DOI: 10.1007/s10535-013-0368-3 BIOLOGIA PLANTARUM 58 (1): 147-152, 2014

Involvement of phospholipase A2 in the release of silymarin to the culture medium of *Silybum marianum* cell suspensions

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Abstract

In suspension cell cultures of *Silybum marianum,* methyl jasmonate (MJ) stimulated the accumulation and release of silymarin (Sm) to the culture medium. This study shows that phospholipase A2 (PLA2) plays a role in the release of Sm in elicited cultures. PLA2 activity increased in cell suspensions treated with MJ. Addition of aristolochic acid (AA) or bromo*enol* lactone (BEL) compounds that inhibit PLA2 activity impeded silymarin release. The addition of linoleic or linolenic acid reversed the inhibitory action of AA. Fatty acids (FAs) stimulated Sm release when added alone to control cultures. By contrast, oleic acid and saturated FA were ineffective in emulating MJ action.

Additional key words: elicitation, fatty acids, methyl jasmonate, secondary metabolites.

Introduction

Plant cell cultures represent an alternative to natural plants for the production of medicines, food additives, and fine chemicals. In addition, some plant cultures constitutively secrete a variety of secondary metabolites into the medium (Berlin *et al.* 1988, Kauss *et al.* 1994).

The study of the mechanisms and regulatory processes that control the release of molecules is of great technological importance since exploiting the secretion process of plant secondary metabolites would allow them to be accumulated in a higher content and be more easily isolated.

Elicitation with methyl jasmonate (MJ) is one of the best approaches for increasing the production of secondary metabolites (Repka *et al.* 2004, Namdeo *et al.* 2007, Zhao *et al.* 2013). MJ has also been found to stimulate secretion in cultures of some species like *Taxus canadensis* (Roberts *et al.* 2003), *Calendula officinalis* (Wiktorowska *et al.* 2010), or *Thevetia peruviana* (Zabala *et al.* 2010). However, there is still insufficient knowledge of how MJ induce the release of secondary metabolites. As plant cell membranes are highly vulnerable to stress, some authors explain the elicitor-induced excretion of secondary metabolites as a consequence of changes in cell osmotic potential, membrane permeability, electric potential, and even cell lysis (Vasconsuelo and Boland 2007). Screening jasmonate responsive genes in *Arabidopsis* reveals that phospholipases (PLs) are up-regulated by MJ treatment and the increased activities of these enzymes result in the modification of lipid constituents of the membrane and generation of one or more products that are able to recruit or modulate specific target proteins (Meijer and Munnik 2003).

Roles for PLs in secretion processes have been proposed in animal systems but remain controversial. Evidence from several groups implicates phospholipase D (PLD) activity in transport-vesicle formation and secretion in mammalian cell, as well as in secretory processes in yeast (Choi *et al.* 2002, Staneva *et al.* 2004). In plants, although less explored, it has been shown that PLD activity and its product, phosphatidic acid (PA), and phospholipase A2 (PLA_2) are also involved in vesicle trafficking and secretion (Li and Xue 2007, Lee *et al.* 2010).

Concerning plant secondary metabolites, several studies indicate association of PLs with the plant response to elicitors (Muñoz-Sanchez *et al.* 2012). However, few reports connect PLs activation with the

Submitted 17 December 2012, *last revision* 23 May 2013, *accepted* 27 May 2013.

Abbreviations: AA - aristolochic acid; BEL - bromo*enol* lactone; FA - fatty acid; MJ - methyl jasmonate; NBD-PC - (1-hexadecanoyl-2-[N-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl) aminohexanoyl]-sn-glycero-3-phosphocholine); PA - phosphatidic acid; PL - phospholipase; PLA2 - phospholipase A2; PLD - phospholipase D; Sm - silymarin.

Acknowledgements: This work was financed by Ministerio de Ciencia e Innovación (BFU2008-02876/BFI) and Consejería de Educación de la Junta de Castilla y León (SA153A11-2) Spain.

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secretion of metabolites.

