposta de defesa da mamona (*Ricinus communis*) contra predadores, analisaram-se as alterações nas atividades de fosfolipases A_2 (PLA_2) em extratos de folhas de plantas, submetidas à injúria mecânica após diferentes intervalos de tempo. Observou-se que os níveis dessa enzima aumentaram de forma significativa (cerca de duas vezes) 15 e 60 min após o ferimento. As atividades que foram induzidas foram sensíveis ao ácido aristolóquico (10 mM), um inibidor específico para PLA_2. A enzima induzida após 15 min foi denominada $Rc\text{-}PLA_2$ I e apresentou uma massa molecular aparente, determinada por SDS-PAGE, de 40 kDa. A proteína induzida após 60 min, $RcPLA_2$ II, apresentou uma massa molecular de 14 kDa. A seqüência N-terminal parcial da Rc-PLAII mostrou homologia com PLA_2 de arroz e olmo. As fosfolipases foram parcialmente purificadas por associação da cromatografia de filtração em gel, seguida por eluição das bandas que apresentaram atividade enzimática após separação por PAGE não desnaturante.
Palavras-chave: fosfolipase A_2, mamona, *Ricinus communis*, sinalização em planta; mamona

INTRODUCTION

The castor bean contains about 50% oil, which has special characteristics such as high viscosity, stability to heat and pressure, low freezing point, and ability to form waxy substances upon chemical treatments. Such properties turn this oil very attractive to modern industry, due to its application in the manufacture of explosives, varnishes, lubricants, dyes, plastics, fertilizers, leather, candles and cosmetics (Turner et al., 2004; Conceição et al., 2005). As energy demands increase and fossil fuels are limited, a lot of research efforts have been directed towards alternative renewable fuels. Castor oil has been identified as a very important potential source for the production of biodiesel in Brazil (Pinto et al., 2005). Therefore, the Brazilian Government developed a specific program called Probiodiesel in order to promote the development of national renewable energy sources. With this scenario in mind, an increase in the cultivation of

castor bean plant is expected nationwide and studies on the defense mechanisms against insect and pathogen pests of our model plant are getting higher relevance.

Plants respond to wounding caused by mechanical stress or by chewing herbivores through the synthesis of an array of defense proteins that facilitate wound healing and provide protection against further wounding or pathogen attacks (Ryan, 2000). A better knowledge of the molecular mechanisms responsible for plant defense is needed and has been recently invoked. Many of the accompanied responses are mediated by jasmonates. The term jasmonate is used for the cyclopentanone compound, jasmonic acid (JA), its methyl ester (JAME) and derivatives such as JA amino acid conjugates (Schaller et al., 2005). In tomato, mechanical wounding leads to expression of the prosystemin gene within vascular bundles (Jacinto et al., 1997). This polypeptide is processed into a peptide of 18 amino acid residues, called systemin (Pearce et al., 2002), which is released into the apoplast from damaged cells and transported through the phloem to distal leaves. In the target cells, systemin can activate the recently cloned membrane-located receptor (Scheer and Ryan, 2002) to initiate a signaling cascade in which linolenic acid is released intracellulary to initiate the formation of oxygenated fatty acids, collectively named oxylipins. Increased free fatty acid levels were observed in cultured cells of several plant species treated with fungal elicitors (Mueller et al., 1993), e.g. in tomato and *Ricinus communis* leaves in response to wounding (Conconi et al., 1996; Ryu and Wang, 1998). Besides this increase in free fatty acids, in tomato leaves and seedlings of broad bean, soybean, sunflower and pepper, phosphatidic acid systemically accumulates in response to wounding, released from lipids presumably by the actions of wound-inducible phospholipases of types A, D and/or C (Lee et al., 1997). However, although the participation of lipases in JA-mediated gene expression has been suggested and a detailed understanding of the signaling pathways that regulate induced responses to insect feeding have been reviewed (Edwards and Singh, 2006), little is known about the presence and the characteristics of such phospholipases.

