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Research article

Phospholipidic signaling and vanillin production in response to salicylic acid and methyl jasmonate in *Capsicum chinense* J. cells

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ABSTRACT

The phospholipidic signal transduction system involves generation of second messengers by hydrolysis or changes in phosphorylation state. Several studies have shown that the signaling pathway forms part of plant response to phytoregulators such as salicylic acid (SA) and methyl jasmonate (MJ), which have been widely used to stimulate secondary metabolite production in cell cultures. An evaluation was made of the effect of SA and MJ on phospholipidic signaling and capsaicinoid production in *Capsicum chinense* Jacq. suspension cells. Treatment with SA inhibited phospholipase C (PLC) (EC: 3.1.4.3) and phospholipase D (PLD) (EC: 3.1.4.4) activities *in vitro*, but increased lipid kinase activities *in vitro* at different SA concentrations. Treatment with MJ produced increases in PLC and PLD activities, while lipid kinase activities were variable and dose-dependent. The production of vanillin, a precursor of capsaicinoids, increased at specific SA or MJ doses. Preincubation with neomycin, a phospholipase inhibitor, before SA or MJ treatment inhibits increase in vanillin production which suggests that phospholipidic second messengers may participate in the observed increase in vanillin production.

1. Introduction

The phospholipid signal transduction system consists of the generation of messengers by phospholipid hydrolysis, phosphorylation or dephosphorylation. When a signal is perceived, phospholipase C (PLC) is activated and hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), generating two products: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG).

IP_3 is a second messenger that can be diffused into the cytosol and recognized by a specific receptor in the endoplasmic reticulum or other endomembranes. It causes calcium channels to open and consequent liberation of the Ca^{2+} ion into the cytosol [1,2]. Although the role of IP_3 in plants is not fully understood [3] it has been suggested that its phosphorylated versions (IP_4, IP_5 and IP_6) are the active molecules. In animal cells, DAG participates in activation of protein kinases associated with the membrane, and it is phosphorylated to produce phosphatidic acid (PA) through the action of diacylglycerol kinase (DGK) [4]. Phosphatidic acid can also be generated by hydrolysis of phospholipids catalyzed by phospholipase D (PLD). PA is a very important signaling molecule in plants, functioning as a second messenger which modulates the activities of kinases, phosphatases, phospholipases and other proteins involved in membrane traffic, calcium signaling and biotic and abiotic stress response [5,6].

Several studies have demonstrated that phospholipidic signaling is involved in plant response to phytoregulators. A number of secondary messengers produced by phospholipids metabolism are involved in responses to auxins [7], cytokinins [8], jasmonates [9,10], abscisic acid (ABA) [10,11], salicylic acid (SA) [12] and ethylene [13]. Methyl jasmonate (MJ) and SA are regulator compounds involved in several plant development and growth processes. They are present in almost all plant tissues, but levels of these phytoregulators increase in response to stressful conditions [14,15]. Since secondary metabolite accumulation is a common response to stress and both SA and MJ mediate cell response to stress, these compounds have been used as elicitors to increase

Abbreviations: ABA, abscisic acid; BSA, bovine serum albumin; DAG, diacylglycerol; DGK, diacylglycerol kinase; IP_3, inositol 1,4,5-trisphosphate; MJ, methyl jasmonate; PC, phosphatidylcholine; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; PIP4K, phosphatidylinositol 5-phosphate 4-kinase; PLC, phospholipase C; PLD, phospholipase D; PA, phosphatidic acid; SA, salicylic acid; TCA, trichloroacetic acid.

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secondary metabolite production in suspension cell cultures of different species, including *Capsicum* [16,17]. Capsaicinoids, the secondary metabolites responsible for the pungency of chili peppers, occur only in the *Capsicum* genus [18]. Capsaicinoids are a group of acid amides formed by condensation of vanillylamine with a fatty acid. Vanillylamine is derived from phenylalanine through the phenylpropanoid pathway, whereas the acyl moiety is formed from either valine or isoleucine. Their accumulation is highly sensitive to environmental conditions such as light, water and nutrient availability, and pathogen infection [19]. In *Capsicum frutescens* cell cultures, SA and MJ may modify accumulation of capsaicinoid and some biosynthetic intermediaries [17]. Depending on developmental stage, the *Capsicum chinense* cell line used here accumulates low capsaicinoid levels.

The phospholipid signal transduction system is also involved in accumulation of some secondary metabolites in response to elicitors [16]. Increases in PLC activity are linked to higher phytoalexin production in cultured carrot cells [20], pisatin accumulation in pea [21] and scoparone synthesis in lemon [22]. In addition, it has been suggested that PLD activity is involved in elicitor-induced phytoalexin accumulation in rice cells [23]. Finally, treatment of pea tissues with a diacylglycerol kinase inhibitor enhanced phytoalexin accumulation induced by a fungal elicitor [24].

The present study objective was to evaluate if SA and MJ modulate phospholipidic signaling and if this effects capsaicinoid production in *C. chinense* J. cells.