Silymarin (Sm) is a constitutive natural mixture of flavonolignans found in the pericarp of the fruits of *Silybum marianum* that is used as an anti-hepatotoxic agent as well as a nutritional supplement to protect the liver from diseases associated with alcohol consumption and exposure to environmental toxins (Flora *et al.* 1998). In previous papers, it was shown that, although in very low amounts, individual Sm components are found in the extracellular medium of cell cultures of this species. Treatment of cultures with MJ improve Sm production and release into the medium (Sánchez-Sampedro *et al.* 2005). MJ also increases PLD activity and it was suggested that PLD and its product PA mediate Sm secretion to the medium of *S. marianum* cultures (Madrid and Corchete 2010).

Based on these evidences, we further exploited the usefulness of elicitation with MJ for improving Sm extracellular accumulation as a bioassay to explore the possible involvement of PLA2 (EC 3.1.1.4) in Sm release. For this, a pharmacological approach with exogenously added FA and PLA2 specific inhibitors was employed. Experiments were also conducted to determine if elicitation altered PLA2 activity in cell cultures.

Materials and methods

Chemicals, plants and treatments: MJ, aristolochic acid (AA), bromoenol lactone (BEL), fatty acids (FAs), PLA2 from honey bee venom, and PLC from *Clostridium perfringens* were from *Sigma-Aldrich* (St Louis MO, USA). NBD-phosphatidylcholine (NBD-PC) was from *Avanti Polar Lipids* (Alabaster, USA). The cell line used was established from *Silybum marianum* (L.) Gaertn. hypocotyl-derived callus and was grown on Murashige and Skoog (1962; MS) medium supplemented with 30 g dm^{-3} sucrose, 1 mg dm^{-3} 2,4-dichlorophenoxyacetic acid, and 0.5 mg dm^{-3} benzyladenine at pH 5.6. Cultures were shaken at 90 rpm in darkness and subcultured every 2 weeks. Cell viability was checked by differential staining with fluorescein diacetate (Widholm 1972).

For experiments, 100 cm^3 flasks containing 20 cm^3 of the medium were inoculated with 2 g (fresh mass) of cells taken from the previous subculture. After 3 d, when cells have already started the active growth phase, elicitation was performed with 100 µM MJ prepared as a stock solution in DMSO. FA, AA, and BEL were also dissolved in DMSO. Commercial PLs were dissolved in water. Controls received equivalent volumes of solvent. After 48-h treatment, Sm was extracted from the extracellular medium and analysed by HPLC as described below. Experimental work was performed at least in triplicate and results are expressed as means ± SD. Differences were tested for significance using Duncan's multiple range test (DMRT).

Flavonolignan analysis: The culture medium was separated from the biomass by filtration and flavonolignans were extracted three times with two volumes of ethylacetate. The combined extracts were dried in vacuum below 40 ºC and resuspended in 1 cm^3 of methanol. Naringenin (0.1 mg) was used as internal standard. Analysis was performed by HPLC with a *Spherisorb ODS-2* (5 µm) reversed-phase column (4.6 × 250 mm). The mobile phase was a mixture of 34 volumes of methanol and 66 volumes of acetic acid/water (5/55, v/v), rate of 1 cm^3 min^{-1}, and detection at 277 nm. Identification of flavonolignans was achieved by comparison with commercial standards and by LC MS (*MSD trap XCT* and *LC 1100* both from *Agilent*® (Madrid, Spain), in a *Spherisorb S3 ODS2* column (2 × 100 mm, 3.5 µm) in E.S.I (-) mode under the same conditions as reported for HPLC analysis of flavonolignans.