Phospholipases potentially involved in plant signal transduction have been studied indirectly or directly by different research groups. In tomato leaves, a wound-inducible PLA_2 activity was reported by Narváes-Vásquez et al. (1999), who evoked it as involved in plant defense signaling process since accumulation of lysophosphatidylcholine occurred in wounded leaves. Jung and Kim (2000) identified and purified a membrane-associated PLA_2 with 48 kDa from broad bean leaves. This protein was activated by calmodulin and the authors suggested a role of this enzyme in the plant cell response. It was also reported that patatin-like enzymes with phospholipase A_2 activity contribute to the accumulation of oxylipins in tobacco mosaic virus-infected tobacco leaves (Dhondt et al., 2000). Furthermore, the cDNAs for three different patatin-like enzymes were cloned and the correspondent genes were seen to be activated in response to virus infection. In a more recent report, Holk et al. (2002) isolated four cDNA sequences from Arabidopsis, which are members of the patatin-related PLA gene family with potential functions in plant signal transduction (Holk et al., 2002). Patatins are acidic proteins of *ca.* 40 kDa, originally purified from potato tubers, which were found to have phospholipase activity (Senda et al., 1996).

In the present study, two phospholipases A_2 from castor bean, $RcPLA_2$ I with 40 kDa and $RcPLA_2$ II with 14 kDa were purified. The early activation of both activities after mechanical wounding suggests their involvement in plant defense signal transduction. Moreover, progress in this area of study is expected to offer a substantial, rational contribution to biotechnological approaches in crop protection.

MATERIAL AND METHODS

Plant material and mechanical wounding: Castor bean (*Ricinus communis* L. cv. IAC-226) plants were obtained from the Instituto Agronômico de Campinas, São Paulo State, Brazil. Plants were grown in plastic pots and maintained in environmental chambers for 17 h under 300 µmol (photons) m^{-2} s^{-1} at 28°C, and for 7 h in the dark at 18°C. When the plants were at the three-leaf stage (18-21 d), their leaves were submitted to a mechanical injury using a hemostat. The period of treatment is given in the figure legend.

Protein extraction: At specific intervals after mechanical injury, leaves were collected, frozen immediately in liquid N_2 and ground to a fine powder. The proteins were

extracted with 50 mM Tris-HCl at pH 8.0, 5 mM $CaCl_2$, and 0.06% NP-40 buffer (1:3, w/v). The slurry was centrifuged at 10,000 g for 30 min at 4ºC. The supernatant was centrifuged at 100,000 g for 60 min at 4ºC with an ultracentrifuge (Himac CP 85β, Hitachi, Japan). The supernatant was treated with $(NH_4)_2SO_4$ to a final concentration of 55% (w/v) and stirred for 1 h at 4°C. Precipitated proteins were pelleted by centrifugation at 10,000 g for 10 min at 4ºC and ressuspended in 50 mM Tris-HCl at pH 8.0, containing 5 mM $CaCl_2$. Ice-cold acetone was added to a final concentration of 45% (v/v) and the extract was left at 4°C for 30 min. Precipitated proteins were removed by centrifugation at 10,000 g at 4ºC for 10 min. The resulting supernatant was used immediately for assay and purification of PLA_2 enzyme. The protein concentration of each sample was measured with a bicinchoninic acid (BCA kit) from Sigma (Sigma Chemical Co., St. Louis, MO, USA), using ovalbumin as standard.

PLA_2 activity assay

Chemicals: 1-palmitoyl-2-[6-[7-nitro-2,1,3-benzoxadiazol-4-yl-amino]-caproyl] phosphatidylcholine (C_6-NBD-PC) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). Aristolochic acid and PLA_2 from bee venom was from Sigma. All other chemicals were of reagent grade.

Activity of PLA_2 was assayed using C6-NBD-PC as substrate according to a previous report (Wittenauer et al., 1984), with some modifications. Briefly, that substrate (41 nM) was dissolved in 50 mM Tris-HCl buffer at pH 8.0 containing 5 mM $CaCl_2$. The reaction was started by addition of crude extract (50 µL for 1.9 mL of reaction buffer) and fluorescence was continuously monitored at 30ºC as a function of time. Fluorescence excitation and emission wavelengths were at 460 nm and 534 nm, respectively, as indicated by the supplier (Avanti Polar Lipids). The calibration curve was established using NBD obtained after hydrolysis of C6-NBD-PC with PLA_2 from bee venom. In some experiments, the enzyme sources and inhibitors were previously mixed and pre-incubated for 20 min at 30ºC, and the reaction was started by the addition of fluorogenic substrate.

