Silymarin secretion and its elicitation by methyl jasmonate in cell cultures of *Silybum marianum* is mediated by phospholipase D-phosphatidic acid

Elena Madrid and Purificación Corchete*

Department of Plant Physiology, Campus Miguel de Unamuno, University of Salamanca, E-37007 Salamanca, Spain

* To whom correspondence should be addressed: E-mail: corchpu@usal.es

Abstract

The flavonolignan silymarin is released to the extracellular medium of *Silybum marianum* cultures and its production can be stimulated by the elicitor methyljasmonate (MeJA). The sequence of the signalling processes leading to this response is unknown at present. It is reported in this work that MeJA increased the activity of the enzyme phospholipase D (PLD). Treatment with mastoparan (Mst), a PLD activity stimulator, also enhanced PLD and caused a substantial increase in silymarin production. The application of the product of PLD activity, phosphatidic acid (PA) promoted silymarin accumulation. Altering PLD activity by introducing in cultures *n*-butanol (*n*BuOH), which inhibits PA production by PLD, prevented silymarin elicitation by MeJA or Mst and also impeded its release in non-elicited cultures. Treatment with iso-, sec- or tert-*butanol* had no effect on silymarin production. The exogenous addition of PA reversed the inhibitory action of *n*BuOH, both in control and MeJA-treated cultures. These results suggest that the enzyme PLD and its product PA mediate silymarin secretion to the medium of *S. marianum* cultures.

Key words: Cell cultures, elicitation, phospholipase D, phosphatidic acid, signalling, silymarin.

Introduction

Silymarin is a constitutive natural compound composed of an isomeric mixture of the flavonolignans silychristin, iso-silychristin, silydianin, silybin A, silybin B, isosilybin A, and isosilybin B (Morazzoni and Bombardelli, 1995; Kim *et al.*, 2003; Lee and Liu, 2003). Silymarin accumulates mainly in the external cover of fruits of the milk thistle *Silybum marianum* (L.) Gaertn, and the levels can vary on a dry weight basis from 1% to 9% in selected varieties (Omidbaigi and Nobakht, 2001). Although its action in the plant is presently unknown, extracts of the fruit are used for the treatment of liver diseases and liver cirrhosis (Valenzuela *et al.*, 1986; Flora *et al.*, 1998) and clinical tests have extended its therapeutic application to other diseases such as cancer since there is strong preclinical evidence for silymarin’s anticarcinogenic effects, including the inhibition of cancer cell growth in human prostate, skin, breast, and cervical cells (Post-White *et al.*, 2007).

Silymarin levels in *in vitro* cultures are much lower than those detected in the fruit and its individual components are predominantly found in the extracellular medium. Elicitation is one of the best approaches for enhancing the production of secondary metabolites from medicinal plants and it has been shown that elicitors affect plant secondary metabolism by modulating the rates of biosynthesis, accumulation, and/or vacuolar transit, turnover, and degradation (Barz *et al.*, 1990). This strategy has also been applied to *S. marianum* cultures and it has been demonstrated that treatment of suspensions with methyljasmonate (MeJA) improved silymarin production and its release into the culture medium to a level about 3-fold higher than that of the control (Sánchez-Sampedro *et al.*, 2005a). The sequence of signalling processes that follows perception of the elicitor by plant cells and ends in the synthesis and transport of secondary metabolites has been the subject of extensive studies and continues to be a topic of active research (Zhao and Sakai, 2003; Zhao *et al.*, 2005). In most studies, calcium influx and a transient calcium pulse in the cytosol have been shown to be necessary and sufficient for defence gene
activation and phytoalexin accumulation. In addition, it has been demonstrated that molecules such as H₂O₂ produced as a result of an oxidative burst (Zhao et al., 2001), are also directly involved in elicitor-induced metabolite synthesis. All these events are suggested to be dependent on protein phosphorylation/dephosphorylation mechanisms that resemble those described in animal systems (Romeis et al., 1999).