Enzyme activities: Activity of PLA2 was assayed using NBD-PC as substrate. For measurement of *in vivo* PLA2 activity, aliquots of 2 cm^3 of suspended cells were preincubated with 2.5 µg cm^{-3} NBD-PC in a multiwell plate at 4 ºC for 4 h. Plates were then incubated at 25 ºC with MJ at different concentrations for the indicated times. NBD-labeled FAs were extracted with 2 cm^3 of chloroform/ethanol (1:2, v/v). Chloroform (0.5 cm^3) and 2 M KCl (0.5 cm^3) were added, vortexed, and centrifuged at 15 000 *g* for 5 min and the lower lipid phase dried under vacuum. The dried phase was dissolved in 0.02 cm^3 of chloroform/methanol (95:5, v/v) and analyzed by thin layer chromatography (TLC). Samples were spotted onto TLC silica gel G plates and developed with chloroform/ methanol/acetic acid (95:5:0.5, v/v/v). Labeled lipids were visualized under UV radiation, the spots corresponding to NBD-FA were scraped from the plates and placed in 0.6 cm^3 of chloroform/methanol/water (5:5:1, v/v/v), vortexed, and centrifuged at 15 000 *g* for 5 min. The fluorescence (excitation 460 nm, emission 534 nm) was measured in a *Perkin–Elmer* (Madrid, Spain) *LS 50B* spectrofluorimeter.

Determination of *in vitro* PLA2 activity was done according to Wittenauer *et al.* (1984) with some modifications. In brief, PLA2 was extracted from cells (fresh mass 1 g), homogenized with 3 cm^3 of extraction buffer (50 mM Tris-HCl, 5 mM $CaCl_2$ at pH 8.0) and centrifuged at 10 000 *g* and 4 ºC for 30 min. Protein content was determined by the method of Bradford (1976). The standard assay mixture (1 cm^3) contained 0.05 cm^3 of NBD-PC (24 nM) dissolved in the extraction buffer and a varied volume of the enzyme extract. The reaction was started by addition of the crude extract and fluorescence was continuously monitored at 30 ºC as a function of time. Fluorescence excitation and emission wavelengths were 460 nm and 534 nm, respectively. The calibration curve was established using NBD obtained after hydrolysis of C6-NBD-PC with PLA2.

Results

In order to test whether the PLA2 activity was altered by the treatment of the cultures with MJ, the *in vivo* PLA2 activity was measured in the cells prelabelled with NBD-PC, incubated in the pure medium or the medium with MJ (100 μM), and then lipids were extracted, separated by TLC, and the fluorescence of the spots corresponding to the fluorescent hexanoic acid recorded. The constitutive PL activity of the *S. marianum* cultures was rather high as demonstrated by hydrolysis of NBD-PC to NBD-DAG and NBD-FA. Since only FA at position 2 in the NBD molecule bears a fluorescence label, the PLA2 activity was detected solely with this substrate. Additionally, an unidentified lipids were found (some minor fluorescent spots appeared on the plates) but they were not investigated further (Fig. 1). Although NBD-PC was degraded with time, accumulation of fluorescent NBD-FA was higher in the MJ-treated cultures. The differential accumulation of this metabolite in the

