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Metabolomic alterations in elicitor treated *Silybum marianum* suspension cultures monitored by nuclear magnetic resonance spectroscopy

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Abstract

A comprehensive metabolomic profiling of *Silybum marianum* (L.) Gaernt cell cultures elicited with yeast extract or methyl jasmonate for the production of silymarin was carried out using one- and two-dimensional nuclear magnetic resonance spectroscopy. With these techniques we were able to detect both temporal quantitative variations in the metabolite pool in yeast extract-elicited cultures and qualitative differences in cultures treated with the two types of elicitors. Yeast extract and methyl jasmonate caused a metabolic reprogramming that affected amino acid and carbohydrate metabolism; upon elicitation sucrose decreased and glucose levels increased, these changes being dependent on "*de novo*" protein synthesis. Also dependent on protein synthesis were the increase seen in alanine and glutamine in elicited cultures. Yeast extract differentially acted on threonine and valine metabolism and promoted accumulation of choline and α-linolenic acid in cells thus suggesting its action on membranes and the involvement of the octadecanoid pathway in the induction of silymarin in *S. marianum* cultures. Phenylpropanoid metabolism was altered by elicitation but, depending on elicitor, different phenylpropanoid profile was produced.

The results obtained in this study will permit in the future to identify candidate components of the signalling pathway involved in the stimulation of the constitutive pathway of silymarin.

Keywords: *Silybum marianum*; Flavonolignans; Metabolic difference; Methyl jasmonate; Yeast elicitor; Nuclear magnetic resonance spectroscopy

1. Introduction

Silymarin is a constitutive natural compound which accumulates in the fruits of the milk thistle *Silybum marianum*, and is composed of an isomeric mixture of the flavonolignans silychristin, isosilychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B (Morazzoni and Bombardelli, 1995; Kim et al., 2003; Lee and Liu, 2003). Although its physiological function in the plant is presently unknown, extracts of the fruit are widely used for therapy of liver diseases and liver cirrhosis (Valenzuela et al., 1986; Flora et al., 1998). In addition to its antioxidant properties, stimulating protein synthesis, and cell regeneration, silymarin is known to inhibit chemically induced carcinogenesis in a number of animal organs and shows direct anticarcinogenic activity against several human carcinoma cells (Zhao et al., 1999; Xiaolin et al., 1999; Yanaida et al., 2002; Agarwal et al., 2003). Tissue cultures derived from this species could be an alternative for the production of flavonolignans, however, up to now, few studies have been conducted and in all of them production was lower than in the fruit (Becker and Schrall, 1977; Ferreiro et al., 1991; Cacho et al., 1999).

Elicitors from physical or chemical origin have been widely employed to increase a target natural product formation in plant cell cultures and this strategy has been effective in stimulating the production of many chemical classes of secondary metabolites, such as terpenoids (Bostock et al., 1982), coumarin derivatives (Conrath et al., 1989), alkaloids (Tyler et al., 1989), and flavonoids (Tamari et al., 1995). Besides its biotechnological importance, elicitation has also been employed to dissect plant–microbe interactions and plant defence responses as well as signalling pathways involving elicitors (Scheel, 1998). In

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this sense, multiple components on the signal transduction pathway(s) have been described to regulate gene expression and biochemical changes; among them the involvement of jasmonic acid in the signal-transduction cascade that mediates a diverse suite of elicitor-induced responses is well documented (Sembdner and Parthier, 1993; Sudha and Ravishankar, 2002).

As recently published (Sánchez-Sampedro et al., 2005), elicitation is also effective on a secondary pathway which leads to the production of constitutive compounds which are not phytoalexins. Treatment of suspensions with a crude extract of yeast elicitor (YE) improved production of silymarin and caused the release of silymarin into the culture medium to a level about three-fold higher than that of the control. Jasmonic acid potentiated the yeast extract effect and one of the jasmonic acid derivatives, methyl jasmonate (MeJA), strongly promoted the accumulation of silymarin thus indicating that the octadecanoid pathway is presumably involved in elicitation responses. Methyl jasmonate seemed to act in a number of steps of the metabolic pathway of flavonolignans and its stimulating effect was totally dependent of "*de novo*" protein synthesis. Chalcone synthase activity was slightly enhanced by methyl jasmonate; however, there did not appear to be a temporal relationship between silymarin accumulation and increase in enzyme activity. Also, this increase was not blocked by the protein synthesis inhibitor cycloheximide (Sánchez-Sampedro et al., 2005). Therefore, the lack of knowledge of an up (or down)-regulated enzyme in this metabolic route makes this system very complex for analysing hypothetical signalling pathways. In this sense, monitoring of metabolite profile, as a way to uncover the system, might be thought to permit simultaneous analyses of compounds that play important roles in metabolism and physiology. Examining the general effects on whole metabolome caused by elicitation in cellular systems may allow establishing a link among the different sets of reactions presumably altered by elicitors (Sumner et al., 2003).