For gel activity assays, extracted proteins were resolved by 12% native PAGE (Davis, 1964). After electrophoresis, the gel was washed three times for 10 min with buffer A. The fluorogenic substrate, C6-NBD-PC, was added to a final concentration of 5 µM and after 60 min incubation, the gel was observed in an UV chamber. The gel was photographed using the Image Master^R VDS (Thermal Imaging System FTI-500, Pharmacia Biotech, Sweden).

Gel electrophoresis: Proteins were analyzed in 12 or 15% (w/v) SDS polyacrylamide gel following the methodology described by Laemmli, (1970). The gels were run using a Mini-Protean system from BioRad according the manufacturer's instructions. For native gels, the SDS was omitted from all solutions.

Purification of PLA_2: The extracts of wounded leaves were loaded onto a Sephacryl S-200 column pre-equilibrated with 50 mM Tris-HCl at pH 8.0 containing 5 mM $CaCl_2$ and eluted at a flow rate of 0.5 mL min^{-1}. Fractions (0.5 mL) were collected and assayed for PLA_2 activity. Specific fractions were chosen to be analyzed for gel activity. The protein band with biological activity was excised and the protein was electro-eluted using an Electro-Eluter from BioRad according the manufacturer's instructions. Ammonium bicarbonate (50 mM) was used as elution buffer. The eluted proteins were concentrated in a spin-vacuum for further analysis.

Sequencing analysis: The N-terminal sequence was determined using an automated protein sequencer PPSQ-10 (Shimadzu, Japan) and the sequences were submitted to automatic alignment.

The N-terminal partial amino acid sequences of isolated proteins were obtained on a Shimadzu PPSQ-10 Automated Protein Sequencer using the Edman degradation principle. Phenylthiohydantoin derivatives (PTH amino acids) were detected at 269 nm after separation on a reverse phase C18 column under isocratic conditions, according to the manufacturer's instructions. The polypeptide sequences obtained were submitted to automatic alignment using a BLAST search (Altschul et al., 1990) of the Protein Data Bank (Sussman et al., 1998).

RESULTS

Detection of wound-inducible Phospholipase A_2 activity in castor bean leaves: In order to investigate the involvement of PLA_2 in responses to stress and defense

in castor bean plants, a time course of constitutively expressed PLA_2 activity was determined in leaf tissue extracts. The values for this enzymatic activity stayed constant until 6 h (Figure 1), at the level of *ca.* 7 nmol NBD mg^{-1} protein min^{-1}. In Figure 1 it is demonstrated that mechanical wounding triggered a rapid induction of PLA_2 activity. An increase of about two fold occurred in 15 min, and at 30 min the PLA_2 activity decreased to near the control level. Interestingly, the activity increased again after 60 min and decreased again at 120 min, although the activity was still above control level at the end of the time course. Figure 1 also shows that the inducible PLA_2 activity was strongly inhibited by aristolochic acid, an alkaloid from the *Aristolochia* species that is a specific inhibitor of secretory PLA_2 (Vishwanath et al., 1987); this inhibitor had little effect on the constitutive activity, however.

Purification of Phospholipase A_2 and physicochemical characterization: Protein extracts from castor bean leaves obtained 15 and 60 min after wounding were fractionated using gel filtration chromatography on a Sephacryl-S-200 column. The column effluent was monitored at 280 nm and the PLA_2 activities of the fractions were determined using 6-NBD phosphatidylcholine as substrate. The protein elution profile for both extracts was very similar (Figure 2A,B). However, with regard to the PLA_2 activity, two peaks with activity were observed, fractions 9 and 13, in extracts obtained 15 min after wounding (Figure 2A). In contrast, only one peak with PLA_2 activity, fraction 13, was observed in extracts obtained 60 min after wounding, suggesting that this PLA_2 isoform is inactive or absent at this time point. The fractions enriched in PLA_2 activity (fractions 9 and 13, obtained 15 and 60 min after wounding, respectively) were submitted to native PAGE.