In previous work with *in vitro* *S. marianum* cell cultures, it was shown that an external source of calcium or alterations in internal calcium fluxes were not required for the elicitation to occur. The increase in silymarin induced by elicitation was suppressed neither by common inhibitors of protein phosphatases nor by protein kinase inhibitors. No H₂O₂ generation was detected at any time after elicitation; also diphenylene ionisation, a potent inhibitor of NAD(P)H oxidase, did not block silymarin production in elicited cultures (Sánchez-Sampedro et al., 2008). The signalling mechanism by which elicitors increase silymarin in *S. marianum* therefore remains unknown. In view of the intricate network of pathways mediating stimuli, signalling pathways other than those studied needed to be explored. By using one- and two-dimensional nuclear magnetic resonance spectroscopy, an elevation of choline in elicited *S. marianum* cultures was detected (Sánchez-Sampedro et al., 2007). Choline, generally present in a bound form as the phospholipid phosphatidylcholine (PtCho), is a vital component of cell membranes in most eukaryotes and comprises 40-60% of the total phospholipid content of non-plastid membranes in plants. Although there is no evidence for this, in our cultures the choline increase could possibly be due to an elicitor effect on membranes.

Phospholipase D (PLD) cleaves phospholipids into phosphatidic acid (PA) and free-head groups such as choline. It is believed that PLD and its product PA, (which can also be generated via the combined action of phospholipase C (PLC) and diacylglycerol kinase (DGK)), play important roles in various plant responses including those related to silymarin secretion (Yang et al., 2007). PA is believed that PLD and its product PA, (which can also be generated via the combined action of phospholipase C (PLC) and diacylglycerol kinase (DGK)), play important roles in various plant responses including those related to silymarin secretion (Yang et al., 2007). PA is believed.

**Materials and methods**

**Chemicals and plant material**

MeJA and PA (1,2-di-octanoyl-sn-glycerol-3-phosphate (OcPA), 1,2 di(cis-9-octadecenoyl) sn-glycerol-3-phosphate (OIPA), PA from egg yolk lecithin (PA), prepared from 1-α-phosphatidylcholine by hydrolysis with cabbage phospholipase D, according to the manufacturer), and phospholipase D enzyme from cabbage were from Sigma-Aldrich (St Louis MO, USA). NBD-phosphatidycholine (NBD-PtCho) was from Avanti Polar Lipids (USA).

The cell line used was established from *Silybum marianum* (L) Gaertn hypocotyl-derived callus. The growth medium was the same as that described in Sánchez-Sampedro et al. (2005a). Cultures were shaken at 90 rpm and subcultured every 2 weeks.

Cell viability was checked by differential staining with fluorescein diacetate (Widholm, 1972).

**Treatments**

Elicitation was performed with 100 μM MeJA, prepared as a stock solution in ethanol (concentration of ethanol in cultures was 0.05%) 3 d after subculture. Controls received equivalent volumes of solvent. After 48 h treatment, silymarin was extracted from the extracellular medium and analysed by HPLC as described in a previous report (Sánchez-Sampedro et al., 2005b). Experimental work was performed in triplicate.

**PLD assays**

PLD *in vitro* activity was essentially analysed as in Ritchie and Gilroy (1998) after Wang et al. (1993). In brief, PLD was extracted from cells (1 g FW), homogenized with 1 ml extraction buffer (50 mM TRIS-acetate, 5 mM EDTA, pH 8.8, 1 mM dithiothreitol, 50 mM CaCl₂, 0.25 mM SDS, 5 mM phenylmethyl sulphonyl fluoride and centrifuged at 4000 g for 10 min. Protein concentration was determined by the method of Bradford (1976).

