

Cellulase elicitor induced accumulation of capsidiol in *Capsicum annum* L. suspension cultures

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Abstract When growth-phase cell suspension cultures of *Capsicum annum* were treated with cellulase-elicitor preparation at 3 µg/ml, the level of capsidiol was transiently increased in the culture media rather than in the cells reaching its maximum approx 24 h after treatment. With methyl jasmonate it took 18 h. Elicitor treatment doubled phospholipase A₂ (PLA₂) activity but simultaneous treatment with aristolochic acid, a PLA₂ inhibitor, inhibited sesquiterpenoid accumulation as well as PLA₂ activity. Mastoparan, a G protein activator, treatment also increased PLA₂ activity and capsidiol production. Taken together, the present study shows that induction of capsidiol production in the *C. annum* is mediated by PLA₂ activation.

Keywords *Capsicum annum* · Cellulase · Elicitation · Octadecanoid pathway · Phospholipase A₂

Introduction

Plant cells may defend themselves from the attack of pathogens by strengthening defensive barriers, producing reactive oxygen species, accumulating antimicrobial

phytoalexins, by sacrificing themselves (hypersensitive response), or by synthesizing proteinase inhibitors (Blechert et al. 1995). These plant defense responses are initiated by the recognition of a molecule, an elicitor, at the recognition site or receptor presided in plasma membrane of the cell (Mandujano-Chavez et al. 2000). Several signalling pathways have been implicated in the elicitation of these responses including phospholipases (Schweizer et al. 1997).

Phytoalexin production in the plant cells is also mediated by a series of signal molecules and is characterized by a transient increase of the product. In treatment with jasmonic acid or methyl jasmonate to suspension cell cultures, expression of phenylalanine ammonia lyase is increased and this is related to phytoalexin production, as a type of plant self-defense mechanism (Hano et al. 2005; Whitehead et al. 1987). Treatment with methyl jasmonate also induces overproduction of taxane compounds in suspension cultures of *Taxus* (Kim et al. 2005). Thus, jasmonic acid may be useful to increase the yield of pharmacologically active chemicals and may be used to examine secondary metabolism regulation in plant.

Phospholipase A₂ (PLA₂) is involved in plant defense mechanisms and plays an important role in elicitor-induced defense responses. Elicitor treatment rapidly elevated the cellular level of free linolenic acid, and the time course for the accumulation of linolenic acid and linoleic acid was correlated with those for the accumulation of jasmonic acid and expression defense genes (Jung and Kim 2000).

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The present results show that the production of a sesquiterpenoid from the *Capsicum annuum* suspension culture was induced by cellulase elicitor and the induced compound, capsidiol, was mainly accumulated in the culture media. The results also show that the elicitation may be mediated by a second messenger, jasmonic acid, linked by preceding PLA₂ activation.

Materials and methods

Suspension culture

The surface of *Capsicum annuum* seeds were sterilized with ethanol (70% v/v, 15 s) and sodium hypochlorite (2.5% w/v, 20 min), followed by a thorough rinse with sterile distilled water, and then germinated on Murashige-Skoog (MS) solid media in 500 ml Phytacone culture flasks. Flasks were maintained at 25°C under fluorescent light (16 h photoperiod). Callus was induced from the root of 4-week old seedlings on MS media containing 2,4-dichlorophenoxyacetic acid (1 mg/l), kinetin (0.1 mg/l) and sucrose (30 g/l) in the dark and maintained on the same media at 25°C in the dark by subculturing at 4 week intervals. Cultures were established with 8 g (fresh wt) callus in 100 ml MS medium containing 2,4-dichlorophenoxyacetic acid (0.5 mg/l) in 250 ml Erlenmeyer flasks. Subculturing was every 4 weeks and at 110 rpm at 25°C in the dark.

Assay for phospholipase A₂ (PLA₂) activity

PLA₂ activity was assayed using 2-[1-¹⁴C]arachidonoyl-glycerophosphatidylcholine (Amersham, UK) as a substrate by the method of Kim et al. (1994).