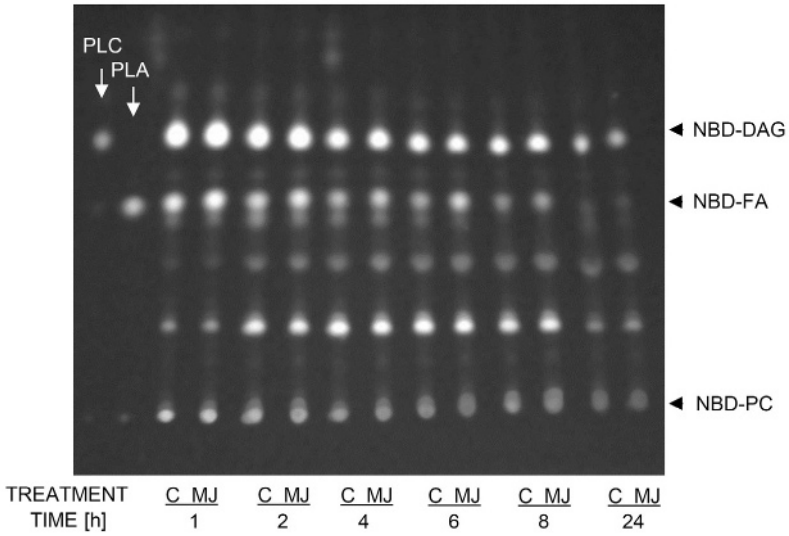

Fig. 1. PLA2 activity, measured as NBD-FA formation, in cell cultures of *Silybum marianum* treated with MJ. The fluorescence image of a TLC plate shows NBD-products after incubating the cell cultures without (C) or with 100 μM MJ for different time periods. The TLC image also shows NBD-products formed by incubating NBD-PC with 5 units of commercial PLC (*arrow*) from *Clostridium perfringens* or commercial PLA (*arrow*) from bee venom at 30 ºC for 30 min. The positions for NBD-PC, NBD-FA, and NBD-diacylglycerol (DAG) are indicated.

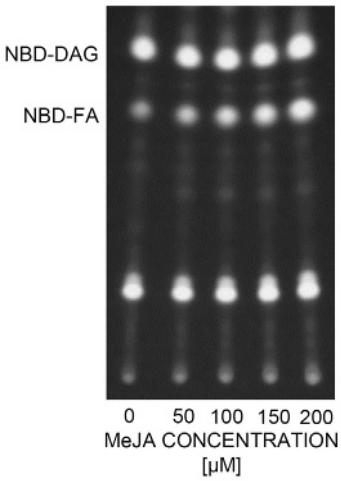

Fig. 2. Changes in content of NBD-FA in cell cultures of *Silybum marianum* in response to several concentrations of MJ. The fluorescence image of a TLC plate shows NBD-FA after incubating the cell cultures with different concentrations of MJ. The cells were loaded with NBD-PC as described in the Materials and methods. Samples were taken 6 h after the MJ treatment.

elicitor-treated samples is indicative of an activa-tion of PLA2. The difference could be noticed 1 - 2 h after the treatment and lasted over the studied period (Fig. 1). The rate of fluorescent FA increased with increasing doses of MJ (Fig. 2).

Phospholipase activity against NBD-PC was also measured by an *in vitro* assay using the cell extracts obtained from the MJ-elicited cultures at different times. The enzymatic activity stayed constant until 30 min after the elicitation (Fig. 3). Then, a progressive increase was detected up to 8 h of the MJ treatment and the activity remained high for the rest of the period studied.

To assess if the increase of the PLA2 activity was associated with the MJ-induced secretion of Sm compounds, the control and MJ-elicited cultures were treated with the PLA2 inhibitors AA and BEL. Both inhibitors are frequently used to identify PLA2 roles in cells (Rosenthal *et al.* 1989, Winstead *et al.* 2000). AA at concentration of 10 μM blocked Sm accumulation in the MJ-elicited cultures. BEL also had an inhibitory effect but concentration of 20 mM was necessary to supress extracellular Sm accumulation caused by its elicitation with MJ. The basal extracellular Sm content

seen in the control cultures was also reduced with the AA and BEL treatments, but the extent of their inhibitory effect in the absence of MJ was less than their effect on the MJ-elicited accumulation (Table 1). This result confirmed that the MJ-induced Sm accumulation was sensitive to the PLA2 inhibitors.

In the *S. marianum* cells in suspension, saturated FAs, which were represented by palmitic acid, miristic acid, and stearic acid, were the most abundant (75 %). Among the unsaturated FAs, palmitoleic acid, linolenic acid, linoleic acid, and oleic acid were the main representatives (Cacho *et al.* 2012). The addition of linoleic or linolenic acids at concentration of 100 µM enhanced the secretion of Sm, whereas miristic, palmitic, stearic, and oleic acids were ineffective. Inclusion of NBD-PC (100 µg per flask) into the cultures for 48 h did not alter Sm production, thus indicating that the C6 FA was also ineffective (Fig. 4). It should be noted that growth of the cells treated with MJ or FA for 48 h was not measurably affected at the concentrations employed (data not shown). However, the addition of FA partially rescued the inhibition of the Sm accumulation induced by 10 µM AA in both the control and MJ-treated cultures (Fig. 4).

