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Transport of flavonolignans to the culture medium of elicited cell suspensions of *Silybum marianum*

Daniel Prieto, Purificación Corchete*

Department of Plant Physiology, Faculty of Biology, University of Salamanca, 37007 Salamanca, Spain

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ABSTRACT

Cell suspension cultures of *Silybum marianum* are able to excrete silymarin compounds into the medium upon elicitation with methyl jasmonate or cyclodextrins. Knowledge of transport mechanism is important to understand Sm metabolism and to develop strategies aimed at increasing production by means of cell cultures. For these reasons, a pharmacological approach was undertaken in this work in order to elucidate the possible mechanism involved in the release of this class of secondary metabolites into the extracellular medium of suspensions.

Treatment with an ionophore or NH_4Cl displayed little effect in elicited cultures, thus indicating that secondary transport, which uses electrochemical gradients, is not involved in the release.

Several inhibitors of ABC transporters showed differential effects. Sodium ortho-vanadate, a typical suppressor of ATPase activity, was highly toxic to cultures even at very low concentrations. The common Ca-channel blocker verapamil did not influence extracellular secondary metabolite accumulation. Glybenclamide and probenecid, both effective inhibitors of ABCC-type ABC transporters, strongly reduced silymarin secretion. A partial cDNA, SmABC1, which showed similarity to ABCC-type ABC transporters, was isolated by RT-PCR from silymarin-producing cultures. SmABC1 expression was enhanced by methyljasmonate and cyclodextrins.

Brefeldin A, a fungal metabolite which affects vesicular trafficking by preventing GTP/GDP exchange, inhibited release in a dose dependent manner.

These results suggest that excretion of silymarin and their precursors is a transporter-dependent active transport and that yet another mechanism involving a vesicle trafficking system seems to participate in driving this class of secondary metabolites to the extracellular compartment.

Introduction

Silymarin (Sm) is a group of flavonolignans that are accumulated in the external cover of the seeds (achene fruits) of the milk thistle *Silybum marianum*. This plant species is one of the oldest and most widely used in European traditional medicine, and has mainly been used for hepatic disorders. The antioxidative, anti-lipid peroxidative, antifibrotic, anti-inflammatory, membrane stabilizing, immunomodulatory and liver regenerating properties of Sm make it useful for hepatoprotection in hepatobiliary diseases and in hepatotoxicity due to drugs (Fraschini et al., 2002). New activities based on the specific receptor interactions have been reported, and there is a growing interest in its anticancer and chemopreventive effects which have been demonstrated in a large variety of illnesses of different organs, *e.g.* prostate, lungs, CNS, kidneys, pancreas and also the skin protection (Kren and Walterova, 2005; Gazak et al., 2007).

Sm is formed by oxidative coupling of the dihydroflavonol taxifolin (Tx) and the phenylpropanoid coniferyl alcohol (CA). The multiple orientation possibilities for coupling of the CA moiety to Tx give rise to the regioisomers of the Sm mixture silychristin, isosilychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B (Morazzoni and Bombardelli, 1995; Kim et al., 2003; Lee and Liu, 2003). Despite the number of articles concerning Sm chemistry and pharmacological uses, its biogenesis has been poorly investigated. To date, only one gene of the pathway has been cloned (GenBank: JN182806.1, unpublished). Apart from the lack of information on biosynthetic enzymes, the precise Sm compartmentation is also unknown.

Compared with the whole fruit, Sm production in plant cell cultures is very low (0.05–0.4% dry weight *versus* 1–3% in fruits) (Cacho et al., 1999; Alikaridis et al., 2000; Hasanloo et al., 2008); therefore, its biotechnological production is not commercially feasible.

Abbreviations: Brf, brefeldin A; CA, coniferyl alcohol; CD, cyclodextrin; Glb, glybenclamide; Gr, gramicidine; MeJA, methyl jasmonate; PB, probenecid; Sm, silymarin; Tx, taxifolin; Ver, verapamil.

* Corresponding author. Tel.: +34 923294531; fax: +34 923294515.
E-mail address: corchpu@usal.es (P. Corchete).

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We have documented previously that elicitation is a good strategy to increase Sm production (Sánchez-Sampedro et al., 2005). In these studies it was determined that methyljasmonate (MeJA) or cyclodextrins (CD), induced the release of Tx and CA with almost all of the isomers of the Sm mixture being also detected in the extracellular medium (Sánchez-Sampedro et al., 2005; Belchi-Navarro et al., 2011).

Most approaches to enhance secondary metabolite yields using plant cell cultures have been focused on the optimization of its biosynthesis. The study of other post biosynthetic events, like chemical or enzymatic modifications, transport, storage/secretion and catabolism/degradation are also biotechnologically relevant. Secretion of secondary metabolites is of particular interest since it avoids toxic effects in the cells and also facilitates downstream processing if cell cultures are to be used routinely for the commercial production (Navia-Osorio et al., 2002). Knowledge of transport mechanisms are still scarce, but their study is a developing research area due to its relevance to understanding metabolic fluxes and to develop strategies aimed at increasing secondary metabolite production.