Isolation and identification of capsidiol

Media (30 l) of the suspended cells, 24 h after treatment with cellulase (3 µg/ml) from *Trichoderma viridae* (Sigma), was extracted three times with an equal volume of ethyl acetate. Upon removal of the solvent in vacuo, the ethyl acetate extract yielded 1.1 g of material. The extract was subjected to column chromatography (cc) over silica gel (3 × 30 cm)

eluted successively with chloroform/methanol (100:1, 50:1, 20:1, 10:1, 5:1, 2:1, 1:1, 0:1 v/v) to afford seven fractions (I–VII). The subfraction III was further applied to a Sephadex LH-20 column with methanol as an eluent, which resulted in the isolation of capsidiol which was identified by ¹H-NMR and ¹³C-NMR analyses.

Quantification of elicited compounds

A batch of suspension cells cultured in 150 ml Erlenmeyer flasks was treated with cellulase elicitor from *Trichoderma viridae* and other chemicals. The ethyl acetate extracts of the media were subjected to gas chromatography (GC) analysis. Analytical GC was carried out in a chromatograph fitted with a flame ionization detector (FID) and a 2.5 mm × 30 m DB-5 capillary column (J & W Scientific, USA). Helium was used as the carrier gas at a flow rate of 30 ml/min. The column was operated in a split mode, the split ratio being 100:1. The oven temperature was programmed 180–300°C at a gradient of 4°C/min. The injector and detector temperatures were 300°C, respectively. All quantifications were performed in triplicate.

Results and discussion

Capsidiol was isolated as a white powder from media of suspended cell elicited with cellulase. The mass spectrum of purified compound exhibited a molecular ion at *m/z* 236. By comparison of ¹H-NMR and ¹³C-NMR data, the purified compound was identified as capsidiol (data not shown) (Stillman et al. 1981). Capsidiol production was increased after treatment of cellulase (3 µg/ml) and methyl jasmonic acid (100 µM) by 40 and 10 times to control cells, respectively (Fig. 1). Tetcyclacis, a cytochrome P450 inhibitor, specifically inhibits jasmonic acid biosynthesis (Saito et al. 2006), and it inhibited capsidiol accumulation by elicitors (Fig. 1). These data showed that jasmonic acid is involved in the capsidiol synthesis and cytochrome P450 is functioned at the upstream of jasmonic acid.

Capsidiol is an eremophilane-type sesquiterpene that has been isolated from many solanaceous species. It is synthesized by tobacco and pepper plants in response to various stimuli such as UV irradiation,

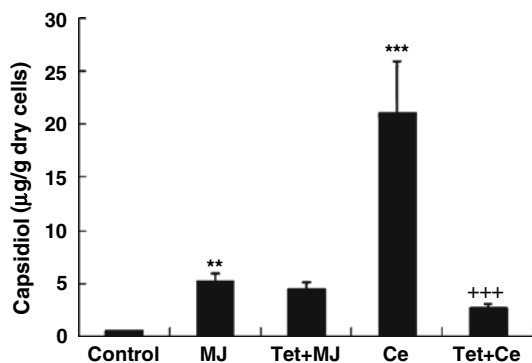


Fig. 1 Accumulation of capsidiol in suspension cultures of *C. annuum* after methyl jasmonic acid (100 µM) or cellulase (3 µg/ml) treatment. Tetcyclacis (0.5 µM), a cytochrome P450 inhibitor that specifically inhibits jasmonic acid biosynthesis, inhibited capsidiol accumulation by cellulase. MJ; methyl jasmonic acid treated, Tet + MJ; methyl jasmonic acid treated after tetcyclacis (0.5 µM) treatment, Ce; cellulase-treated, Tet + Ce; cellulase-treated after tetcyclacis treatment. The values shown are means ± S.D. of three experiments. ** $P < 0.01$, *** $P < 0.001$ vs. control cells and +++ $P < 0.001$ vs. cellulase-treated cells (ANOVA)

biotic elicitors—cellulase, yeast extract, fungus extract, and abiotic elicitors—CuSO₄, MnCl₂ (Vogeli and Chappell 1990; Zhao et al. 2004). Capsidiol has antifungal and antimicrobial activities (De Marino et al. 2006). Also, capsidiol inhibited contraction induced by the agonists, histamine, acetylcholine, bradykinin, and barium chloride in the isolated guinea-pig ileum (Nashiri et al. 1993).

