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Research article

Role of reactive oxygen species and proline cycle in anthraquinone accumulation in *Rubia tinctorum* cell suspension cultures subjected to methyl jasmonate elicitation

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ABSTRACT

Elicitors are compounds or factors capable of triggering a defense response in plants. This kind of response involves signal transduction pathways, second messengers and events such as Reactive Oxygen Species (ROS) generation, proline accumulation and secondary metabolite production. Anthraquinone (AQs) biosynthesis in *Rubia tinctorum* L. involves different metabolic routes, including shikimate and 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathways. It has been proposed that the proline cycle could be coupled with the pentose phosphate pathway (PPP), since the $NADP^+$ generated by this cycle could act as a cofactor of the first enzymes of the PPP. The end-product of this pathway is erithrose-4-phosphate, which becomes the substrate of the shikimate pathway. The aim of this work was to study the effect of methyl jasmonate (MeJ), a well-known endogenous elicitor, on the PPP, the proline cycle and AQs production in *R. tinctorum* cell suspension cultures, and to elucidate the role of ROS in MeJ elicitation. Treatment with MeJ resulted in AQs as well as proline accumulation, which was mimicked by the treatment with a H_2O_2-generating system. Both MeJ-induced effects were abolished in the presence of diphenyliodonium (DPI), a NADPH oxidase inhibitor (main source of ROS). Treatment with the elicitor failed to induce PPP; therefore, this route did not turn out to be limiting the carbon flux to the shikimate pathway.

1. Introduction

Elicitation is a well-known strategy that has been widely used to enhance secondary metabolite production in plant systems. The treatment of plant cells with an elicitor results in physiological changes and an onset of a defense response, which involves the expression of genes associated with secondary metabolite accumulation [1–3]. It has been observed that some substances normally produced by plant cells in a defense response as signal molecules can also trigger the same kind of response when applied exogenously. Some examples of these compounds, known as endogenous elicitors, are jasmonic acid (JA) and its methyl ester (methyl jasmonate, MeJ), as well as salicylic acid (SA) [3].

The defense response begins with the interaction between the elicitor and a receptor localized in the plasmatic membrane or in the cytosol of the plant cell [1,2]. This process leads to the activation of effectors and to the amplification of the signal by a cascade of second messengers, which finally results in the establishment of a defense response that includes secondary metabolite production.

Production of ROS is a common event in defense responses [4], being superoxide ($O_2^{\cdot -}$) and peroxide (H_2O_2) the most abundant species. They are mainly produced by membrane-bound NADPH oxidase and, to a lesser extent, by apoplastic peroxidase [1]. These compounds exhibit a wide variety of actions, including hypersensitivity reactions, cell death, cell wall reinforcement, gene activation and induction of defense compounds [1]. $O_2^{\cdot -}$ and H_2O_2 have been described as signals involved in phytoalexin induction in different plant species [5]. It has been reported that, in tomato, H_2O_2 is involved in the defense response to different elicitors, including MeJ [4].

Abbreviations: AQs, anthraquinone(s); DPI, diphenyliodonium; G6PDH, glucose-6-phosphate dehydrogenase; GDH, glutamate dehydrogenase; GI, growth index; GOD, glucose oxidase; H_2O_2, peroxide; ICDH, isocitrate (NADP) dehydrogenase; ICS, isochorismate synthase; JA, jasmonic acid; MeJ, methyl jasmonate; MEP, 2-*C*-methyl-D-erythritol-4-phosphate; MES, 2-N-morpholino ethanesulphonic acid; $O_2^{\cdot -}$, superoxide; PPP, pentose phosphate pathway; ROS, reactive oxygen species; SA, salicylic acid.

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Accumulation of proline in response to different kinds of stress (drought, high salinity, UV irradiation, heavy metals, and oxidative and biotic stress) is well documented, being described to exhibit antioxidant activity, not only by reacting with ROS but also because it can stabilize and protect detoxifying enzymes [6]. This amino acid also contributes to cell recovery after stress by providing electrons to the respiratory chain and by acting as a nitrogen and carbon source [6,7]. In addition to this, proline and its degradation products have been described to induce the expression of genes related to pathogen defense [8]. It has also been reported that, under stress conditions, proline accumulation is a consequence of an increase in its biosynthesis from glutamate [7].

The defense response can also induce the pentose phosphate pathway (PPP), in order to feed secondary metabolite routes with carbon skeletons (Fig. 1). It could also provide antioxidant protection against ROS by producing NADPH. Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), the first enzyme of this pathway, has a protective role against oxidative stress in cell types that have no other NADPH-producing pathways [9].

It has been proposed that the PPP could be coupled to the proline cycle that occurs in the mitochondria and the cytosol [10]. The model suggests that the first enzymes in the PPP could use the $NADP^+$ generated by the conversion of proline in the cytosol, thus driving the carbon flux towards the shikimate and other secondary metabolite pathways.