Experimental data suggests that although transport of some secondary metabolites can occur by passive diffusion, as it is the case for camptothecin (Sirikantaramas et al., 2007), intracellular and intercellular transport of secondary metabolites are mediated in most cases by transporter proteins energized directly by MgATP (ABC proteins) (Yazaki, 2006), or by transporters energized by chemiosmotic coupling (MATE) (Yazaki et al., 2008). Yet another mechanism involving a complex vesicle trafficking system has been suggested to drive phytochemicals to their accumulation sites (Grotewold, 2004; Yazaki, 2005).

ABC transporters form a large superfamily of proteins whose diversity is believed to be related with the ample variety of secondary metabolites in plants, with many members of this family possibly being involved in the complex compartmentation of this class of compounds. The MATE family is also a large family with 56 members identified in the arabidopsis genome. However, only a few ABC or MATE transporters have been characterized concerning the transported substrates. These include, for instance, the berberine ABC transporters *Cj*ABCB2 of *Coptis japonica* (Shitan et al., 2013) or the anthocyanin ABC transporter *Zm*MDR3 of *Zea mays* (Goodman et al., 2004) and, among the MATE members, the Arabidopsis MATE transporter TT12 for flavonoids (Marinova et al., 2007) or the *antho*MATE1 an *antho*MATE3 of *Vitis vinifera* for anthocyanins (Gomez et al., 2009).

The extracellular accumulation of Sm compounds seen in elicited cell cultures of *S. marianum* plant species offers a good opportunity to initiate studies aimed at defining whether a specific transport mechanism is involved on Sm release. Although cell cultures are limited in that many of the phenotypic and functional characteristics of the original tissue may be lost due to culture conditions and the absence of endogenous factors and signals, these cell systems can provide specific information on the type of transporter(s) involved.

Despite showing poor specificity, the use of known transporter inhibitors or/and metabolic inhibitors is a common method of investigating particular transporter characteristics in cell biology. In the present work, this pharmacological approach was employed to study the effect of various inhibitors of transport processes on Sm excretion in cell cultures elicited with MeJA or CD.

Materials and methods

Cell cultures

Silybum marianum cell cultures were derived from cotyledon explants. Suspensions were routinely subcultured every 2 weeks by transferring approximately 7 g wet weight of cells of the previous culture, generated after mixing three parental flasks, to 50 mL of Murashige and Skoog medium (Murashige and Skoog, 1962), supplemented with 30 g/L sucrose, 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.5 mg/L benzyl adenine at pH 5.6. Cultures were incubated in the dark at 25 °C and shaken at 90 rpm.

Chemicals and culture treatments

Chemically pure heptakis (2,6-di-O-methyl)-β CD was purchased from Duchefa (Spain). All other compounds used in this study were from Sigma–Aldrich (Spain).

For experiments 3 g wet weight 14-day old cells were transferred to 100 mL flasks containing 20 mL medium and incubated for three days. Prior to the addition of CDs or MeJA (final concentration 30 mM or 100 μM, respectively), cultures were treated with inhibitors for 1 h. Stock solutions of sodium orthovanadate and NH_4Cl were prepared in water. Sodium orthovanadate was depolymerized before use according to the method of Goodno (1979). Probenecid (PB), glibenclamide (Glb), gramicidine (Gr), and verapamil (Ver) were dissolved in DMSO. Brefeldin A (Brf) was prepared in ethanol. A maximum of 20 μL of each solution was added to cultures. Controls received 20 μL of each solvent. Ethanol or DMSO did not affect secondary metabolite production or cell viability at this concentration. The viability of the cells was tested by staining with fluorescein diacetate according to Widholm (1972).

Flavonolignan analysis

At the required time periods, 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 vacuo* below 40 °C and resuspended in 1 mL methanol. Samples received 0.1 mg naringenin respectively as internal standard before extraction. Analysis was performed by HPLC in a Spherisorb ODS-2 (5 μm) reversed-phase column (4.6 mm × 250 mm). The mobile phase was a mixture of 34 volumes of methanol and 66 volumes of acetic acid:water (5:55, v/v) at 1 mL/min and detection at 277 nm (Sánchez-Sampedro et al., 2005). Identification of CA, Tx and Sm was achieved by comparison with commercial standards and by LC MS (MSD trap XCT and LC 1100 both from Agilent®), in a Spherisorb S3 ODS2 column (2 mm × 100 mm, 3.5 μm) in E.S.I. (−) under the same conditions as reported for HPLC analysis of flavonolignans.

Chromatograms of the ethylacetate extracts revealed a prominent peak at a retention time of 6.1 min. Mass spectra showed the presence in the same peak of a main representative ion $[M]^-$ at *m*/*z* 178.8 which correspond to CA and one minoritaire at 303.2. Molecular weight of Tx is 304.3 therefore the ion at 303.2 probably represented this flavonoid. However, due to its limited presence, its levels could not be quantified throughout this work and in the results, absolute values were given only for CA. Peaks at retention times other than 6.1 showing a molecular ion $[M]^-$ at *m*/*z* 481.2 corresponded to Sm.

The amount of flavonolignan type metabolites in the culture medium was separately given as the phenol precursor CA, and Sm, this latter represented as a mixture of isomers.

Isolation and quantification of a fragment of an ABC-like gene from cultured S. marianum cells

RNA was isolated using the Nucleospin RNA Plant kit (Macherey-Nagel, Germany) followed by quantification using a NanoDrop spectrophotometer (ND1000). RNA was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen, CA, USA). PCR was carried out with Taq DNA polymerase (Kappa Taq), using the DNA-RNA