Phospholipase A₂ (PLA₂) plays a crucial role in signal transduction of plant cells. Elicitor treatment elevated a cellular level of free linolenic acid. Linolenic acid is known to serve as the precursor of jasmonic acid and other octadecanoid-derived chemical mediators that stimulate the defense-related gene expression (Jung and Kim 2000). Thus, the release of linolenic acid by a PLA₂ is thought to be an important step in the plant defense mechanisms. We tried to elucidate the effect of elicitation on PLA₂ activity. Activation of the PLA₂ by elicitation, early stage of the octadecanoid pathway was investigated. The activity of PLA₂ was increased by 3.8-times 1 h after treatment with cellulase (3 µg/ml) to *C. annuum* cells (Fig. 2a). The production of the capsidiol was also increased at 24 h after treatment of cellulase to the same batch of the cells. Even after elicitation by cellulase, if the cells were treated with aristolochic acid (50 µM), a PLA₂

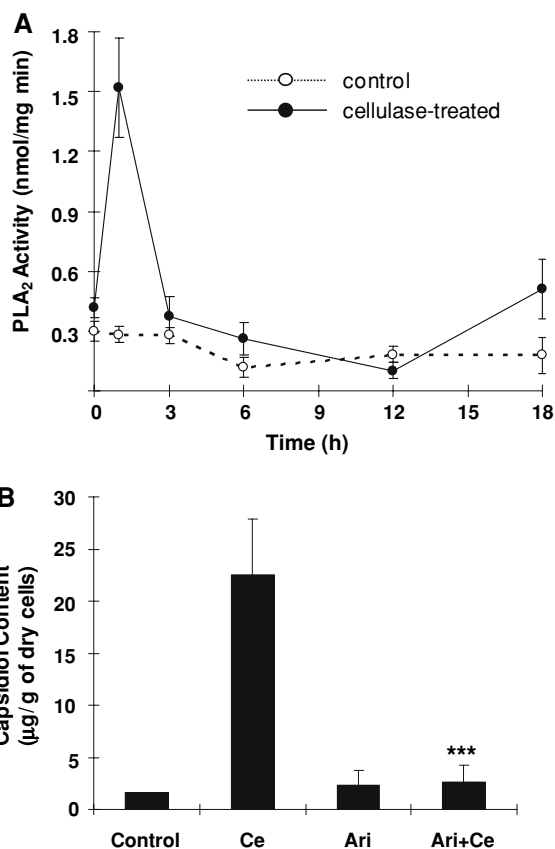


Fig. 2 (a) PLA₂ activation after cellulase (3 µg/ml) treatment. (b) The effect of PLA₂ activation on the capsidiol production by elicitation. Ce; cellulase-treated, Ari; aristolochic acid (50.0 µM) treated, Ari + Ce; cellulase-treated after aristolochic acid treatment. The values shown are means ± S.D. of three experiments. *** $P < 0.001$ vs. cellulase-treated cells (ANOVA)

inhibitor, capsidiol production was not increased (Fig. 2b). The present results implicated that PLA₂ was involved in the phytoalexin synthesis by elicitation. It was reported that PLA₂ activity was increased after treatment of yeast extract in *Scutellaria baicalensis* suspension cultures (Yoon et al. 2000). It suggested that PLA₂ activation is involved in capsidiol production by elicitation.