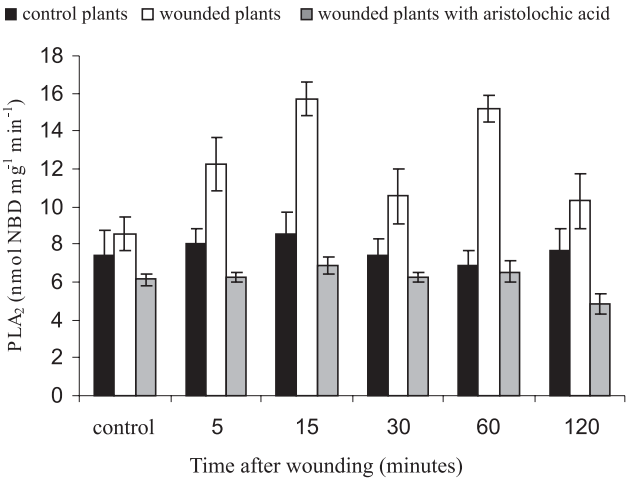

Figure 1. Specific activity of PLA_2 in *Ricinus communis* leaves in response to mechanical wounding. Protein extracts were assayed for pospholipolytic activity with and without aristolochic acid (PLA_2 inhibitor), as described in methods. Assays were performed using 6-NBD-phosphatidylcholine as substrate. Values are means of five independent experiments. Bars indicate SD.

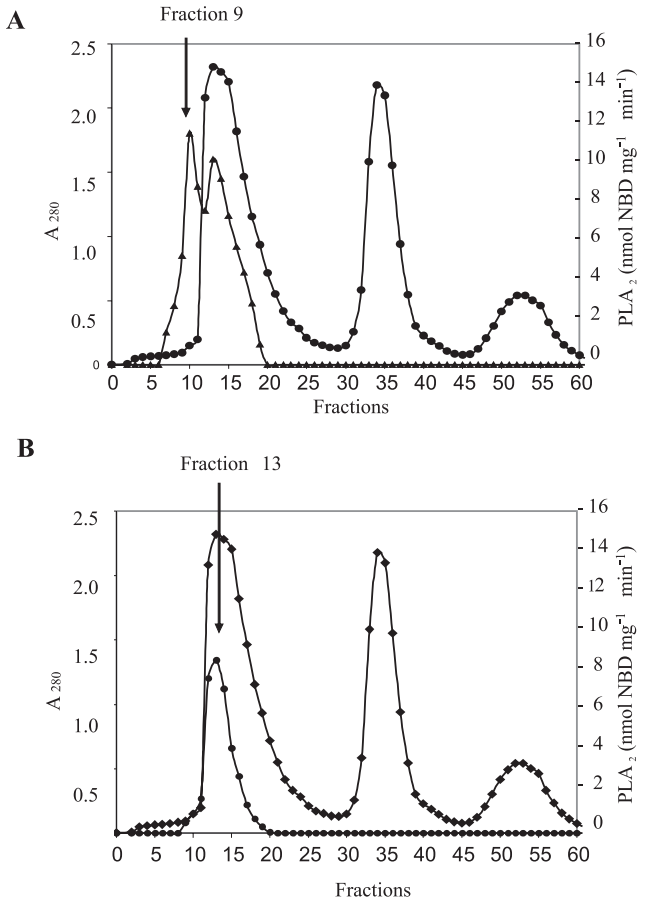

Figure 2. Elution profile of gel filtration chromatography on Sephacryl S-200. Protein extracts obtained 15 min **(A)** or 60 min **(B)** after wounding were loaded onto the column pre-equilibrated with 50 mM Tris-HCl (pH 8.0). Elution was carried out with the same buffer at a flow rate of 0.5 mL min^{-1}. The collected fractions (0.5 mL) were monitored for protein concentration (♦) and assayed for PLA_2 activity (●) using 6-NBD- phosphatidylcholine as substrate.

After electrophoresis, the PLA_2 activity was determined by incubation of the gels with fluorogenic substrate. One fluorescent band with very high mobility was observed in fraction 9, which matched with a protein band revealed in a replica gel stained with Coomassie Blue (Figure 3A). The protein electro-eluted from this gel region (from another replica gel) was homogeneous, as revealed by SDS-PAGE analysis, and named $RcPLA_2$ I (Figure 3B). The N-terminal amino acid sequence of the $RcPLA_2$ I could not be directly analyzed on the protein sequencer using Edman degradation, indicating that its N-terminal residue is blocked.