Anthraquinones (AQs) are secondary metabolites with interesting therapeutic activities [11]. In *Rubia tinctorum* L., their biosynthesis involves several metabolic pathways (see Fig. 1): on one hand, the ring C is originated from an IPP unit derived from the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway. On the other hand, α-ketoglutarate and isochorismate are condensed to produce *o*-succynilbenzoate, which is the precursor of A and B rings. Isochorismate is synthesized from chorismate, the end product of shikimate pathway, by the isochorismate synthase (ICS) [12]. Many authors have reported an induction in AQs production in *R. tinctorum* cell suspension cultures in response to the treatment with different elicitors: chitosan [13], fungal polysaccharides, shear stress [14], JA and SA [15], and MeJ [12]. It was observed that the defense response against chitosan was regulated by the final activation of mitogen activated protein kinase (MAPK). This response involved a rise in intracellular Ca^{2+} concentration and the activation of phospholipase C (PLC), phosphoinositide 3′–OH–kinase (PI3K) and proteinkinase C (PKC), whereas the adenilate ciclase (AC)/cAMP/proteinkinase A (PKA) cascade was not involved [16].

In this work, we studied the effect of MeJ elicitation on AQs production, the PPP and proline cycle, and also the role of ROS in the defense response. For the latter purpose, an inhibitor of the NADPH oxidase (diphenyliodonium, DPI) was used.

2. Results and discussion

2.1. Assays with MeJ and DPI

In order to evaluate the effect of MeJ elicitation on *R. tinctorum* suspension cultures, and to elucidate the role of ROS in response to this elicitor, different experiments were performed. Besides control and MeJ treatment, DPI was added (to both non-treated and MeJ-treated cells). This compound acts as an inhibitor of the NADPH oxidase (one of the main sources of ROS). Finally, a treatment with a H_2O_2-generating system was accomplished by the addition of both glucose and glucose oxidase (GOD, EC 1.1.3.4). This enzyme converts glucose into gluconic acid and produces H_2O_2.

2.1.1. Effect on pH, growth index (GI) and cell viability

When media pH values were compared (Table 1), it was found that both MeJ treatments (MeJ alone and MeJ–DPI), showed an

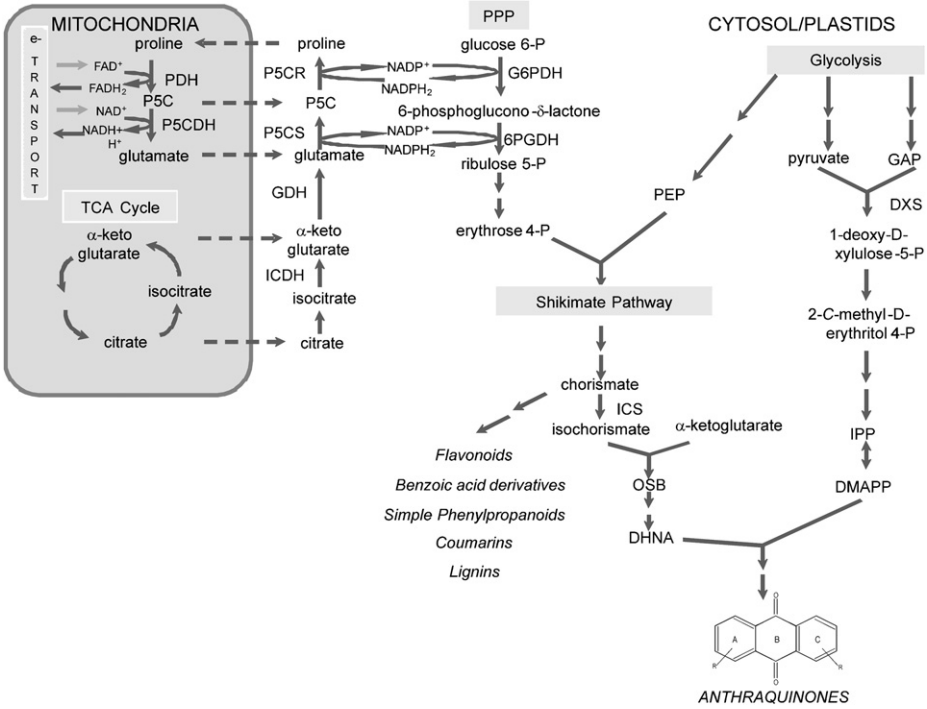

Fig. 1. Schematic illustration of the metabolic pathways mentioned in this work. 6PGDH: 6-phosphogluconate dehydrogenase; DHNA: 1,4-dihydroxy-2-naphthoic acid; DMAPP: 3,3-dimethylallylpyrophosphate; DXS: 1-deoxy-D-xylulose 5-phosphate synthase; G6PDH: glucose-6-phosphate dehydrogenase; GAP: glyceraldehyde-3-phosphate; GDH: glutamate dehydrogenase; ICDH: isocitrate (NADP) dehydrogenase; ICS: isochorismate synthase; IPP: isopentenyl-5-pyrophosphate; OSB: *o*-succinylbenzoic acid; -P: phosphate; P5C: pyrroline-5-carboxylate; P5CDH: P5C dehydrogenase; P5CR: P5C reductase; P5CS: P5C synthetase; PDH: proline dehydrogenase PEP: phosphoenolpyruvate; PPP: pentose phosphate pathway; TCA: Tricarboxylic acids.