The standard assay mixture contained 20 mM MES/NaOH (pH 6.5), 50 mM CaCl₂, 0.25 mM SDS, 5 μl 1-hexadecanoyl-2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]hexanoyl-sn-glycerol-3-phosphocholine (NBD-PtCho) fluorescent substrate (1.2 nmol), 1% (v/v) nBuOH and a varied volume of extract; the final volume of the reaction was 50 μl. The reaction was initiated by the addition of the substrate and incubated at 30 °C for 30 min with shaking (100 rpm). The reaction was stopped by the addition of 150 μl chloroform:methanol (1:2, v/v). Chloroform(50 μl) and 50 μl 2 M KCl were added, the mixture was vortexed and centrifuged at 15 000 g for 2 min. The phases were separated and 100 μl chloroform was added to the aqueous phase, vortexed, and centrifuged again at 15 000 g for 2 min, and the lower chloroform phases from each step pooled. Each sample was dried under a stream of N₂ and 20 μl chloroform:methanol (95:5, v/v) added. PLD activity was measured as the production of NBD-phosphatidylbutanol (PtBuOH) and NBD-PA in each sample, determined by TLC, as described below.

For *in vivo* measurement of PLD, aliquots of 2 ml suspended cells were preincubated with 25 μg ml⁻¹ NBD-PtCho in a multwell plate at 4 °C for 4 h. Plates were then incubated at 25 °C with nBuOH (0.2% v/v) for 20 min prior to treatment with MeJA (10, 100, 200, or 300 μM) or Mastoparan (Mst) (10 μM) for the indicated times. NBD-labelled PA and PtBuOH were extracted with 2 ml chloroform:methanol (1:2 v/v). Chloroform (300 μl) and 500 μl 2 M KCl were added, vortexed, and centrifuged for 5 min at 15 000 g and the lower lipid phase dried under vacuum. The dried
Results

Phospholipase activity against NBD-PtCho was measured first by an in vitro assay using cell extracts obtained from MeJA-elicited cultures at different times. Lipids were extracted and separated by TLC, and fluorescence spots were analysed; the reporter alcohol nBuOH at 1% was also introduced in the assay as a specific measure of PLD activity.

At the concentration of the substrate and incubation time employed in in vitro assays, two fluorescent spots could be quantified which correspond to NBD-PA and NBD-PtBuOH, respectively, fluorescence corresponding to PLC or phospholipases A1 and A2 activity products being negligible. PLD activity increased substantially in elicited cultures (Fig. 1), as demonstrated by the rise in NBD-PtBuOH. The fluorescence increase was detected after 30 min of MeJA treatment, reached a maximum at 6 h and remained high for the rest of the period studied. Experiments done by introducing 10 μM of Mst, a known stimulator of PLD activity in plants, into control cultures (Munnik et al., 1995; de Vrije and Munnik, 1997) resulted in a more marked increase of PLD activity than that observed in MeJA-elicited cultures (see also Fig. 1). When the inactive Mst analogue 17 was used, no particular increase in PLD products was observed (data not shown).

To evaluate in vivo PLD activity, cells, labelled with NBD-PtCho at 4 °C for 4 h, were incubated with medium, MeJA (100 μM) or Mst (10 μM), and transferred to 25 °C; nBuOH at 0.2% was also included in all treatments. After different time periods, lipids were extracted, separated by TLC, and the fluorescence of spots recorded. A significant increase in the production of NBD-PA in the presence of Mst occurred at 30 min and also the appearance of NBD-PtBuOH in cells confirmed that the PLD pathway was active. At longer times, both fluorescence spots were also noted in control and MeJA-treated cells, the fluorescence intensity in this latter treatment being higher than in the controls. As shown in Fig. 2, the NBD-PA and NBD-PtBuOH formation in Mst- or MeJA-treated cells increased up to 6 h and gradually decreased until 24 h but remained higher than in the control. The fluorescence intensity of NBD-PtBuOH in in vivo experiments was much lower than that detected in in vitro assays, in which 1% nBuOH was included. It should be noted that, in certain plant systems, high doses of nBuOH are employed for the in vivo determination of PLD activity without impairing cell viability (i.e. 0.5% in cultured tobacco cells (Dhonukshe et al., 2003) or 0.75% in leaves of Craterostigma plantagineum (Frank et al., 2000)).