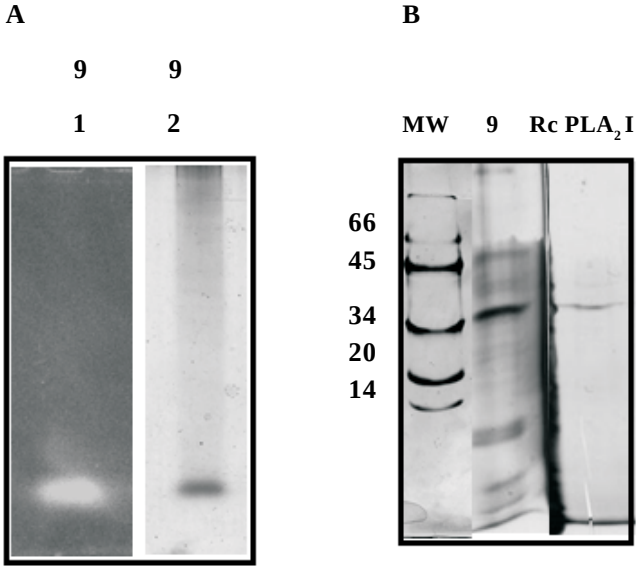

Figure 3. (**A**) 12% native-PAGE analysis of the fraction 9 obtained by gel filtration chromatography on Sephacryl S-200. Proteins (30 µg) were loaded in each lane. Lane 1: the protein band with PLA_2 activity determined using 6-NBD-phosphatidylcholine as substrate; Lane 2: a replica gel stained with Coomassie Brilliant blue-R. (**B**) 15% SDS-PAGE analysis. Lane 1: 30 µg of protein from fraction 9; Lane 2: the purified phospholipase (Rc-PLA_2 I) obtained after electro-elution of the protein band with PLA_2 activities observed in (**A**). The proteins were visualized by silver staining.

For fraction 13, obtained 60 min after wounding, one fluorescent band with very low mobility was observed when assayed for PLA_2 activity in the gel and a matching band was also observed in a replica gel stained with Coomassie Blue (Figure 4A). The protein electro-eluted from this band was also homogeneous, presented a

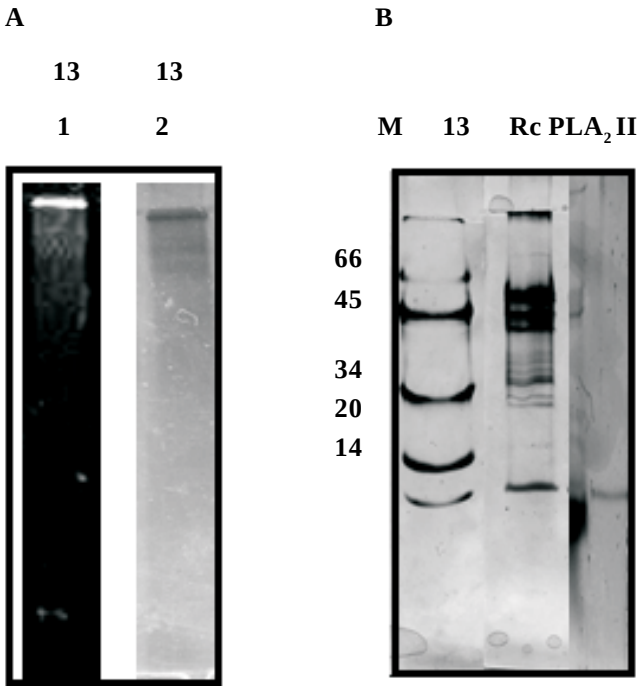

Figure 4. (**A**) 12% native-PAGE analysis of fraction 13 obtained by gel filtration chromatography on Sephacryl S-200. Proteins (30 µg) were loaded in each lane. Lane 1: the protein band with PLA_2 activity revealed using 6-NBD-phosphatidylcholine as substrate; Lane 2: a replica gel stained with Coomassie Brilliant blue-R. (**B**) 15% SDS-PAGE analysis. Lane 1: thirty µg of protein from fraction 13; Lane 2: the purified phospholipase (Rc-PLA_2 II) obtained after electro-elution of the protein band with PLA_2 activities observed in (**A**). The proteins were visualized by silver staining.

molecular weight of 14 kDa and was named $RcPLA_2$ II (Figure 4B). The purified protein was also present in fraction 13, as demonstrated by SDS-PAGE analysis (Figure 4B). Figure 5 shows that the N-terminal partial sequence of $RcPLA_2$ II presented high homology with

RC-PLA_2 II	**L N V - - Q A I A - T A**
Elm PLA_2	**L N V G V Q A T G - T S**
Rice PLA2 (Deduced)	**L N I G - D L L G S T P**

Figure 5. Alignment of the N-terminal amino sequence of the purified *Ricinus communis* Rc-PLA_2 II with *Ulmus glabra* (Elm) and deduced amino acid sequence of Rice EST sequence D47653.