In many cases, phospholipase A₂ activation is stimulated by G protein activation. We investigated relationship between G protein activation and PLA₂ activity increase in case of *C. annuum*. Mastoparan, a G protein activator, treated cells were harvested at each time. These cells were homogenized with optimized buffer. Then we estimated PLA₂ activity of protein preparation. The PLA₂ activity was increased to the

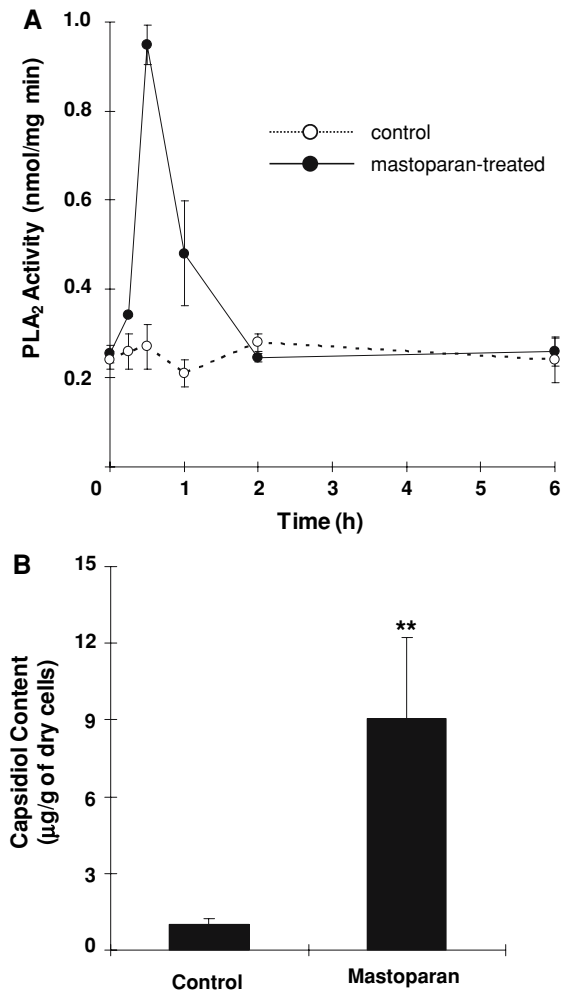


Fig. 3 Involvement of G protein in PLA₂ activation by cellulase elicitation. (a) The effect of mastoparan (25.0 µM), a G protein activator, treatment on PLA₂ activity. (b) The effect of mastoparan, a G protein activator, treatment on capsidiol production. The values shown are means ± S.D. of three experiments. ** $P < 0.01$ vs. control cells (ANOVA)

peak 30 min after mastoparan treatment and then decreased to the control level (Fig. 3a). Mastoparan also increased the amount of capsidiol production by 10 times after 18 h treatment (Fig. 3b). These data provided that PLA₂ activation by elicitation is occurred via G protein activation and PLA₂ activation is necessary for the production of capsidiol via signal cascades. Mastoparan, a toxin from wasp venom, is a potent stimulator of exocytosis from diverse mammalian cells. Mastoparan increases the GTPase activity and the nucleotide binding of several purified GTP-binding

regulatory proteins (G proteins) whose function is to couple cell surface receptors to intracellular mediators. G proteins use a common mechanism to couple receptor to effector. When a G protein binds GTP, it is converted to an active state such that it can stimulate its effector protein. Hydrolysis of bound GTP to GDP by the G protein terminates that activation. The agonist-liganded receptor promotes both the dissociation of bound GDP and the subsequent binding of GTP, thereby increasing the time that the G protein spends in the active state (Higashijima et al. 1988). As shown in Fig. 3, mastoparan increased PLA₂ activity and capsidiol production in *C. annuum* suspension cultures. These results suggested that PLA₂ activation by elicitation is occurred via G protein activation and PLA₂ activation is necessary for the production of capsidiol via signal cascades.

Taken together, these data suggested that elicitor induced capsidiol production in *C. annuum* suspension cultures is at least mediated by PLA₂ involved in octadecanoid pathway.

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