Is there a role of PLD and PA in MeJA-induced silymarin accumulation?

As MeJA elicitation or Mst treatment stimulated PLD activity, the question arises as to whether this response was associated with silymarin production. Therefore, a set of experiments were conducted in cultures.

The addition of Mst to suspensions strongly enhanced silymarin production (Fig. 4) and the increase was higher than that induced by MeJA. Treatment with the same concentration of the inactive analogue Mst 17 was ineffective. When cultures were treated with nBuOH 20 min prior to the addition of MeJA or Mst, the alcohol, at 0.2% concentration, diminished silymarin accumulation in elicited cultures. Staining with the vital dye fluorescein diacetate revealed no loss of viability in nBuOH-treated cells (data not shown). Treatment of cultures with the alcohol alone also impeded silymarin secretion into the culture medium.

The inclusion of sec-BuOH, iso-BuOH or tert-BuOH, alcohols that do not inhibit PLD-catalysed PA formation, in both control (data not shown) and MeJA treated cultures, did not modify silymarin production.

To gain further insight into the role of PLD, a study was carried out to establish whether exogenously supplied PA could reverse the inhibitory action of nBuOH or could emulate the elicitor effect of MeJA on the induction of silymarin. For this, cultures were treated with PA from egg yolk lecithin, OcPA, and OIPa, added alone to 0.2% nBuOH-treated cultures and to cultures treated simultaneously with MeJA and nBuOH. As shown in Fig. 5, individually, PA (100 μg ml−1 culture), or 100 μM OcPA or OIPa stimulated silymarin production with no apparent preference for any of the PA employed; the concentrations of the different PA were also sufficient to reverse the inhibitory action of the primary alcohol on the secretion of silymarin induced by MeJA (Fig. 5).
Discussion

Signal-activated phospholipases and lipid kinases together represent the molecular basis for the growing field of lipid signalling in plants. The activity of these enzymes is stimulated upon receptor ligation by agonists, resulting 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). Much recent evidence suggests that PLD plays a role in multiple cellular and physiological processes including signal transduction, vesicular trafficking, cytoskeletal rearrangements, membrane remodelling, and membrane lipid degradation (Munnik et al., 1998; Wang, 2004; Testerink and Munnik, 2005). PLD converts PtCho to choline and PA which may affect several plant processes via various intracellular targets and by influencing the biophysical state of lipid membranes (Bargmann and Munnik, 2006).

Because PLD catalyse a transphosphatidylation reaction utilizing short-chain primary alcohols as phosphatidyl-group acceptors, the formation of phosphatidylalkohols has served as a convenient and sensitive marker for PLD activation in cultured cells. Furthermore, alcohols have been used to probe the involvement of PLD in various regulatory processes, because of their ability to decrease PA formation by shunting phosphatidyl moieties into phosphatidylalkohols (Ella et al., 1997).

By using this approach, it is shown in this paper that production of PA by PLD is necessary for the release of silymarin compounds into the culture medium of Silybum marianum suspended cells and that silymarin elicitation by MeJA seems to occur via induced PLD activity. Primary alcohols, which in some systems stimulate rather than inhibit PLD activity, may modify other elements besides PLD in the membrane environment (Wang, 2000; Hirase et al., 2006), thus, an effect associated with an alcohol treatment needs to be interpreted with caution because it may result from other alcoholic effects, such as increases in lipid hydrolysis, changes in lipid composition, and/or release of lipid head groups that have regulatory functions (Chapman, 2004). In addition, PA is also produced through sequential action of PLC and DGK (Testerink and Munnik, 2005). However, the lack of effect of secondary and tertiary alcohols on silymarin production/secretion, and the emulation of the described responses by exogenously added PA.
Phospholipase D-phosphatidic acid as mediators of silymarin secretion in cell cultures of *Silybum marianum*