PLA_2 enzymes from elm (Stahl et al., 1998) and rice (Stahl et al., 1999).

DISCUSSION

Different defense signaling pathways and points of cross-talk between them have been described for plant-insect or microbe interactions, but many of the early signal transduction events remain unknown. Phospholipid-derived molecules are emerging as novel second messengers in plant defense signaling. Recent research has begun to reveal the signals produced by the enzymes phospholipase C, phospholipase D and phospholipase A_2 and their putative downstream targets (Munnik and Meijer, 2003).

Wounded leaves induce rapid and transient changes in the lipid composition of cell membranes (Conconi et al., 1996; Lee et al., 1997; Ryu and Wang, 1998). These changes include a decrease in the content of polar lipids (Conconi et al., 1996; Ryu and Wang 1998) and the elevation of levels of lysolipids (Conconi et al., 1996; Lee et al., 1997), phosphatidic acid (Ryu and Wang, 1998; Lee et al., 1997), and free fatty acids, predominantly linolenic acid (LA) (Conconi et al., 1996; Ryu and Wang, 1998). Linolenic acid can stimulate the plasma membrane H^+-ATPase and it is the precursor of phytodienoic acid and JA (Vick and Zimmerman, 1984), which are potent signaling molecules inducing transcriptional activation of systemic wound-responsive genes in tomato leaves (Farmer and Ryan, 1992; Bergey et al., 1996). Thus, the characterization of PLA_2 involved in intracellular signaling events is a key step in the activation of the defensive genes.

We, herein, describe the detection and isolation of two wound-inducible PLA_2 enzymes from castor bean leaves. The increase in PLA_2 activity was seen to be biphasic, peaking at 15 min, decreasing at 30 min, and then increasing again to reach a maximum at ~1 h. These results were similar to those previously reported by Narváez-Vásquez et al. (1999) in tomato plants. These authors attributed the first peak of the biphasic response as a result of an initial hydraulic signal caused by breaching the vascular system of the plant, and the second increase in PLA_2 activity was attributed to the systemic signal that was released at the wound site.

In our system, using protein extracts from the first induced peak, a PLA_2 with 40 kDa was obtained and named $RcPLA_2$ I. Since the size of this enzyme is similar to that of the so-called patatin-like proteins and it possesses the characteristic acidic feature, (inferred from its very low mobility under native PAGE; Figure 3A), we speculate that the $RcPLA_2$ I may be a patatin-like PLA_2. Comparative amino acid sequence data from patatin and $RcPLA_2$ I have yet to be analyzed. A PLA_2 related to patatin, described by Dhondt et al. (2000), was shown to have a potential role in transducing the defense response during the hypersensitive response in tobacco plants. Another patatin-like lipase (VLPAT1) was reported to be possibly involved in the signal transduction of drought stress in the cowpea (*Vigna unguiculata*) plant (Matos et al., 2001). More recently, Holk et al. (2002) described several patatin-related phospholipases from Arabidopsis with a potential function in plant signal transduction.

The search for the wound-induced PLA_2 in castor bean leaves, observed during the second induction peak, yielded a protein with 14 kDa, called $RcPLA_2$ II. The N-terminal sequence of this enzyme showed a high degree of homology with PLA_2 isolated from elm (*Ulmus glabra*) seeds (Stahl et al., 1998) and PLA_2 from rice obtained from the deduced EST sequence D47653 (Stahl et al., 1999). The small molecular weight PLA_2 (15 kDa) purified from elm seeds is related to animal secreted PLA_2, however it is not related to patatin. To date, few examples of plant PLA_2 related to animal secreted PLA_2 have been reported (Kim, 1994; Stahl et al., 1998), indicating the lack of information regarding the diversity of phospholipase isoforms and their physiological roles.

The catabolic activity of phospholipases in plants is important for general lipid metabolism, and for releasing lipid mediators for regulation of different cell functions. The three-step methodology employed here to detect and purify $RcPLA_2$ could be an efficient tool for obtaining PLA_2 from other sources and so to increase the understanding of the intracellular information processing system, defense gene transcription and the functional roles of specific phospholipase isozymes in castor bean plant. In addition, such information could be useful to modulate the expression of target proteins in transgenic plants in order to increase their resistance to pest attack in the field.

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