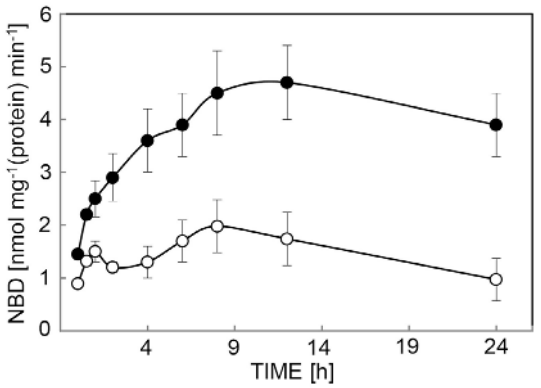

Fig. 3. Quantification of PLA2 activity in *Silybum marianum* cultures untreated (*open circles*) or treated (*filled circles*) with MJ for several time periods. Protein extracts were assayed for activity using NBD-PC as substrate. Values are means of five independent experiments ± SD.

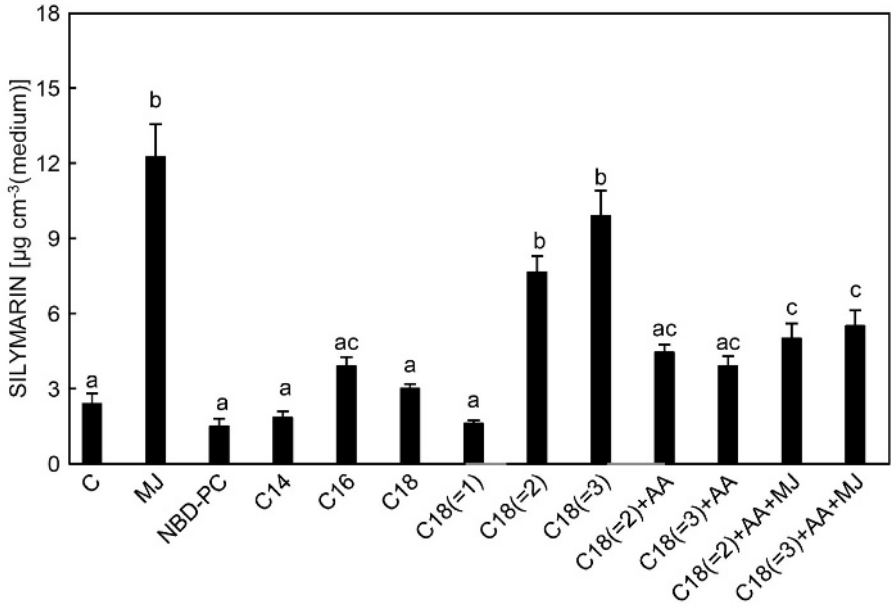

Fig. 4. Effect of different FA on Sm production in *Silybum marianum* cultures. Three-day-old cell cultures were treated with 100 µM MJ or 100 µM miristic (C14), palmitic (C16), stearic (C18), oleic [C18 (=1)], linoleic [C18 (=2)] or linolenic [C18 (=3)] acids. To analyze the effect of FAs in the control or MJ-treated cultures under PLA2 inhibition, the suspensions were preincubated with FA for 20 min and then with 10 µM AA for 20 min before addition of 100 µM MJ or an equivalent volume of DMSO. Sm was extracted from the culture medium after an incubation period of 48 h. The values represent the average of three replicate experiments ± SD. Values followed by the same letter are not significantly different ($P > 0.05$) as determined by Duncan's multiple range test.

Discussion

As mentioned in the introduction, phospholipids seem to be mediators of extracellular Sm accumulation in *Silybum marianum* cell cultures. Further support is given in this study with the observation that PLA2 was necessary for the release of this group of secondary metabolites. Although a mechanism by which Sm accumulates in extracellular medium is presently unknown, the observations that Sm exudation was abolished by the treatment with the PLA2 inhibitors AA and BE and that addition of exogenous FA stimulated silymarin secretion,

Table 1. Effect of inhibitors of PLA2 activity on Sm production [μg cm^{-3}(medium)] in cell cultures of *Silybum marianum*. Three-day-old cell cultures were preincubated for 20 min with AA (1, 5, or 10 μM) or BEL (5, 10 or 20 mM). The cells were subsequently treated with 100 μM MJ. Control cultures received 10 μM AA or 20 mM BEL. Sm was extracted from the culture medium after an incubation period of 48 h. Means of three replicate experiments ± SD are given. Values followed by the same letter are not significantly different ($P > 0.05$) as determined by Duncan's multiple range test.