2. Results

2.1. C. chinense cell culture cycle

A growth curve for *C. chinense* suspension cells was established by measuring fresh weight during a 24-day period. This cell line exhibits a typical growth curve with a lag phase during the first four days; exponential growth ending at day 7; a linear phase from day 8 to day 18; and a final stationary phase from day 19 to day 24 (Fig. 1). Based on previous research in our laboratory, SA and MJ treatments were applied on day 7 or day 14 of the cell culture cycle.

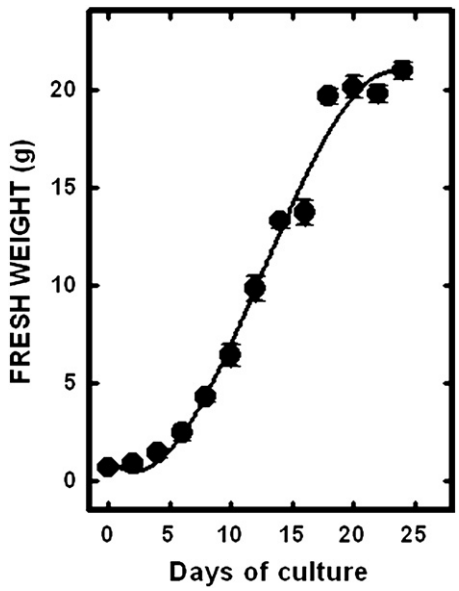

Fig. 1. Culture cycle of *C. chinense* cells. Cells were cultured from day 0 to 24 in MS medium and fresh weight measured every two days.

2.2. Changes in phospholipase activities in response to SA and MJ

The effect of SA and MJ on PLC activity *in vitro* was evaluated in a dose-response experiment. Cells were incubated with different SA or MJ concentrations for 30 min, homogenized and PLC activity assayed in a membrane fraction. SA produced dose-dependent inhibition in PLC activity, with a maximum of 40% inhibition at 200 μM SA (Fig. 2A). PLC activity *in vitro* was increased most when cells were treated with 50 μM MJ, and decreased at higher MJ concentrations (Fig. 3A). The effect of SA and MJ on PLD activity was evaluated as described for PLC, but using soluble extract. Different SA concentrations produced inhibition of 20–30% (Fig. 2B). PLD activity varied depending on MJ levels, with increases of up to 60% at 25, 50 and 100 μM MJ, but a decrease at 200 μM (Fig. 3B).

2.3. PLC and PLD protein levels after SA and MJ treatments

Phospholipase activities *in vitro* varied between SA and MJ treatments, so a Western blot technique was applied to determine if protein levels were modified. Cells were treated with different SA or MJ concentrations for 30 min, the protein fraction isolated, separated by SDS-PAGE and immunobloted. The antibodies (against the catalytic domain of *Coffea canephora* PLC) used to detect PLC protein levels showed PLC immunodetection bands with 45 and 66 kDa. Based on previous results [9,25,26], these bands correspond to PLC proteins. Signal levels did not change significantly in response to SA or MJ treatment (Figs. 4A and 5A). The PLD antibodies (against the purified PLD from *Brassica oleracea*) produced only a single band at 97 kDa with a slight change in response to SA (50–100 μM) treatment (Fig. 4B); however, quantification of these bands showed no statistical difference. No change in this band in response to MJ treatment was detected (Fig. 5B).

2.4. Effect of SA and MJ on lipid kinase activities

Changes in phospholipids phosphorylation state have been reported in response to environmental stresses [12,27]. The effect of SA and MJ on lipid kinase activities *in vitro* in the present system was evaluated by treating the cells for 30 min with different SA and MJ concentrations. Treatment of cells with SA caused an approximately 50% increase in the enzymatic activities responsible for PIP_2 formation (Fig. 6A). Formation of PA was biphasic when cells were treated with different SA concentrations (Fig. 6B); increases were observed at 25, 50 and 200 μM SA, and a decrease at 100 μM SA.

After treatment with different MJ concentrations, inhibition of the enzymatic activities responsible for PIP_2 formation was observed in the 25 and 200 μM MJ treatments (Fig. 7A). Treatment with MJ increased PA formation, with the highest responses in the 25 and 100 μM MJ treatments (Fig. 7B).

2.5. Capsaicinoid production in cells treated with SA or MJ

No capsaicinoids were detected in the assays, although significant levels of vanillin, a late precursor, were observed (Fig. 8). Treatment effects were differential since vanillin only increased in cultures exposed to low MJ (25 μM) or high SA (100 μM and higher) concentrations (Fig. 8A and B). Under the experimental conditions, high SA or low MJ concentrations clearly promoted vanillin synthesis, although once formed the vanillin was not used for capsaicinoid synthesis. Vanillin production under treatment with SA or MJ in the presence of neomycin sulphate (a phospholipase inhibitor) [28] was tested. Preincubation with neomycin for 15 min attenuated vanillin production stimulated by SA and MJ (Fig. 8). Phospholipase activity *in vitro* was completely inhibited under the same conditions (data not shown).