**Fig. 2.** *In vivo* PLD activity, measured as NBD-PtBuOH and NBD-PA formation in cell cultures of *Silybum marianum* treated with MeJA or Mst for different time periods. (A) Fluorescence image of a TLC plate showing NBD-PA and NBD-PtBuOH extracted from cell cultures untreated (treatment 1), treated with 100 μM MeJA (treatment 2) or 10 μM Mst (treatment 3) for the indicated times. (B) Fluorescence image of a TLC plate showing NBD-PA extracted from cell cultures treated with 100 μM MeJA in the presence or absence of 0.2% n-BuOH for the indicated times. Cells were loaded with NBD-PtCho as described in the Materials and methods. (C, D) Quantification of NBD-PA and NBD-PtBuOH levels in cultures treated with MeJA (filled circles) or Mst (filled squares); (open circles) control. NBD-PA levels in the absence of nBuOH in MeJA-treated cultures are not shown since they overlapped with those obtained in the presence of the alcohol. Values represent the average of three replicate experiments ±SD.

**Fig. 3.** Changes in levels of NBD-PA and NBD-PtBuOH in cell cultures of *Silybum marianum* in response to several concentrations of MeJA. (A) Fluorescence image of a TLC plate showing NBD-PA and NBD-PtBuOH extracted from cell cultures treated with different concentrations of MeJA. Cells were loaded with NBD-PtCho as described in the Materials and methods. Samples were taken 6 h after MeJA treatment. (B) Quantification of NBD-PA (filled circles) and NBD-PtBuOH (open circles) levels in cultures treated with several concentrations of MeJA. The values represent the average of three replicate experiments ±SD.

indicate that the elicitor MeJA seems to activate silymarin production through a PLD-dependent increase in PA in the *S. marianum* model. The mechanism by which PLD activity is enhanced by MeJA remains to be investigated. Without excluding that Mst could directly activate PLD as it has been shown for animal cells (Mizuno et al., 1995), Mst-induced PLD stimulation suggests the possibility that PLD is activated by an intermediate such as G protein, as seen in many mammalian PLD-based signal transduction pathways (Cabrera-Vera et al., 2003). G protein activation of PLD has also been reported in *Chlamydomonas* (Arisz et al., 2003), and there are extensive data implicating G proteins as intermediates in signal transduction in plant cells (Assmann, 2002; Mishra...
replicate experiments. The values represent the average of three preincubated for 20 min with 0.2% nBuOH before the addition of OcPA or 100 l M MeJA. 3-d-old cell cultures were treated with 100 l M MeJA, 10 l M Mst, or 10 l M Mst17. Silymarin was extracted from the culture medium after an incubation period of 48 h. The values represent the average of three replicate experiments ± SD.

Fig. 4. Effect of modulators of PLD activity on silymarin production in cell cultures of Silybum marianum. Three-day-old cell cultures were preincubated for 20 min with 0.2% nBuOH, sec-BuOH, iso-BuOH or tert-BuOH or untreated (control). The cells were subsequently treated with water, 100 l M MeJA, 10 l M Mst, or 10 l M Mst17. Silymarin was extracted from the culture medium after another incubation period of 48 h. The values represent the average of three replicate experiments ±SD.

Fig. 5. Effect of different PA on silymarin production in Silybum marianum cultures. 3-d-old cell cultures were treated with 100 l M MeJA, 100 l M lecithin PA (PA), 100 l M dioleoyl PA (OcPA) or 100 l M dioleoylPA (OIP). To analyse the effect of PAs in control or MeJA cultures under PLD inhibition, suspensions were preincubated for 20 min with 0.2% nBuOH before the addition of effectors or MeJA. The values represent the average of three replicate experiments ±SD.

et al., 2006; Zhao and Wang, 2004). In this way, the phospholipase would function as an effector enzyme, transducing the presence of extracellular information into intracellular PA, a second messenger.