Control	C+AA10	C+BEL20	MJ	MJ+AA1	MJ+AA5	MJ+AA10	MJ+BEL5	MJ+BEL10	MJ+BEL20
2.4±0.03a	1.0±0.20a	1.7±0.23a	12.25±2.4b	3.9±0.64	1.0±0.18a	0.3±0.04a	12.35±0.15b	10.5±0.13b	0.85±0.09a

points to an important role of PLA2 in the Sm release.

Additionally, we show that the enhanced Sm release caused by the MJ elicitation seems at least to occur *via* induced PLA2 activity. Activation of PLA2 by biotic elicitors has been observed in several plant cell cultures (Roy *et al.* 1995, Chandra *et al.* 1996, Yoon *et al.* 2000, Laxalt and Munnik 2002), and some studies has suggested that PLA2 is involved in elicitor-induced secondary metabolite accumulation. PLA2 hydrolyses the *sn*-2 fatty acid ester bond of a phospholipid to produce free FA and lyso-phospholipid, and both the products have been able to increase production of secondary metabolites in plant cells (Roos *et al.* 1999, Viehweger *et al.* 2002, Scherer 2010).

By studying transcription, it has been reported that the exposure to MJ stimulates expression of PLA2 genes in sorghum (Salzman *et al.* 2005*)* and *Arabidopsis* (Narusaka *et al.* 2003). Although MJ is one of the best studied elicitors of plant secondary production, few studies have been performed to determine whether phospholipid signaling is connected with MJ-stimulated secondary metabolite production. In a recent paper, Altuzar-Molina *et al.* (2011) have reported that treatment with MJ produced increases in PLC and PLD activities and the production of vanillin, a precursor of capsaicinoids. By contrast, elicitation of *T. cuspidata* with MJ did not markedly increase FA production which suggests that the PLA2 pathway might not contribute to the enhancement of taxol production observed in MJ-induced *Taxus* cells (Yang *et al.* 2008). Other observations show that PLA2 activity is poorly induced in MJ-treated tobacco or tomato leaves (Narváez-Vásquez *et al.* 1999, Dhondt *et al.* 2002). The observed different effects of the PLA2 activation by MJ could reflect the specific gene expression of different types of cells and the complexity of MJ-triggered phospholipase signaling in diverse biological systems.

The stimulating effect of linoleic and linolenic acids on Sm accumulation may be important. Both FAs could be considered as potential elicitors of secondary metabolites in plant tissue cultures. For instance, their biological activity has also been demonstrated in cultures of *Solanum lycopersicum*, *Tinospora cordifolia*, *Erythrina crista-galli*, *Eschscholzia californica*, and *Panax ginseng* (Wu *et al.* 2009). FA or their derivatives can be envisaged as lipidic second messengers. Alternatively, it is also generally thought that the FA-induced promotion of secondary metabolite accumulation is probably due to stimulation of endogenous jasmonic acid biosynthesis *via* an inducible octadecanoid pathway (Weber 2002, Kazan and Manners 2008). Several parameters, such as FA concentration, duration of exposure, age of culture, cell line, or combination with other elicitors should be tested in the near future in order to optimize Sm induction.

Taken together, our results demonstrate that in the *S. marianum* suspension cultures, the accumulation of Sm depended on the activity of PLA2, and implicate that MJ, besides activating a range of downstream signaling events, stimulated secondary metabolite release by acting on this enzyme. Additional biochemical and genomic studies would help to elucidate the importance of PL and their products in the mechanisms underlying Sm release in cell cultures of *S. marianum*.

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