The biochemical status can be, at least partially, viewed by quantitative and qualitative measurements of the whole set of metabolites. The ultimate goal of metabolomic analysis is a non-biased and non-targeted analysis. However, the major limitation of metabolomics is its current inability to comprehensively profile all of the metabolome due in part to the chemical complexity of the plant metabolic pool (Krishnan et al., 2005).

So far, none of the single analytical techniques provides the ability to profile all of the metabolome. A number of analytical technologies have been proposed and applied to profile the metabolome. Among possible analytical methods for metabolomics ^{1}H NMR spectroscopy as a tool has several advantages. It can cover a broad range of metabolites, although its low sensitivity has made it difficult to detect minor metabolites. It is possible to carry out the structure elucidation of unknown or unexpected metabolites, particularly secondary metabolites of plants. The signal intensities of NMR spectra are based on molar concentrations and can therefore be compared directly. These advantages of NMR, together with its speed and robustness of signal value make NMR as a first choice for a 'macroscopic metabolomics' approach guiding a broad profile of the metabolome before doing a 'microscopic' or targeted metabolomic analysis for a detailed small range of metabolites (Choi et al., 2004a,b, 2006).

The present study was designed to evaluate overall metabolic changes in YE-elicited cultures using nuclear magnetic spectroscopy, to compare them with those caused by elicitation with MeJA and to see at what level cycloheximide (CH) interrupted the silymarin elicitation process.

2. Materials and methods

2.1. Cell cultures

S. marianum L. (Gaernt) cell suspensions were established from 3-month-old undifferentiated hypocotyl callus and maintained in darkness in Murashige and Skoog liquid medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose, 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.5 mg l^{-1} benzyl adenine at pH 5.6. Cultures were shaken at 90 rpm and routinely subcultured every 2 weeks.

2.2. Treatment of cultures

For experiments, 250 ml flasks containing 50 ml medium were inoculated with 4 g wet weight cells taken from the previous subculture; treatments were done 3 days after transfer, when cells have already started the active growth phase and results were analysed at the time periods denoted in the Section 3. Cell suspensions were aseptically treated with an autoclaved elicitor isolated from yeast extract by ethanol precipitation as described (Schumacher et al., 1987; 1 mg glucose equivalents ml^{-1} medium). MeJA, prepared as stock solution in ethanol, was employed at a final concentration of 100 μM (concentration of ethanol in cultures were 0.1%). For experiments with the protein synthesis inhibitor CH, 5 μM of the compound was added to cultures 15 min prior elicitation. Controls received equivalent volumes of solvents. Silymarin was extracted and analysed by HPLC as described in previous report (Sánchez-Sampedro et al., 2005). Experimental work was performed in triplicate.

2.3. Extraction of cells

For extraction of cells, deuterated NMR solvents were directly used in order to avoid possible loss or degradation of metabolites (Hendrawati et al., 2006; Liang et al., 2006; Yang et al., 2006). Powdered material of 100 mg from lyophilised cells was transferred into a micro tube. KH_2PO_4 of 750 μl in D_2O buffer (with 0.1%, w/w TSP) and 750 μl of methanol-d_4 were added to the tube followed by vortexing for 1 min and sonication for 20 min. The tube was centrifuged at 1000 × *g* for 20 min at 25 °C. The extract was transferred into a microtube and centrifuged at 17,000 × *g* for 1 min. The supernatant (800 μl) was transferred to a 5 mm-NMR tube.

2.4. NMR measurements

^{1}H NMR spectra were recorded at 25 °C on a 400 MHz Bruker AV-400 spectrometer operating at a proton NMR