In contrast to the rapid activation of PLD in different cell systems treated with elicitors (Yamaguchi et al., 1995; Van der Luit et al., 2000), the PA and PtBuOH increase, measured as the formation of NBD-PA and NBD-PtBuOH in NBD-PtCho-labelled S. marianum cultures treated with MeJA, thus reflecting in vivo PLD activity, was not rapid nor transient, suggesting the induction of transcription rather than the activation of PLD. In other plants like Arabidopsis, genes encoding phospholipases exhibited different temporal patterns of expression during host response to virulent and avirulent pathogen challenge (de Torres Zabela et al., 2002); in rice, McGee et al. (2003) reported from a transcriptional analysis of PLD genes that RPLD1 was induced rapidly but transiently in wounded leaf tissues. RPLD2, also induced by wounding, was present at lower levels but steadily increased over 48 h. Although the functions of the individual PLD genes remain to be determined in plants, variations in transcription, expression, and biochemical activity indicate that these enzymes are regulated at many levels and suggests a tight control of their activity.

The mechanisms by which PA controls different physiological events in plant cells have been poorly investigated to date. PA may mediate cellular processes via several different modes of action. These include binding to its targeted proteins to increase or inhibit their activities; acting as a membrane anchor during the assembly of signalling proteins, or serving as a substrate for the production of other lipid regulators (Testerink and Munnik, 2005; Wang et al., 2006). It has been shown that changes in the PA level affect physical properties of cell membranes and their capacity of vesicle formation. Thus, PA may affect vesicle trafficking associated with exo- and endocytosis (Munnik, 2001; Wang et al., 2006; Roth, 2008).

With respect to this consideration, it should be pointed out that the silymarin components are accumulated in the pericarp of S. marianum achene fruit. During ontogenesis, following the pistil’s organization into fruit and seed, respectively, the biogenesis and accumulation of flavonolignans correlate with the lignification of the seed-coat (Bela, 2007) and therefore, the deposition of silymarin compounds should be mediated by secretion. In this way, the stimulating effect in S. marianum cultures of Mst, a potent secretagogue in animals (Ozaki et al., 1990), the limitation in silymarin release exerted by nBuOH in both control and elicited-cultures, and the demonstration that addition of exogenous PA stimulates silymarin secretion and reversed the inhibitory action of nBuOH, supports a role for PA in the secretion of this group of secondary compounds. Most secondary metabolites serve as communication signals between the plant and other living beings which share the same habitat and, for this purpose, many of these products are secreted to interact with other organisms. When secondary metabolites are synthesized in plant cell cultures, they are either secreted into the surrounding medium or stored intracellularly, which is a major impediment to efficient bioprocess design. Knowledge of the specificity of exportation systems is of great interest because of their basic and industrial relevance. Elicitation has been shown to induce the release of several intracellular compounds into the surrounding medium of cultured cells, as is the case of paclitaxel from Taxus chinensis cell cultures (Zhang et al.,
2007); however, information on secretion mechanisms is still very limited. A number of reports have recognized that PLD and its product PA is a key player in the regulation of secretion in mammals (Cummins et al., 2002), although its precise role remains a research question. In plant cells this is an emerging field and only in recent years have important studies focused on this aspect (Bargmann and Munnik, 2006; Munnik and Testerink, 2009). The results shown in this paper provide additional evidence about the activation of a PLD-PA pathway in an elicited plant cell system and its contribution to the secretion of secondary metabolites. Further lipidomic, biochemical, and genomic studies would help to elucidate the importance of PLD and PA in the mechanisms underlying silymarin elicitation.

Acknowledgement

This work was financed by Ministerio de Ciencia e Innovación (BFU2008-02876/BFI) Spain.

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